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Extensive mitochondrial specific transcription of the *Brassica campestris* mitochondrial genome

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ABSTRACT

We constructed a complete transcriptional map of the 218 kb Brassica campestris (turnip) mitochondrial genome. Twenty-four abundant and positionally distinct transcripts larger than 500 nucleotides were identified by Northern analyses. Approximately 30% (61 kb) of the genome is highly transcribed. In addition, a number of less abundant transcripts, many of which overlap with each other and with the major transcripts, were also detected. If each abundant transcript represents a distinct rRNA or protein species, then plant mitochondria would appear to encode a significantly larger number of proteins than do animal mitochondria. Although B. campestris mitochondrial DNA contains a number of chloroplast DNA-derived sequences, none of these chloroplast sequences appear to be transcribed within the mitochondrion. We determined the positions of 12 genes in the B. campestris mitochondrial genome. The order of these genes in B. campestris is completely different than in maize (1) and spinach (2).

INTRODUCTION

Plant mitochondrial genomes are considerably larger and more complex than those of animals and fungi. Animal mitochondrial genomes exhibit the greatest conservation of organization and size (16-23 kb; refs. 3,4). While fungal mitochondrial genomes are less conserved in size (19-176 kb; refs. 5,6), the largest fungal mitochondrial genome is smaller than the smallest flowering plant mitochondrial genome (218-2400 kb; refs. 7,8). Analysis of flowering plant mitochondrial DNAs (mtDNAs) by restriction mapping indicates that the genomes are composed primarily of unique sequences carried on a collection of different-sized circles that interconvert via recombination between pairs of direct repeats (2,7,9-13). The organization of these genomes is highly variable, even among closely related

species (7,12,13). In contrast to their variability in size and organization, plant mtDNAs are rather uniform in base composition (46-51% GC; ref. 14). Thus, the extra DNA found in plant mitochondrial genomes does not consist of large AT rich regions, as is the case in fungal mtDNA (5).

Does the large size of flowering plant mtDNAs reflect an increased coding capacity compared to animals and fungi? Isolated plant mitochondria synthesize at least 18-20 polypeptides (15), in contrast to the 13 protein coding regions found in animal mtDNA (16). Furthermore, plant mitochondria are known to contain at least four genes not found in animal mtDNA. Two subunits of the ATPase complex, subunit 9 of F_OATPase (17-19) and the alpha subunit of F_1ATPase (20-22), are encoded in the nucleus of animals but in the mitochondria of plants. Plant mtDNA also encodes a ribosomal protein (17) and a 5S rRNA (23) not encoded by animal mtDNA. Of these plant-specific mitochondrial genes, only atp9 is found in fungal mtDNA.

Species in the genus Brassica offer perhaps the simplest system in which to examine the coding capacity of a flowering plant mitochondrial genome. To date, the 218 kb B. campestris mitochondrial genome is the smallest plant mitochondrial genome examined in detail. It exists as three interconverting circles, a 218 kb master chromosome and two subgenomic circles of 135 and 83 kb (7).

In this report we construct a detailed transcriptional map of the B. campestris mitochondrial genome in an effort to identify actively transcribed regions and to make the first measurement of the coding capacity of a plant mitochondrial genome. In addition, we map several known mitochondrial genes on the B. campestris mitochondrial genome and identify their transcripts.

MATERIALS AND METHODS

MtDNA was isolated from six week old green leaves of turnip (B. campestris cv. Purple Top White Globe) by the DNAase I procedure (24). MtRNA was isolated from six week old green leaves and storage roots (i.e. turnips) using the procedure of Stern and Newton (25), except that mitochondria were isolated

from the 20%/30% interface of 20%/30%/52% sucrose step gradients. Chloroplast RNA (cpRNA) was isolated from chloroplasts purified on 30%/52% sucrose step gradients under similar conditions.

RNA (5 micrograms) was electrophoresed in a 1% agarose gel containing 37% formaldehyde, 20 mM MOPS pH 7.0, 5mM NaOAc, 1mM EDTA and transferred to Zetabind filters (AMF Cuono) in 20 x SSC. Hybridizations were conducted at 60°C for 18 hrs using $7x10^4$ CPM of nick-translated probe/ml hybridization solution (1M NaCl, 1% SDS, 5% dextran sulfate). Filters were washed in 2 x SSC (0.3 M NaCl/30mM trisodium citrate) and 0.5% SDS at 60° prior to fluorography for 48 hrs. Chloroplast and cytosolic ribosomal RNAs and HaeIII fragments of phage Øx174 were used as size standards. Filters were stripped prior to rehybridization with five changes of a boiling solution of 0.01% SDS in 0.01 x SSC.

Restriction endonuclease digestions, agarose gel electrophoresis of DNA fragments, bidirectional transfers of DNA fragments from agarose gels to Zetabind filters, labeling of recombinant plasmids by nick-translation, and filter hybridizations were performed as described (26,27). Clones used in heterologous filter hybridizations are listed in Table 1.

RESULTS

Gene Mapping

Gene mapping experiments were performed to facilitate identification of actively transcribed regions of the B. campestris mitochondrial genome. Maize, Oenothera, tobacco and watermelon mitochondrial gene probes (Table 1) were hybridized to filter blots containing PstI, SalI, KpnI and BglI restriction fragments of B. campestris mtDNA. In this manner, the position of nine protein coding genes (atpA, atp6, atp9, cob, coxI, coxII, coxIII, ndh1 and rps13) as well as the 26S and 18S + 5S rRNA genes was determined. The relative map positions of these genes on the three interconverting circles of the genome are shown in Fig. 1, while Fig. 2B indicates their positions with respect to a linear map of the master circle. The orientation of three of the 12 mapped genes was established. Two genes, cob

TABLE 1 GENE PROBES

Gene	Protein	Species	Fragment	Reference
atpA	(a)	Maize	4.2 kb HindIII	(20)
atp6	(b)	Maize	2.7 kb HindIII	(28)
atp9	(c)	Maize	2.2 kb XbaI	(18)
coxI	(d)	Oenothera	2.9 kb EcoRI/PstI	
coxII	(e)	Maize	2.8 kb HindIII	(29)
coxIII	(f)	Oenothera	1.1 kb EcoRI/PstI	(31)
5' cob	(g)	Oenothera	0.8 kb EcoRI/HindIII	(31)
3' cob	(g)	Oenothera	1.5 kb EcoRI/HindIII	(31)
18S-5SrRNA		Maize	6.0 kb BamHI	(32)
26S rRNA		Maize	14 kb BamHI	(33)
5' ndh1	(h)	Watermelon	726 bp BamHI/HindIII	(34)
3' ndh1	(h)	Watermelon	2.3 kb XhoI	(34)
rbcL	(i)	Pea	1.2 kb PstI/HindIII	(35)
rps13	(j)	Tobacco	1.3 kb PstI/BamHI	(17)

(a) Alpha subunit of F_1 ATPase Complex
(b) Subunit 6 of F_0 ATPase Complex
(c) Subunit 9 of F_0 ATPase Complex
(d) Cytochrome Oxidase Subunit I
(e) Cytochrome Oxidase Subunit II
(f) Cytochrome Oxidase Subunit III
(g) Apocytochrome b of Subunit II
(h) Subunit 1 of NADH Dehydrogenase Complex I
(i) Large Subunit of Ribulose-1,5-bisphosphate carboxylase
(j) Ribosomal Protein S13

and ndh1, were oriented using 5' and 3' gene probes in conjunction with detailed restriction mapping. The orientation of the Brassica 26S rRNA gene was inferred based on restriction sites shared with two 26S genes of known orientation, from maize (33) and Oenothera (36).

Transcription of cpDNA Sequences Present in mtDNA

Northern analyses were performed to 1) locate transcriptionally active sequences in the B. campestris mitochondrial genome and 2) measure its overall coding capacity. Clones covering greater than 95% of the B. campestris mitochondrial genome were used to probe Northern blots of mtRNA isolated from

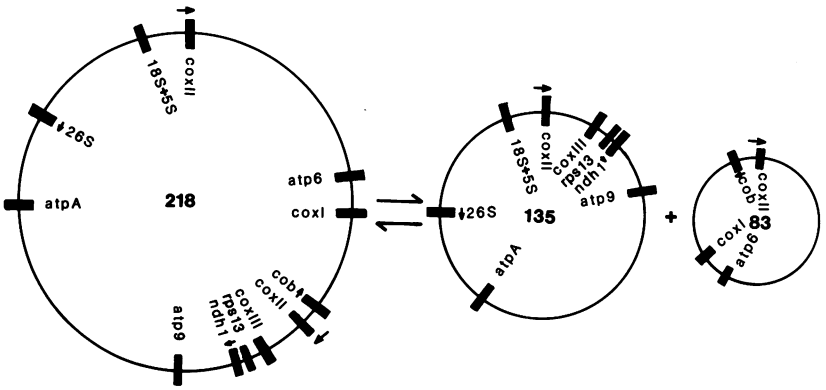


FIG. 1. Gene organization of the B. campestris mitochondrial genome. Solid boxes indicate the position of mapped mitochondrial genes. Inner arrows represent gene orientation, where known. Outer arrows indicate the position and orientation of two copies of a 2 kb repeat. Recombination between these repeats interconverts the largest circle with the two smaller ones (7).

leaves of light grown plants (Fig. 2A). Transcripts below 300 nucleotides (NT) in size were not examined, therefore the positions of the tRNA genes are not shown. A large number of transcripts were detected, revealing a complex transcriptional pattern (Fig. 2A).

Plant mtDNAs in general (2,37-39) and Brassica genomes in particular (40, J. Nugent and J. Palmer, unpublished data) are known to contain a significant number of sequences that are derived from the chloroplast genome. A recent report claims that these cpDNA sequences are transcribed at high levels within the mitochondrion (41). It is therefore important to assess whether any of the transcripts shown in Fig. 2A are derived from these chloroplast sequences and, if so, to distinguish whether such transcripts originate from the chloroplast or mitochondrial copy of the interorganellar duplication element. To this end, clones containing B. campestris cpDNA fragments identified as having homology to B. campestris mtDNA were used to probe Northern blots of B. campestris mtRNA. The two transcripts denoted with a "c" in Fig. 2A were the only transcripts detected with both cpDNA and mtDNA clones.

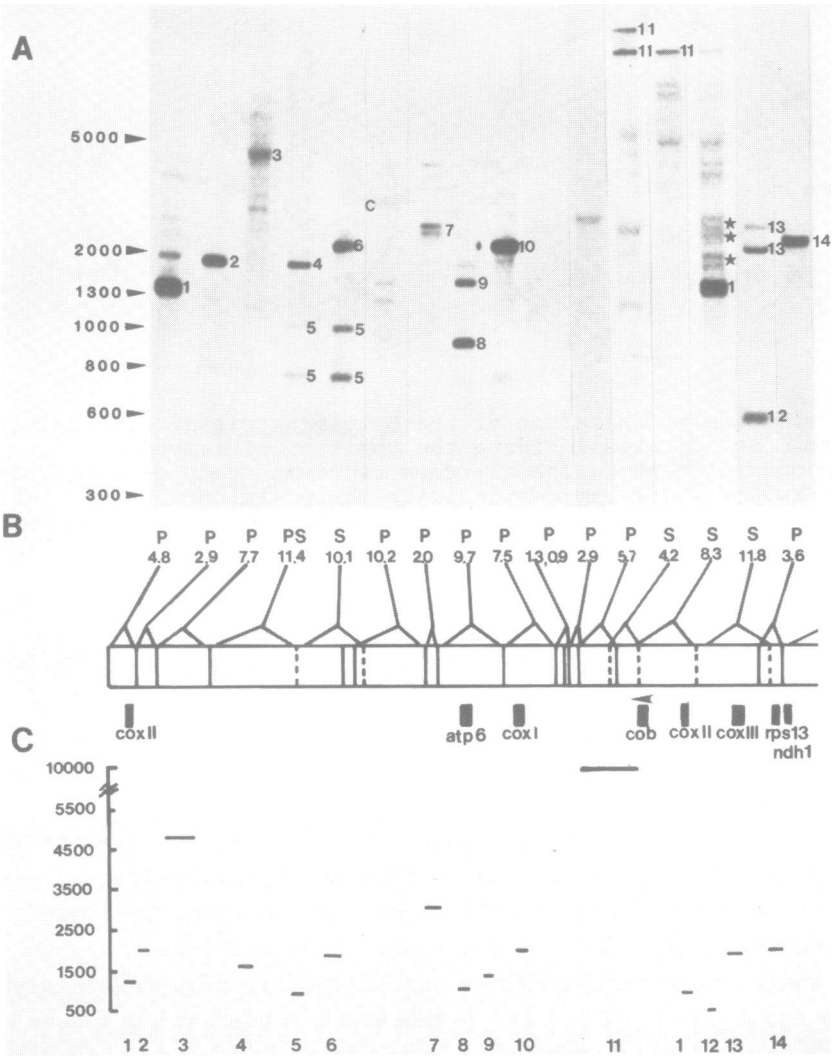
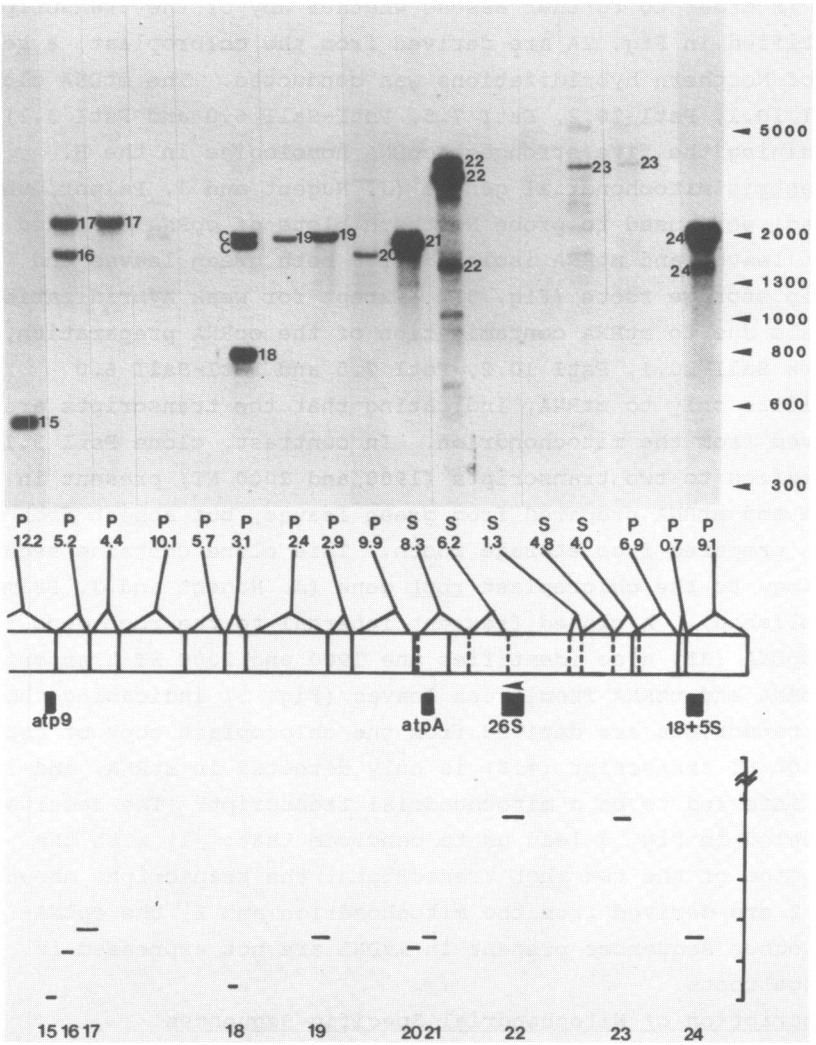


FIG. 2. Northern analysis of the B. campestris mitochondrial genome. A: Mitochondrial RNA (5 ug) from six week old light grown B. campestris seedlings was electrophoresed on 1% agarose/formaldehyde gels, transferred to Zetabind and probed with clones covering greater than 95% of the B. campestris mitochondrial genome. Chloroplast transcripts are identified by a "c". Abundant transcripts are numbered from left to right; cob transcripts are identified with stars. Size scales are in nucleotides. B: Restriction site and gene map of the master chromosome of B. campestris mtDNA, showing the clones used in the hybridization analysis. The complete map for PstI (P; sites depicted by solid vertical lines) is shown while only selected



SalI sites (S; dashed vertical lines) are indicated. The sizes of the clones are shown in kb. The positions of mitochondrial genes determined by Southern analysis are shown by solid boxes. The direction of transcription is denoted by an arrow where determined. C: Mitochondrial transcription map. Size scale in nucleotides is shown to the left. Abundant mitochondrially-derived RNA species only are shown in this diagram. Where two or more abundant transcripts were found to overlap by Northern analysis (Fig. 2A), only the most abundant is shown here. Lines only approximate transcript size. All abundant transcripts, except for numbers 3,4 and 18, were precisely positioned relative to internal restriction sites.

In order to further assess whether any of the transcripts identified in Fig. 2A are derived from the chloroplast, a second set of Northern hybridizations was conducted. The mtDNA clones (SalI 10.1, PstI 10.2, PstI 7.5, PstI-SalI 6.0 and PstI 3.1) containing the five strongest cpDNA homologies in the B. campestris mitochondrial genome (J. Nugent and J. Palmer, unpublished) were used to probe Northern blots of cpRNA isolated from green leaves and mtRNA isolated from both green leaves and turnip storage roots (Fig. 3). Except for weak hybridization signals due to mtRNA contamination of the cpRNA preparation, clones SalI 10.1, PstI 10.2, PstI 7.5 and PstI-SalI 6.0 hybridize only to mtRNA, indicating that the transcripts are derived from the mitochondrion. In contrast, clone PstI 3.1 hybridizes to two transcripts (1900 and 2000 NT) present in cpRNA and mtRNA prepared from green leaves, but absent from mtRNA prepared from storage roots. This clone contains sequence homology to the chloroplast rbcL gene (J. Nugent and J. Palmer, unpublished). A cloned fragment internal to the rbcL gene from pea cpDNA (35) also identifies the 1900 and 2000 NT transcripts in cpRNA and mtRNA from green leaves (Fig. 3) indicating that the transcripts are derived from the chloroplast copy of rbcL. The 800 NT transcript (#18) is only detected in mtRNA, and is thus inferred to be a mitochondrial transcript. The results presented in Fig. 3 lead us to conclude that: 1) with the exception of the two rbcL transcripts, the transcripts shown in Fig. 2 are derived from the mitochondrion and 2) the cpDNA-homologous sequences present in mtDNA are not expressed in storage roots.

Transcription of Mitochondrial Specific Sequences

Once we had accounted for cpRNA contamination we wished to investigate the abundant mitochondrial-specific transcripts. The transcripts shown in Fig. 2A are the result of an initial survey using 34 clones that span the genome. We have arbitrarily divided the transcripts into two groups, major (abundant) and minor. The location of the 24 major transcripts (Fig. 2C), with the exception of transcripts 3, 4 and 18, was determined using overlapping clones and small subclones (data

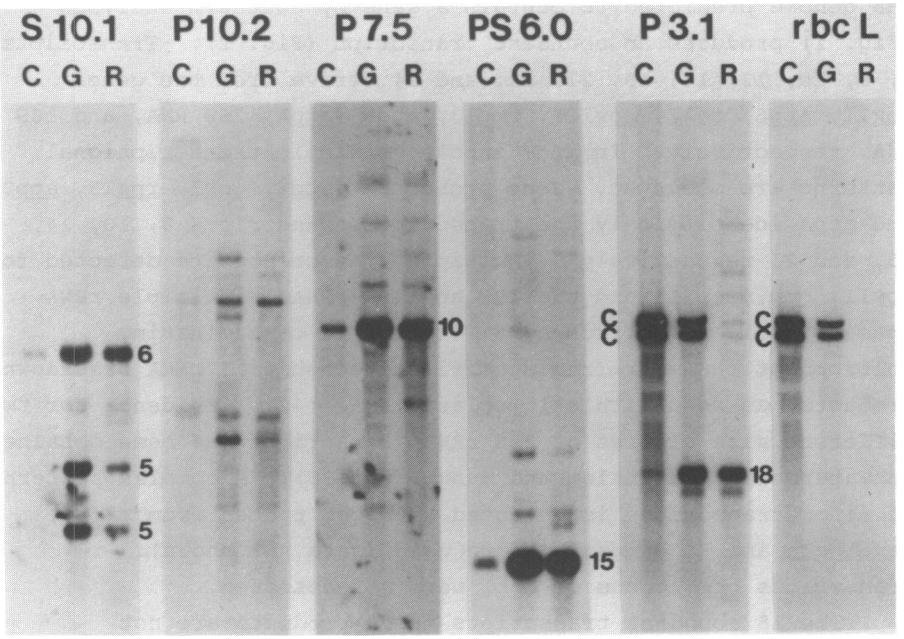


FIG. 3. Analysis of the transcription of cpDNA homologous sequences in the mitochondrion. Five micrograms of cpRNA from green leaves (C) and mtRNA from green leaves (G) and storage roots (R) were electrophoresed on a 1% agarose/formaldehyde gel, transferred to Zetabind and probed as described in Materials and Methods. Five B. campestris clones containing cpDNA homologies (SalI 10.1, PstI 10.2, PstI 7.5, PstI-SalI 6.0 and PstI 3.1) and a clone containing a 1167 bp PstI-HindIII fragment internal to the rbcL gene from pea cpDNA (30) were nick-translated and used successively as probes on two filters. Transcripts are labeled as in Fig. 1A.

not shown). All of these transcripts were positioned relative to internal restriction sites. In this manner, we were able to determine whether multiple transcripts identified by a single clone are positionally distinct. For example, SalI fragment 10.1 identified 3 transcripts, two of which map across the same SalI site (#5) and are positionally distinct from transcript 6 (Fig. 2).

Known mitochondrial genes account for nine of the 24 abundant transcripts. Except for cob and ndh1, each region of

the genome predicted to contain a gene by Southern analysis (Fig. 1) produces an abundant transcript (Fig. 2). Transcripts 1, 8, 10, 13, 14, 15, 21, 22, and 24 derive from the genes coxII, atp6, coxI, coxIII, rps13, atp9, atpA, 26S RNA, and 18S RNA, respectively. In many instances simple transcriptional patterns are observed. Gene probes for atp6, coxI, rps13, atp9 and atpA identify only one transcript (transcripts 8, 10, 14, 15, and 21 respectively). Multiple transcripts are detected for coxII, cob, coxIII and the 18S and 26S rRNAs. Multiple rRNA species could arise from hybridization to contaminating chloroplast rRNAs or from specific mitochondrial rRNA breakdown products, as seen with chloroplast rRNAs (42). Evidence for two different size classes of 26S rRNA transcripts has been obtained in watermelon, muskmelon and mung bean (25). A complex pattern of minor transcripts is detected when cob probes from maize, Oenothera and B. campestris are used (data not shown). Transcripts homologous to ndh1 were not detected.

The 15 abundant transcripts whose products are not identified (#2-7, 9, 11, 12, 16-20, 23) range in size from 500 to 10,000 NT. With the exception of transcript 11, these are in the size range expected for protein coding transcripts. Transcript 11 is noteworthy for a second reason. The total size of the mtDNA clones that hybridize to the largest of the two transcript 11 species is smaller than the transcript itself. The PstI 5.7 kb fragment is the only mtDNA clone that hybridizes strongly to this transcript. This suggests some unusual property of the transcript (e.g., circularity) that may make it migrate anomalously.

The possibility that different mtDNA clones were detecting the same transcript as a result of repeated elements was examined. Southern blots of B. campestris mtDNA were probed with B. campestris mtDNA clones containing the atpA, atp9, coxI and rps13 genes to determine if they contained sequences that are repeated elsewhere on the genome. While some of the clones contain repeated elements, none hybridize to clones that identify unassigned transcripts (data not shown).

DISCUSSION

The transcriptional analysis reported here suggests that the mitochondrial genomes of flowering plants encode a significantly larger number of genes than those of animals. Twenty-four abundant and positionally distinct transcripts have been identified by Northern analysis of B. campestris leaf mtRNA. In contrast, animal mtDNA encodes only 13 polypeptides and two ribosomal RNAs (16). The Brassica mitochondrial genome produces approximately 60 kb of abundant nonoverlapping RNAs whereas the animal genome is only 16-23 kb in total size. Subclones, overlapping fragments and chloroplast DNA clones have been used to determine the position and authenticity of most of the abundant Brassica mtDNA transcripts. The presence of twenty-four abundant mtDNA transcripts is in reasonable agreement with the observation that approximately 20 polypeptides are synthesized in isolated mitochondria. If the abundant transcripts are used as a measure of the B. campestris mitochondrial genome coding capacity, then coding regions represent about 30% of the genome. While plant mitochondrial genomes do encode a significantly higher number of genes than the mitochondrial genomes of animals, their increased size is by no means entirely accounted for by an increased coding capacity.

Simple transcriptional patterns are observed for the protein coding genes atp6, coxI, rps13, atp9, and atpA (transcripts 8, 10, 14, 15 and 21 respectively). The observation that many of the B. campestris mitochondrial genes produce simple transcriptional patterns is in contrast to the more complex patterns observed in other systems. In maize, six of the seven protein-coding genes examined (atp6,28; atp9,18; cob,43; coxI,44; coxII,29; rps13,17) produce multiple transcripts. Only three of the nine protein genes, coxII, coxIII and cob, produce multiple transcripts in B. campestris. The two transcripts (doublet numbered 1 in Fig. 2A) detected by the coxII-containing fragments PstI 4.8 and SalI 8.3 could result if the two copies of the gene are nonidentical. This suggests that the gene is not totally contained within the 2 kb repeat. However, the

possibility that the two transcripts each arise from both copies of the gene can not be excluded. The most complex pattern for an identified gene is observed for cob. At least five RNA species (indicated by stars in Fig. 2A) hybridize to cob probes. In addition, cob RNA appears to be less abundant than transcripts representing other mitochondrial genes.

Transcripts corresponding to ndh1 were not detected, suggesting that it is not expressed in B. campestris mitochondria. The possibility that it is expressed at a much lower level with respect to other mitochondrial genes, or that the message is inherently unstable can not be ruled out. Although ndh1 homologous transcripts were detected in watermelon (34), the proposed structure of the gene has recently been questioned (45). At the present time, sequences homologous to the seven remaining ndh genes present in human mitochondria (46) have not been identified in flowering plant mtDNA, whereas sequences homologous to six of these ndh genes are present in cpDNAs of tobacco (47) and Marchantia (48). One wonders whether the mitochondrial polypeptides might perhaps be synthesized in the chloroplast and exported to the mitochondrion.

Many less abundant, overlapping transcripts were also detected. The nature of the less abundant transcripts has not been investigated; therefore it is not clear if they encode specific gene products or are the result of nonspecific transcription events. While some of these transcripts may result from spurious promoter and termination signals, RNA processing, specific breakdown events, or partial duplication events, the cob transcriptional pattern suggests that some may encode gene products.

RNA excess hybridization results have suggested that a high proportion of the watermelon (70%, 49) and B. napus (100%, 41) mtDNAs are transcribed. Our results indicate that a large percentage of the B. campestris mitochondrial genome is also transcribed, albeit at low levels. They also indicate that a large proportion of the transcripts could arise from noncoding regions of the genome, as has been suggested for the cucurbits (49).

There are numerous examples of chloroplast DNA sequences

present in the mitochondrial genomes of plants (2, 37-39). It has recently been suggested that cpDNA homologous sequences present in the mtDNA of B. napus are transcribed (41). In contrast, our results indicate that the chloroplast sequences present in the mitochondrial genome of B. campestris are not transcribed at detectable levels. Fig. 3 shows that rbcL, a major cpDNA homologous mtDNA sequence, is not expressed in turnip storage roots and its presence in mtRNA from green leaves is most likely due to cpRNA contamination. This illustrates the necessity of assessing whether RNA species hybridizing to mtDNA originate in the mitochondrion or the chloroplast. While we consider it unlikely, we can not rule out the possibility that the mtDNA rbcL sequences are differentially expressed in mitochondria from green leaves and turnip storage roots. Based on our findings, we feel that the results of Carlson et al. (41), who concluded that "chloroplast sequences in mitochondria (of B. napus) transcribe (sic) at levels representative of the mitochondrial genome in general" are artifactual. Perhaps their mitochondria used for in organello RNA synthesis were grossly contaminated with plastids. The effects of such contamination could be exacerbated if the plastids were much more active in the in organello system than the mitochondria.

The 12 genes that have been mapped in B. campestris mtDNA are unlinked and scattered throughout the genome (Fig. 1). They are not clustered into functional units, either on the master chromosome or subgenomic circles. The positions of unassigned abundant transcripts are also consistent with coding regions being randomly scattered throughout the genome. Furthermore, all the genes appear to be independently transcribed. Mitochondrial gene order in B. campestris bears little resemblance to those published for the maize (1) and spinach (2) genomes. In addition, gene order is highly variable among closely related Brassica species as a result of numerous inversions and rearrangements (12, unpublished data). Together, these observations suggest that the order and arrangement of genes in plant mitochondrial genomes is irrelevant to their proper expression and function.

The small, well characterized structure of the B.

campestris mitochondrial genome has allowed us to generate the first global transcriptional map for a plant mitochondrial genome. The results presented here provide insight into the coding capacity and gene organization of a flowering plant mitochondrial genome. The identification of actively transcribed regions provides a basis for further studies concerning the regulation of mitochondrial gene expression during plant development and growth, and on changes in transcriptional patterns that may accompany cytoplasmic male sterility.

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