A tRNA gene mapping within the chloroplast rDNA cluster is differentially expressed during the development of *Daucus carota*

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**ABSTRACT**

*In vivo* analysis of expression of the chloroplast rDNA cluster during somatic embryogenesis of *Daucus carota* (*D. carota*) was performed by Northern-blot analysis with different DNA probes, spanning both the 16S rRNA gene, the 16S –23S rRNA spacer, which contains the two mosaic tRNA genes tRNA^lle^ and tRNA^Ala^, and the region upstream of the 16S rRNA gene, where a tRNA^Val^ maps. We show that expression of both the spacer tRNAs tRNA^lle^ and tRNA^Ala^ is not significantly regulated during development whereas the amount of the transcript corresponding to tRNA^Val^ is not detectable during early embryonic stages and progressively accumulates during late phases. Multiple transcription start sites have been identified upstream of the tRNA^Val^ gene by S1 mapping analysis, which are activated late during the embryogenesis. These data indicate that developmental control mechanisms act on plastid gene expression during embryogenesis in carrot.

**INTRODUCTION**

A characteristic of plant development is the capacity of some sporophytic and gametophytic cells to undergo embryogenesis in culture and to differentiate into a mature plant with the same morphological changes that occur in zygotic embryos (1, 2).

Cell cultures of the carrot (*D. carota*) can be induced to form large numbers of somatic embryos by alteration of the hormone concentration in the culture medium (1, 3). In comparison to zygotic embryos, which are embedded within the ovary, these somatic embryos are accessible to physical manipulations. It is also possible to fractionate embryonic carrot cultures to obtain sufficient material for biochemical analyses of each stage in embryo development (4). We have used *D. carota* to study plastid gene expression during embryo development in flowering plants. In developing plants, chloroplasts are derived from small proplastids (5). The biogenesis of proplastids into mature chloroplasts is the most significant morphogenetic change in leaf formation, and initial efforts to analyze the control of plastid gene expression have concentrated on their transcriptional regulation (6, 7). At present little is known about the mechanisms that modulate the differential gene expression in plastids during the development. The control of chloroplast gene expression operates on several stages, namely transcription, post-transcription, translation and post-translation (8, 9). Recent studies suggest that gene expression is mainly controlled at the post-transcriptional level (7, 10).

In particular specific mRNAs for *psbA* (component of photosystem II), *psaA* – *psaB* (photosystem I), *rbcL* and *atpBE* (ATP synthase) were described to accumulate in response to light and during leaf development, as a consequence of post-transcriptional events leading to their stabilization (11, 12, 13). Two transcription units in the spinach plastid genome, however, show changes in their transcriptional activities during chloroplast development and leaf maturation. One is the gene for tRNA^lys^ (*trnK*), and the other corresponds to an operon containing several genes for ribosomal proteins (11). The ribosomal RNA genes (rDNAs) in higher-plant chloroplasts are clustered and arranged like in *Escherichia coli* (*E. coli*) in the order 16S –23S – 4.5S – 5S rRNA with an interspersion of transfer RNA (tRNA) genes (14, 15, 16, 17). Like most of the chloroplast genes, rDNA genes are transcribed polycistrionically (7, 18). Transcriptional regulation of plastid rDNA clusters differs from protein coding genes during chloroplast development (13, 19). This is logic since ribosome assembly is one of the crucial processes preceding the expression of plastome-encoded photosynthetic genes and since

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ribosomes are stable complexes compared with the unstable mRNAs. At the present time, however, no experimental data has been obtained to explain differential gene expression in chloroplasts.

In order to understand the regulatory mechanisms which affect rRNA synthesis in chloroplasts during plant embryogenesis, we have cloned and analyzed the promoter region of the carrot chloroplast rDNA cluster. This DNA clone, which is presented here, encodes the chloroplast tRNA\textsuperscript{Val}\textsubscript{(GAC)} as well as the start of the 16S rRNA gene and contains sequences similar to the Pribnow box and the −35 region found in E. coli promoters (20, 21). In vivo transcription of the DNA fragment containing this region reveals that the tRNA\textsuperscript{Val}\textsubscript{(GAC)} gene is differentially expressed during somatic embryo development.

**MATERIALS AND METHODS**

**Plant material and culture conditions**

Carrot cultures were initiated from seedling hypocotyls of *D. carota* c. v. ‘S.Valery’ and maintained by transferring every 14 days 2 ml of packed cell volume (after centrifugation at 200 × g) in 50 ml of fresh Gamborg’s B5 medium supplemented with 0.5 ml/1 2,4 dichlorophenoxyacetic acid (2,4 D) and 0.25 ml/l 6-benzylaminopurine (6 BAP). Cultures were kept at 24°C on a rotary shaker (80 rpm) under continuous light (500 lux). To induce embryogenesis at the 8th day of subculturing, cultures were filtered through two nylon sieves with 120 μm and 50 μm pore size (22). Cell clumps (collected on the second filter) were washed several times with hormone-free medium and resuspended in the same medium at a density of 5000 c.u./ml (cellular units/ml). The different embryonic stages were purified according to (23), with the difference that the globular stages were isolated at day 3 and day 6.

**Bacterial strains and growth conditions**

*Escherichia coli* (E. coli) strain DH5 α amp\textsuperscript{+} and plasmid pGEM-3Zf(+) were used to construct the genomic library. Conditions for subcloning of restriction enzyme fragments from the carrot genomic clones, bacterial transformation, media and growth conditions for bacteria have been described previously (24, 25).

**DNA procedures**

Plant DNA was isolated either from dark grown 5 to 6 days old seedlings or from growing calli, according to (26), modified by (27). Standard molecular techniques were applied according to (28).

**Construction of a carrot genomic library**

High molecular-weight DNA was partially digested with *Hind*\textsubscript{III} and size fractionated on a 10 to 40% sucrose gradient in 100 mM NaCl–10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA. Gradient fractions (5 to 10 kb) were pooled, dialyzed against 10 mM Tris–1 mM EDTA (pH 7.4), extracted with 2-butanol and ethanol precipitated. DNA fragments were cloned into pGEM-3Zf(+) Hind\textsubscript{III} digested vector DNA using T4 ligase. *E. coli* transformation was done according to (25) and transformants were plated on LB medium containing 100 μg of ampicillin and 50 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) per ml.

**Colony hybridization with labelled RNA probes**

Transformants were replica plated on 5 LB plates containing ampicillin. After 4–5 h of growth at 37°C hybridization was performed with a 32P-labelled total RNA extract from the following developmental stages of carrot: undifferentiated cells, embryoid phases (globular, heart- and torpedo-shaped) and early plantula. Transformants whose DNA hybridized differentially with total RNA from embryoids were further analyzed.

**RNA procedures**

Total plant RNA was isolated by a LiCl small-scale procedure (29). All aqueous solutions were treated with diethylpyrocarbonate prior to autoclaving. *In vitro* 5'-32P-labelling of total RNA was modified according to (30). The reaction mixture (50 μg RNA in 50 μl of H\textsubscript{2}O; 50 μl of 0.1 M Na\textsubscript{2}CO\textsubscript{3} solution) was incubated at 50°C for 50 min. The solution was neutralized by 100 μl of HCl (0.05 M) and adjusted to 0.05 M Tris–HCl pH 7.6. To label the 5'-ends of the RNA with T4 polynucleotide kinase and γ-32P-ATP, 1 volume of 20 mM MgCl\textsubscript{2} and 10 mM 2-mercaptoethanol was added. The reaction mixture was transferred to a tube containing 0.05 mCi of γ-32P-ATP (specific activity approximately 3000 Ci/mmmole). After addition of T4 polynucleotide kinase (1.5 units), the mixture was incubated for 30 min. at 37°C for 30. SDS was added to a final concentration...
of 0.5% and labelled RNA was separated from free γ-32P-ATP by Sephadex G-50 gel filtration. Specific activity was approximately 4–8×10^6 cpm/μg RNA. Electrophoresis of RNA was done in 1.5% agarose gels with formaldehyde (31). RNA transfer to hybond membranes (Amersham) and hybridization with 32P-labelled fragments were carried out at 42°C in a buffer containing 50% formamide, 5×SSC, 0.1% SDS, 2×Denhardts, 20 mM sodium orthophosphate pH 6.5, 50 μg/ml sonicated calf thymus DNA. After hybridization the filters were washed for 20 min. at room temperature in 1×SSC, 0.1% SDS, followed by three washes of 20 min. each at 68°C in 0.2×SSC and 0.1% SDS according to (28). RNA slot-blot analysis was performed according to (28). The quantitative analysis of transcripts was performed by counting the radioactive bands excised from the filter.

RNA/DNA-hybridization, S1 nuclease digestion, and analysis of the hybrids on polyacrylamide denaturing gels were performed according to (32, 33).

**In vitro transcription**

Supercoiled plasmid DNA was transcribed in vitro according to the following procedure. The reaction mixture (20 μl) contained 300 ng DNA, 0.4–0.8 μg E.coli RNA polymerase (Boehringer) and was preincubated for 10 min. at 37°C in a solution of 20 mM Tris-acetate (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 4 mM Mg-acetate and 150 mM KCl. The elongation reaction was carried out in the presence of a 200 μM concentration each of ATP, CTP, GTP and UTP, and 10 μg per ml rifampicin for 20 min. The reaction was stopped by 90% of 0.3% (w/v) SDS and 10 μg yeast tRNA in 50 mM EDTA. The sample was extracted twice with a 1:1 mixture (v/v) of water-saturated phenol and chloroform/isoamyl alcohol and then precipitated with 0.3 M Na-acetate (pH 5) and 2.5 vol. of ethanol. The pellet was collected and dissolved in water. The unlabelled transcripts were analyzed by primer extension.

**RESULTS**

Cloning and characterization of the chloroplast rDNA cluster

Our aim was to isolate genes differentially expressed during the somatic embryogenesis. The different embryoid stages: globular, heart-shaped and torpedo-shaped were obtained according to (23).

A HindIII genomic library was constructed in the vector pGEM3Zf(+). A replica plating experiment was performed to screen the library with different labelled RNA probes extracted from undifferentiated cells, from different stages of carrot embryoids, and from plantula. Clone p46 was analyzed by restriction enzyme analysis (Figure 1A). Fragments A, B, C, and D were totally, fragments E and F partially sequenced (34).

Comparison of our sequences with the EMBL data bank revealed a similarity of 95–98% between *D. carota* and *Nicotiana tabacum* and *Zea mays* (14, 35). The physical and genetic map of the chloroplast rDNA cluster from *D. carota* is shown in Figure 1A. The genetic map was deduced from the comparison with *Nicotiana tabacum* and *Zea mays*.

Northern blot analysis of the transcripts from the rDNA cluster during embryogenesis

To analyze possible embryo-regulated transcripts within the rDNA cluster, we have performed a Northern blot analysis using probes spanning different regions of the cluster (Figures 2 and 3). Total RNA was extracted from the following developmental stages: 1) undifferentiated cells, 2) globular, 3) heart-shaped, 4) torpedo-shaped, and 5) plantula and analyzed by Northern blot hybridization with the DNA probes indicated in Figure 1A. In Figure 2 a signal for the tRNAVal(GAC), which was undetectable in the cellular stage, increased from globular phase to plantula when compared with the amount of 16S rRNA detected with the same probe d and with probe a in Figure 3A. The size of the
Table 1. Quantitative analysis of tRNA gene expression in the rDNA cluster

<table>
<thead>
<tr>
<th></th>
<th>trnV</th>
<th>trnA</th>
<th>trnA</th>
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<tr>
<td>Undifferentiated cells</td>
<td>54</td>
<td>2560</td>
<td>2368</td>
</tr>
<tr>
<td>Globular</td>
<td>126</td>
<td>2783</td>
<td>2454</td>
</tr>
<tr>
<td>Heart-shaped</td>
<td>345</td>
<td>2966</td>
<td>2665</td>
</tr>
<tr>
<td>torpedo-shaped</td>
<td>1063</td>
<td>3289</td>
<td>2857</td>
</tr>
<tr>
<td>Early plantula</td>
<td>3178</td>
<td>3462</td>
<td>3076</td>
</tr>
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20 μg of total RNA, extracted from the following developmental stages: undifferentiated cells, globular, heart-shaped, torpedo-shaped, and early plantula were analyzed by slot-blot (see Materials and Methods) with three oligonucleotides complementary to the nucleotide sequences of the genes trnV, trnl, and trnA (the oligonucleotides are indicated as trnV, trnl, and trnA, respectively). The quantitative analysis was performed by directly counting the radioactivity of the bands excised from the filter, and the value expressed in cpm.

Figure 3B two additional faint bands are visible. The slower migrating band very likely corresponds to intron 1; the faster migrating band corresponds in size to a non-functional maturation product located between the 16S rRNA 3'- and tRNA^Val^ (GAC) 5'-maturation sites (Figure 1A).

High resolution mapping of transcripts from the chloroplast rDNA cluster during embryo development

In order to investigate the possible mechanisms of embryoregulated tRNA^Val^ (GAC) expression, a detailed transcript map of the tRNA^Val^/16S rRNA region was obtained by S1 nuclease mapping and primer extension (Figures 4 and 5). A 781 bp HindIII–Aval DNA fragment, 5’-end-labelled at the Aval site (Figure 1B and lane 7 of Figure 4), was used as a probe in an S1 mapping experiment (Figure 4). RNAs used in this experiment were extracted from the different developmental phases mentioned earlier. In addition to a signal corresponding to the mature 16S rRNA, two faint bands were observed. The faster migrating one (Pi) was present in the same amount during all developmental phases, whereas the slower migrating band (tRNA^Val^ (GAC)) was only detectable in the early plantula. The sizes of these two bands were determined on the basis of the sequencing ladder which was run in parallel (lanes 8 and 9).

The Pi transcript starts from the promoter in the tRNA^Val^ (GAC)–16S rDNA intergenic region (Figure 6) previously identified in Z.mays (37, 38). The transcript indicated as tRNA^Val^ (GAC) has the 5’-end coincident with the 5’-end of the mature tRNA^Val^ (GAC); asterisks mark possible minor processing sites.
PI, P2 and P3 detected in the early plantula
Two minor heterogeneity of spurious transcription start points was identified, of which coincide in size with the transcripts of carrot chloroplast DNA could be demonstrated by this method. Previous reports have shown that this enzyme has been previously tested for the absence of contaminating nuclease activities. Previous reports have shown that this enzyme recognizes promoters upstream of the trnV gene (37, 39). Tohdoh et al. (39) have shown that there is one strong transcription initiation site in the tobacco rDNA leader sequence located 20 to 26 bp upstream of the trnV gene (GAC). This second hypothesis is not unlikely, since it has been shown that transcription of the trnV gene is driven by upstream promoter sequences (37, 39).

The experiment shown in Figure 5 confirmed the data of the Northern blot analysis (Figure 2) and demonstrated an embryo-specific transcription activation in the region upstream of the trnV gene, leading to the expression of trnV(GAC). We were not able, however, to discriminate between processing events and transcription initiation in this region. Therefore, the nature of the transcripts P1, P2 and P3 was further investigated by in vitro transcription assays, using the 781 bp HindIII–4Avl fragment as a template and purified E. coli RNA polymerase, which has been previously tested for the absence of contaminating nuclease activities. Previous reports have shown that this enzyme recognizes promoters upstream of the trnV gene (37, 39). Putative transcription start points in the region upstream of the trnV gene of carrot chloroplast DNA could be demonstrated by this method. The result of this experiment is shown in Figure 5, lane 7. A heterogeneity of spurious transcription start points was identified, the more prominent of which coincide in size with the transcripts P1, P2 and P3 detected in the early plantula in vivo. Two minor RNA species (* and **), possibly processed products, were only detected in vivo.

**DISCUSSION**

The gene for trnV(GAC) is approximately 300 bp upstream of the start of the 16S rRNA gene in maize (40), tobacco (39) and carrot (34). The trnV(GAC) and the 16S rRNA are transcribed as a part of the rDNA operon (8, 37, 41). Putative promoters were also identified in the region upstream of the trnV gene both in tobacco (39) and maize (37). An additional strong and well conserved promoter was also found between the trnV(GAC) and 16S rRNA genes in these species. The significance of multiple promoters of the rDNA cluster is unknown. Developmentally regulated promoter switching was hypothesized (37, 41, 42). In the attempt to isolate developmentally regulated genes in D. carota, we have cloned the chloroplast rDNA cluster. Northern blot analysis revealed that the expression of the trnV gene is embryo-regulated, since this trnV(GAC) is not detectable in the undifferentiated cellular phase and increases from globular stage to early plantula. In contrast, expression of the spacer RNA genes trnL and trnA parallels that of the 16S rRNA. The two-fold increase in the amount of tRNAs tRNAVal and tRNAAla and the 16S rRNA was also detected during the development from the undifferentiated cellular phase to early plantula. The increase of the amount of 16S rRNA is due to modulation of its stability (13).

This result was also confirmed by our S1 mapping analysis with a probe spanning the trnV–16S rDNA region (Figure 4). This experiment revealed the presence of the previously mapped promoter in the intergenic region between trnV and the 16S rDNA (37, 39), whose activity remained substantially constant during the embryogenetic process. In addition to this transcription start site, this analysis identified an embryo-regulated transcript, whose 5'-end coincided with the mature 5'-end of tRNAVal(GAC). The detection of this molecule with a probe 5'-end labelled at a restriction site within the 16S rRNA gene again demonstrated that the trnV(GAC) gene is transcribed as a part of the rDNA operon (8, 37, 41). We suppose that this transcript represents a processing intermediate of trnV(GAC). Alternatively, the transcription initiation site of the trnV gene could coincide with the first nucleotide of the mature tRNAVal(GAC). This second hypothesis is not unlikely, since it has been shown that transcription of the trnV gene is driven by upstream promoter sequences (37, 39).

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Furthermore, RNA polymerase binding experiments have shown that the maize rDNA leader sequence upstream of the trnV gene contains two binding sites (14), and the second one coincided with the transcription initiation site detected in the tobacco sequence. The 5'-leader regions of maize and tobacco rDNA have more than 90% sequence identity back to, but not beyond, a point 73 bp preceding the trnV gene.

We conclude that the embryo-regulated transcript observed is a processing intermediate of trnV(GAC). Three putative transcription start sites, P1, P2 and P3, located 105, 41, and 16 nucleotides upstream of the trnV gene, respectively, were identified by primer extension. They map in a region of minor
evolutionary conservation, as shown by comparison of the sequence with the maize and the tobacco sequence. The three corresponding transcripts, which are absent in the undifferentiated cellular phase, progressively accumulate during embryogenesis. Since the *E.coli* RNA polymerase recognizes chloroplast promoters in *vitro* (7, 8, 37), we could demonstrate that P1, P2 and P3 are promoters rather than processing signals. Purified *E.coli* RNA polymerase was able to drive transcription from these promoters but at a low rate. The absence of any similarity with the prokaryotic consensus −35 and −10 regions could account for the low transcription rate (43).

Our data suggests that the developmentally regulated expression of the tRNA\(_{\text{Val}}^{\text{GAC}}\) is dependent on the differential activity of upstream promoters. Both transcriptional competence of the template and limiting RNA polymerase levels could play regulatory roles. Subunit composition and site of synthesis of the chloroplast RNA polymerase, however, are still controversial. Different purification procedures led to the hypothesis that chloroplasts of algae and higher plants may contain at least two different RNA polymerase activities which are distinguishable by their preference for specific genes and their biochemical properties (44). One type of RNA polymerase synthesizes ribosomal RNA (45, 46, 47) and consists of a single polypeptide chain (48). Subsequently, more complex chloroplast RNA polymerases have been described (49, 50 51), which are able to transcribe tRNA- and mRNA genes (42, 52). Some of the RNA polymerase subunits may be encoded by the chloroplast genome (53, 54). This finding, however, is in contrast to a previous report (5) that all chloroplast RNA polymerase subunits are nuclear encoded. Our finding that transcription of the *trnV* gene is developmentally regulated, allows some considerations of the problem of RNA polymerase. Since the presence of a functional and efficient protein synthesizing machinery is a prerequisite for the expression of chloroplast genes in all developmental stages, it is difficult to believe that (a) chloroplast encoded subunit(s) of RNA polymerase may be involved in starting the transcription of the *trnV* gene. It is still conceivable that a totally entirely nuclear encoded enzyme is responsible for this developmentally regulated transcription. This could provide a mechanism by which the nucleus controls organellar transcription by regulating the expression of a tRNA which is an essential part of the chloroplast protein synthesizing machinery.

It is important to note, that tRNA\(_{\text{Ala}}^{\text{UGC}}\) and tRNA\(_{\text{Leu}}^{\text{GAD}}\) are needed in all cell types, since they are the only or major tRNA isoacceptors in chloroplasts. The tRNA\(_{\text{Val}}^{\text{GAC}}\) isoacceptor, which recognizes the codons GUC and GUU may not be needed in all cells, depending on the codon usage, since another tRNA\(_{\text{Val}}^{\text{GAC}}\) isoacceptor is encoded in the chloroplast genome. Based on sequence homology, G+C-content and codon usage, Shimada and Sugiiura (56) have divided the chloroplast genes in three groups: genetic system genes, photosynthesis genes and NADH-dehydrogenase genes. Based on the data of these authors (56) we have compared the relative frequency of codons recognized by tRNA\(_{\text{Val}}^{\text{GAC}}\) with those specified by the isoacceptor tRNA\(_{\text{Val}}^{\text{UAC}}\) in genes belonging to these three groups in different plant species. In NADH-dehydrogenase genes the ratio between the number of codons recognized by tRNA\(_{\text{Val}}^{\text{GAC}}\) and the number of codons recognized by the isoacceptor tRNA\(_{\text{Val}}^{\text{UAC}}\) is about 1.5. In the other two classes the ratio between the two codons is approximately 1. If tRNA\(_{\text{Val}}^{\text{GAC}}\) is involved in developmental regulation, the expression of a developmentally regulated gene could be modified by its relative content of GAC versus UAC codons.

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