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Update section

Short communication

A divergent plastid genome in *Conopholis americana*, an achlorophyllous parasitic plant

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Abstract

We have used heterologous probes to investigate the degree of sequence conservation in the plastid genome of *Conopholis americana*, a totally achlorophyllous angiosperm which exists as a root parasite on red oaks. Although *Conopholis* is completely nonphotosynthetic, it retains a plastid genome in which certain regions, including that which contains the ribosomal RNA genes, are highly conserved. Other regions, including those containing the genes for numerous photosynthesis proteins, are either absent or highly divergent. We also find that the 16S and 23S ribosomal genes of the *Conopholis* plastid are transcribed and processed, implying a potentially functional genetic apparatus. These results are in agreement with findings reported recently for a related root parasite, *Epifagus virginiana* (dePamphilis and Palmer, 1990). Furthermore, the plastid genome is maintained in high copy number in fruit tissue, whereas mature seeds have an approximately 10-fold lower copy number.

Introduction

Approximately 20% of the 96 identified genes of the plastid genome encode photosynthetic functions [8, 20, 26, 28]. The vast majority of the remaining genes encode products which are directly involved in the maintenance and function of the genetic apparatus itself, including (in most plants) the genes for 3 subunits of chloroplast RNA polymerase, a full complement of rRNA genes, at least 30 tRNA genes, and 19 ribosomal protein genes. Although there are an additional 30 unidentified open reading frames, it is clear that an enormous proportion of the plastid genome is composed of: (1) photosynthesis genes, or (2) the means to express those genes (i.e., transcription and translation functions).

There are, however, certain angiosperms which have abandoned the photosynthetic habit completely, and instead exist as obligate parasites on other plants [14]. One such family of plants is the Orobanchaceae, a family of obligate root parasites which are totally achlorophyllous. It has recently been demonstrated [5] that a member of this family, *Epifagus virginiana*, has a highly reduced plastid genome lacking genes for photosynthesis and chlororespiration. We have used heterologous probes to investigate the plastid genome of another genus of Orobanchaceae, *Conopholis americana*, and find a similarly diver-

gent plastid genome. The data presented here indicate that the plastid genome of *Conopholis* has regions which are either absent or highly divergent. Other regions are highly conserved. In addition, we show that the 16S and 23S rRNA genes are expressed and that the plastid genome copy number is developmentally regulated. Taken together, the results from both *Epifagus* and *Conopholis* raise the question of whether the plastid genome in the Orobanchaceae is simply an evolutionary vestige, or plays an active role in the biology of the plant.

Materials and methods

Plant material

Conopholis americana was collected from a wooded area at the University of Wisconsin-Milwaukee Field Station in Saukville, WI, and from a wooded area in the southern part of Milwaukee County. The plant material was frozen in liquid nitrogen immediately after collection, and was stored at − 80 °C until DNA and RNA extractions were performed.

Nucleic acid isolation

Total cellular DNA was isolated from frozen plant tissue using a cetyltrimethylammonium bromide (CTAB) extraction procedure [19] as modified by Doyle and Doyle [6]. Maize plastid DNA was isolated using the procedure of Kolodner and Tewari [11]. Plasmid DNA was isolated by alkaline lysis [3], followed by CsCl banding in a Beckman VTi 65.2 vertical rotor. The RNA isolation procedure was a modification of the procedure described by Parish and Kirby [24].

Restriction digests

DNA was digested with restriction enzymes purchased from Bethesda Research Laboratories, Gaithersburg, MD. Digests were carried out under conditions recommended by the manufacturer.

Gel electrophoresis

DNA electrophoresis was done in 0.8% agarose gels in TAE buffer (0.04 M Tris-acetate, 2 mM EDTA) plus 1 μg/ml ethidium bromide. RNA electrophoresis was done in 1% agarose gels in 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 0.66 M formaldehyde [4]. To each 20 μl RNA sample 1 μl of a 10 mg/ml solution of ethidium bromide was added prior to loading on the gel [7].

Gel blots

DNA and RNA gels were blotted onto nitrocellulose membranes (BA85, Schleicher & Schuell, Keene, NH) using an LKB 'VacuGene' vacuum blotting apparatus.

Radioactive labelling of DNA

DNA was labelled with ^{32}P by nick translation [25], using reagents purchased from Bethesda Research Laboratories. Unincorporated deoxyribonucleotides were removed from the nick translation mixture using Sephadex G-50 spin columns purchased from 5 Prime-3 Prime, West Chester, PA.

Hybridizations and autoradiography

Nitrocellulose filters were prehybridized and hybridized using standard procedures [17]. Hybridizations were performed in 50% formamide, 5 × SSC, for 16–24 hours at 42 °C. Washes were done in 0.5 × SSC and 0.1% SDS at 25 °C (DNA : DNA hybridizations) or 0.1 × SSC and 0.1% SDS at 65 °C

(DNA : RNA hybridizations). Autoradiography was done at −80 °C, using Kodak XAR-5 film and DuPont Cronex intensifying screens.

Cloning of Conopholis *plastid DNA*

Total cellular DNA was digested with *Bam* HI and electrophoresed in several lanes on a 0.8% agarose gel. Bands enriched in plastid DNA, as judged by ethidium bromide staining and hybridization with heterologous plastid probes, were excised from the gel, electroeluted into dialysis tubing, and cloned into the plasmid vector pGEM3 (Promega, Madison, WI) using standard procedures [1, 17]. Clones of plastid DNA sequences were identified by their ability to hybridize to cloned lettuce plastid DNA fragments.

Results

A clone library of *Sac* I fragments of the lettuce plastid genome [9] was used to compare the degree of sequence conservation in the plastid genome of *Conopholis* with those of two other angiosperms, tobacco and maize. The 15 lettuce *Sac* I fragments, representing approximately 95% of the lettuce plastid genome, are shown on the linear map in Figure 1. The cloned fragments were radioactively labelled and hybridized to *Hin*d III digests of total cellular DNA from *Conopholis*, tobacco, and maize. The hybridizations (Fig. 1), demonstrate that certain regions of the *Conopholis* plastid genome are as highly conserved as the corresponding regions of tobacco and maize plastid DNA. The most conserved regions are those within the inverted repeat. In striking contrast, fragments outside the inverted repeat (notably fragments 6, 8, 10, 11, 12, and 14) showed weak hybridization to *Conopholis* plastid DNA, when compared with the hybridization signals obtained from tobacco and maize. These lettuce clones

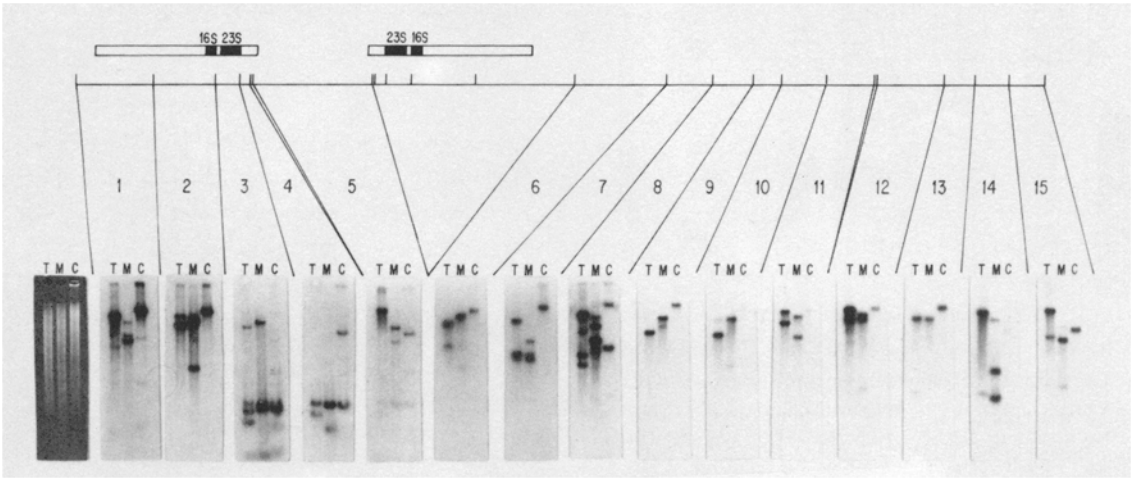

Fig. 1. Hybridization of cloned lettuce chloroplast DNA fragments to total cellular DNA from tobacco, maize, and *Conopholis*. Total cellular DNA from tobacco (T), maize (M), and *Conopholis* (C) was digested with *Hin*d III. The restriction digests were loaded onto a 0.8% agarose gel in 15 sets of 3 lanes, with 2 μg of DNA per lane. After electrophoresis, the gel was blotted onto nitrocellulose, cut into 15 identical sections, and probed with cloned *Sac* I fragments of lettuce chloroplast DNA (gift of Dr R. Jansen). The first panel shows 3 lanes of the ethidium bromide stained gel. The remaining panels show the hybridization with each lettuce *Sac* I fragment, numbered consecutively from 1 to 15. The map (adapted from Jansen and Palmer [9]) shows the position of each *Sac* I fragment on a linearized lettuce chloroplast genome. Also indicated on the map are the positions of the inverted repeats, showing the ribosomal RNA genes.

contain numerous genes encoding photosynthetic functions [9]. Consistent with the findings in *Epifagus* [5], cloned probes for specific photosynthesis genes showed little or no hybridization (data not shown).

The major transcription unit found within the inverted repeat is the rRNA gene cluster [21], which is expressed in *Epifagus* [5]. Since clones from the inverted repeat of the lettuce plastid genome hybridize strongly to *Conopholis* DNA, it was of interest to investigate the structure and potential expression of plastid rRNA genes in *Conopholis*. A 7.2 kb *Bam* HI fragment of *Conopholis* plastid DNA which hybridizes to lettuce plastid rRNA genes was cloned into the plasmid vector pGEM3. The cloned fragment was mapped and subcloned, and the subclones were labelled with ^{32}P and used to probe gel blots of *Conopholis* total cellular RNA isolated from fruit tissue. The results, shown in Fig. 2, indicate expression of both 16S and 23S genes. Three of the six subclones hybridized to 16S rRNA, while four of them hybridized to 23S rRNA, along with specific additional fragments. The multiple fragments hybridizing to the 23S probe are typical of plastid 23S rRNA, which has 'hidden breaks' [13, 15]. At the hybridization and wash stringencies used (hybridization at 42 °C in 50% formamide and 5× SSC and wash at 65 °C in 0.1× SSC), no cross-hybridization with nuclear or mitochondrial rRNAs was detected.

The *Conopholis* DNA used in the experiment shown in fig. 1 was isolated from fruit tissue. Restriction digests of total cellular DNA from *Conopholis* fruits reveal visible plastid DNA bands superimposed on the nuclear smear, indicating a high plastid DNA copy number. This finding was surprising, because such abundant plastid DNA is more typical of photosynthetic leaf cells. In order to investigate whether plastid DNA copy number is developmentally regulated in *Conopholis*, total cellular DNA was isolated from a different developmental stage, the mature seed. Equal amounts (2 μg) of fruit and seed DNA were digested with *Hind* III (Fig. 3A, lanes 1 and 2) and *Xba* I (Fig. 3A, lanes 3 and 4), electrophoresed on 0.8% agarose, blotted onto nitrocellulose, and probed with radioactively labelled maize plastid DNA (Fig. 3B). The results

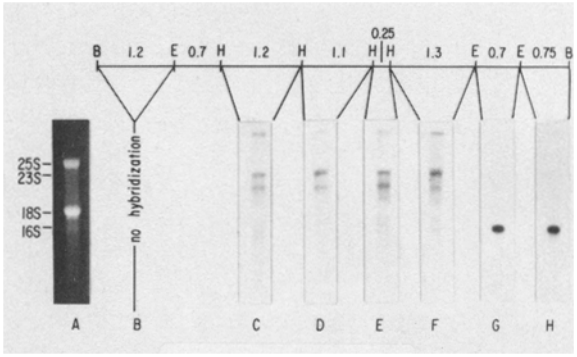

Fig. 2. Hybridization of cloned fragments of *Conopholis* plastid DNA to gel blots of *Conopholis* total cellular RNA. Shown is a restriction map of the 7.2 kb *Bam* HI fragment which hybridizes to the inverted repeat of lettuce chloroplast DNA (B, *Bam* HI; E, *Eco* RI; H, *Hind* III). Below the map are the ethidium bromide-stained RNA gel (panel A) and the results of hybridizations of seven subcloned fragments of the original 7.2 kb fragment (panels B through H). Total cellular RNA was isolated from *Conopholis* fruit tissue, fractionated on a 1% agarose/formaldehyde gel, and blotted onto nitrocellulose. Hybridizations were done in 5× SSC and 50% formamide at 42 °C, and posthybridization washes were done in 0.1× SSC at 65 °C.

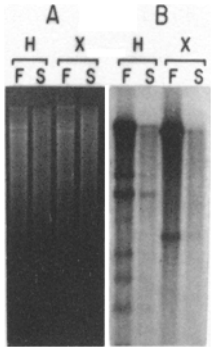

Fig. 3. Hybridization of total cellular DNA from fruits (F) and seeds (S) of *Conopholis* with maize chloroplast DNA. Two micrograms of total cellular DNA from *Conopholis* fruits and seeds were digested with *Hind* III (H) and *Xba* I (X), fractionated on a 0.8% agarose gel, blotted onto nitrocellulose, and probed with maize chloroplast DNA. Panel A: ethidium bromide stained gel. Panel B: autoradiograph of the hybridized filter.

demonstrate that plastid DNA constitutes a significantly higher proportion of the total cellular DNA in fruits than in seeds. Densitometry scans (not shown) indicate an approximately 10-fold greater concentration of plastid DNA in fruit tissue, as compared to seeds.

Discussion

Although the plastid genomes of green plants vary in size from approximately 120 kb [12, 23] to over 200 kb [22], they have a remarkably constant gene content (to within $< 1\%$) and gene arrangement [8, 20, 26, 28]. Heterotrophic plants, however, have been exposed to a rather different set of selective constraints in the recent evolutionary past than green plants. One would predict that a plant which has lost its dependence on photosynthesis might experience the subsequent loss of functional genes encoding photosynthesis proteins. The mapping of the *Epifagus* genome [5] confirmed this hypothesis, and the findings presented here in *Conopholis* imply that plastid genome reduction is a general feature in the Orobanchaceae.

As is the case in *Epifagus*, plastid 16S and 23S rRNA genes are transcribed in *Conopholis*. Although rigorous demonstration of their participation in protein synthesis awaits further experimentation, the presence of the 16S and 23S rRNAs demonstrates that: (1) the plastids have an intact transcription apparatus, (2) the rRNA gene cluster has an intact promoter, and (3) the sequence of the primary transcript allows for correct processing. These facts argue that some plastid genetic activity has been preserved in the Orobachaceae through evolutionary time, even though photosynthesis is no longer one of the plastid's activities. Interestingly, the plastid genome of *Epifagus* lacks 3 of the 4 RNA polymerase genes found in most plastid genomes, demonstrating that the missing genes are not required for transcription of the rRNA gene cluster.

We have considered the possibility that the hybridization of lettuce plastid DNA that we see in *Conopholis* total cellular DNA represents a transfer of plastid DNA sequences to the *Conopholis* nucleus. There is ample evidence of recent intercompartmental nucleic acid traffic in the plant cell [2, 16, 27, 29], and it is an essential feature of the endosymbiotic theory of plastid evolution [18]. Furthermore, in *Epifagus*, the *psaA* gene can be detected, but it does not map to the plastid genome, indicating that it now resides elsewhere in the cell [5]. However, the change in the copy number of the hybridizing sequences that we detect in different developmental stages of *Conopholis* argues that those sequences occupy a cellular compartment separate from the nucleus. Although our experiments do not distinguish between a change in genome copy number per plastid and a change in plastid number per nucleus, it is clear that the number of plastid genome copies per cell differs in fruits and mature seeds. This does not rule out the possibility that certain sequences not detected in our experiments may have been translocated to other compartments.

Although the plastid evolved as a photosynthetic organelle, it has numerous other functions which are essential to the plant, including fatty acid synthesis, starch synthesis, nitrite reduction, amino acid metabolism, and sulphate reduction [10]. Therefore, it is not surprising that nonphotosynthetic plants have retained plastids. However, although these other plastid functions are necessary for the survival of the plant, there are as yet no specific plastid-encoded structural genes which have been identified as being essential to these processes. It is not obvious, therefore, why these nonphotosynthetic plants should retain a plastid *genome*. It is clear that in the Orobanchaceae, the plastid genome has been retained and it is expressed. In *Conopholis*, furthermore, the plastid genome is maintained in high copy number in fruit tissue, as compared with seeds. It will be extremely interesting to find out what functions, if any, are served by the plastid genomes of achlorophyllous plants.

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