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Mapping the chloroplast genome of triazine resistant canola

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Summary. The chloroplast of a triazine resistant weed biotype of *Brassica campestris* (bird's rape) has been transferred by repeated back-crossing into an agriculturally important strain, 'Tower', of *Brassica napus* to form a triazine resistant cultivar of canola, 'Triton', that is low in both erucic acid and thioglucosinolate. In this report, the *B. campestris* derived chloroplast chromosome of *B. napus* (cv 'Triton') has been cloned into bacterial plasmids and physically mapped for eight restriction enzymes: Apa I, Bam HI, Bgl I, Hind III, Pst I, Pvu II, Sac I and Xho I. The genes for *rRNA, rbcL, cytF, atpA, atpB, atpE, atpH* and the triazine resistance gene, *psbA* were located on the map by heterologous hybridization. The directions of transcription for most of these genes were determined by reverse heterologous hybridization.

Key words: Chloroplast DNA map – *Brassica napus* – Triazine resistance – Canola

Introduction

The 'Triton' cultivar of *Brassica napus* (oilseed rape) is an agriculturally important, triazine resistant strain of canola that is low in both erucic acid and thioglucosinolate. This cultivar was produced by repeated back-crossing of the *B. napus* cultivar 'Tower' onto a triazine resistant biotype of *B. campestris* (bird's rape) with 'Tower' as the recurrent pollen parent (Beversdorf et al. 1980). Thus, 'Triton' effectively contains the nuclear genome of 'Tower' in a *B. campestris* cytoplasm. This has been verified by a study of the restriction enzyme digestion patterns of chloroplast DNA from different members of the *Brassica* complex (Erickson et al. 1983). *B. napus* (n = 19) is thought to be an amphiploid resulting from a cross between *B. oleracea* (n = 9) and *B. campestris* (n = 10) (U 1935). The restriction enzyme digestion patterns of chloroplast DNA from most *B. napus* cultivars were more similar to those of *B. oleracea* than those of *B. campestris,* indicating that B. campestris was probably the pollen donor in the cross that formed the original amphiploids. The restriction enzyme digestion patterns of 'Triton' chloroplast DNA, however, were identical to those of *B. campestris.* Thus, *B. napus* (cv 'Triton') has the chloroplast genome of its triazine resistant ancestor.

The transfer of triazine resistance with the chloroplast of *B. campestris* is consistant with the observation that triazine resistance has been traced to a mutation in the gene for a chloroplast polypeptide of 32,000 d (Steinback et al. 1981; Pfister et al. 1981). This protein, the Q_B protein, binds a plastoquinone molecule which functions as the secondary electron acceptor on the reducing side of the PS II reaction center. Triazine herbicides (as well as several other classes of herbicides) bind to the Q_B protein and thus displace the plastoquinone molecule (Velthuys 1981). In higher plants, herbicide resistance has been traced to a single base pair change in the chloroplast DNA coded *psbA* gene which results in a serine to glycine amino acid substitution (Hirschberg and McIntosh 1983; Hirschberg et al. 1984).

In this communication, we report that the *B. campestris* derived chloroplast chromosome of *B. napus* (cv 'Triton') has been cloned and physically mapped for eight restriction enzymes: Apa I, Bam HI, Hind III, Pst I, Pvu II, Sac I and Xho I. The gene responsible for triazine resistance, *psbA,* has been located and the direc-

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tion of transcription determined. In addition, the genes *atpA, atpB, atpE, atpH, cytF, rbcL* and *rRNA* have been mapped by heterologous hybridization and the direction of transcription for most of these genes determined by reverse heterologous hybridization.

Materials and methods

Chloroplast DNA cloning

Chloroplast DNA was isolated from *B. napus* (cv 'Triton') as described by Erickson et al. (1983) or by a modification of the method of Bookjans et al. (1984). Chloroplast DNA clones banks were constructed by cloning Bam HI or Pst I digested ctDNA fragments into plasmids pCB4 (Gendel et al. 1983) or pBR322 (Bolivar et al. 1977), respectively. Plasmid pCB4 is a shuttle vector capable of replication in both *E. coli* and the cyanobacterium *Anacystis nidulans* R2. Following transformation into *E. coli* strain HB101, recombinant plasmids were screened, identified and isolated according to Ko et al. (1983).

Physical mapping

B. napus ctDNA was digested by Apa I, Bam HI, Bgl I, Eco RI, Hind III, Pst I, Pvu II, Sac I and Xho I according to the supplier's instructions. The digests were electrophoresed on 0.7% agarose submarine gels. DNA fragments were transferred to nitrocellulose filters (S&S, BA85) according to Southern (1985) or by the bidirection modification described by Maniatis et al. (1982). The Bam HI clone bank was employed as the major source of radioactive probes for homologous DNA hybridization. Other probes came from the Pst I clone bank or ctDNA fragments isolated from low melting temperature agarose gels according to Ko et al. (1984). DNA probes were nicktranslated using [α-^{32}P] deoxycytidine 5'-triphosphate (Amersham) and a nick translation kit (Bethesda Research Labs). Hybridization was performed as described by Maniatis et al. (1982).

Gene mapping

The gene probes used in this report are listed in Table 1. Entire recombinant plasmids or restriction fragments isolated from low melting temperature gels were nick translated and hybridized as described above. To determine the direction of gene transcription, the plasmids listed in Table 1 were cleaved with suitable restriction enzymes and, following gel electrophoresis, the resulting fragments were transferred to nitrocellulose filters. The appropriate cloned fragments of *B. napus* ctDNA were then nick translated and hybridized. Alternatively, for the *rbcL* and *psbA* genes, three *Vicia faba* ctDNA clones (Ko et al. 1984), each of which contain only a part of these two genes, were hybridized to restricted 'Triton' ctDNA to determine gene orientation.

Results

Physical mapping

Digestion of 'Triton' ctDNA with restriction enzymes Pst I, Bgl I, Bam HI, Sac I, Apa I, Pvu II, Eco RI, Hind III and Xho I generates the patterns of restriction fragments seen in Fig. 1. The sizes of these fragments were calculated using Hind III digested lambda DNA as markers and are tabulated in Table 2. The size of the 'Triton' ctDNA was estimated by summing these fragment sizes, yielding a genome size of approximately 152 kb. This value is in good agreement with sizes reported for related species (Vedel and Mathieu 1983; Palmer et al. 1983).

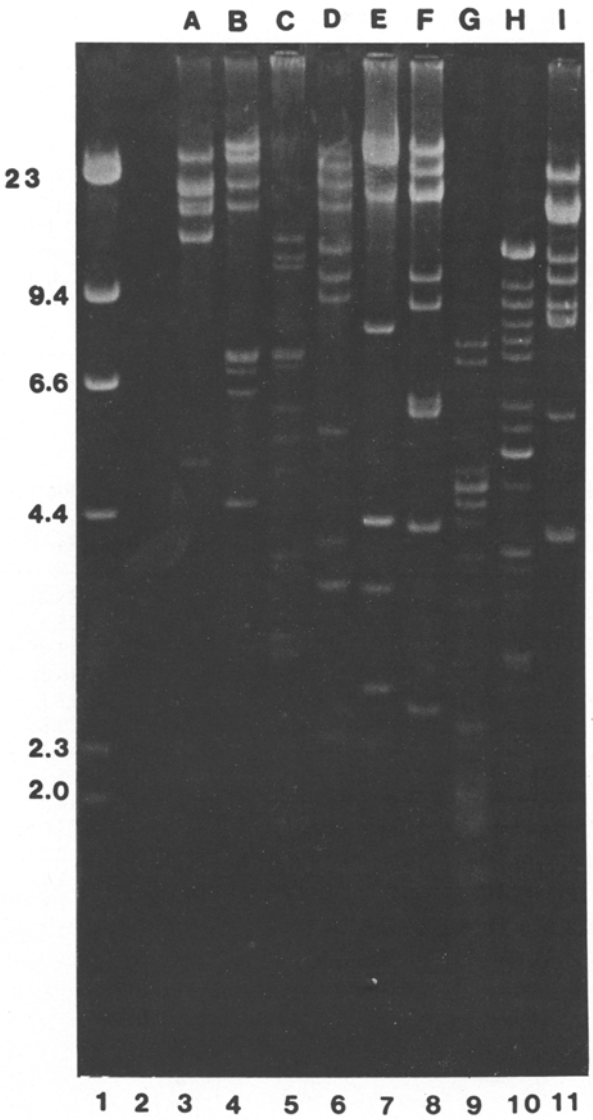

Fig. 1. Gel electrophoresis of restriction endonuclease digests of *Brassica napus* (cv 'Triton') chloroplast DNA. Restriction patterns are shown for Pst I (*lane 3*), Bgl I (*lane 4*), Bam HI (*lane 5*), Sac I (*lane 6*), Apa I (*lane 7*), Pvu II (*lane 8*), Eco RI (*lane 9*), Hind III (*lane 10*), Xho I (*lane 11*) and Hind III digested lambda DNA (*lane 1*). Fragment sizes for the lambda DNA markers are given in kilobase pairs. Each *B. napus* ctDNA digest has been designated by a capital letter that will be used for identification in other figures

Table 1. Heterologous gene probes[a]

	Plasmid	Gene fragment	Gene	Source	Reference
1	pVFP3	15.5 kb Pst I	*rRNA*	broad bean	Ko et al. 1983
2	pMBB	1.83 kb Bgl II	*psbA*	mung bean	Palmer (pers. comm.)
3	pPSP2	3.3 kb Bgl II	*cytF*	pea	Willey et al. 1983
4	pJZA4	2.2 kb Bam-Ava I	*rbcL*	spinach	Erion et al. 1981
5	pSOK6	1.75 kb Kpn-Bgl I	*atpB*	spinach	Westhoff et al. 1981
6	pSOK6	2.6 kb Bgl I-Kpn I	*atpE*	spinach	Westhoff et al. 1981
7	pSOP3	2.4 kb Sal I	*atpA*	spinach	Westhoff et al. 1981
8	pSOP3	1.6 kb EcoR I	*atpH*	spinach	Alt et al. 1983b

[a] Recombinant plasmids were constructed using published data or were generous gifts as indicated by Ko et al. (1984)

Table 2. Restriction enzyme analysis of *B. napus* (cv 'Triton') chloroplast DNA[a]

No.	Pst I	Bgl I	Bam HI	Sac I	Apa I	Pvu II	Hind III	Xho I
1	28.0	42.5	12.8	33.2	37.5	39.0	12.0	23.0
2	19.2 (2)[b]	30.0	11.5	25.2	33.8 (2)	24.0	11.6 (2)	16.2
3	18.0	19.7	10.9	19.9	18.3	17.5	9.8	15.5 (2)
4	15.9	15.8	7.4	16.0	8.1	17.0	8.9	14.8
5	14.8	7.3 (2)	7.3 (2)	12.1	4.4 (2)	10.0	8.2	11.0
6	12.3 (2)	7.2	7.0	9.8	3.5	8.8	7.7	9.8
7	4.9	6.9	6.2	9.1	2.7 (2)	6.2	7.3	8.6
8	2.2	6.3	5.6	5.7	2.4	6.0	6.2	8.5
9	2.0	4.3 (2)	5.2	4.2		5.9	5.8	8.2
10	1.6		4.1 (2)	3.6 (2)		4.3 (2)	5.5 (3)	5.8
11	1.2		3.9	2.2 (2)		2.4 (3)	4.9	4.1
12			3.8	2.1		1.8	4.0 (2)	3.8 (2)
13			3.6	1.8			3.8	1.3
14			3.2 (2)	1.3			3.6	1.2
15			3.0 (3)				3.0	0.7
16			2.7				2.9	
17			2.5				2.7	
18			2.1 (2)				2.2	
19			2.0				2.0	
20			1.9				1.7	
21			1.85 (2)				1.5 (2)	
22			1.6 (3)				1.4 (2)	
23			1.5 (3)				1.3 (2)	
24			1.4 (3)				1.1	
25			1.3 (2)				1.0	
26			1.1 (2)				0.9	
27			1.0 (2)					
	151.6	151.6	153.4	152.0	151.6	152.0	151.8	151.8

[a] Fragment sizes (in kilobase pairs) were determined using Hind III digested lambda DNA as size markers
[b] Nos. in parentheses indicate the number of co-migrating bands

Initially, Bam HI was selected as the primary enzyme for cloning 'Triton' ctDNA. This selection was based on the relatively small size of the largest Bam HI restriction fragments (< 13 kb), which would allow the stable cloning of these fragments in plasmid vectors. As the mapping of the genome proceeded, however, several difficulties arose which necessitated the construction of a second clone bank from Pst I restriction fragments. The primary impetus for this effort was the inability to clone Bam HI fragment 9 (B9). Despite several independent attempts involving four different people and using low melting temperature gel fragment isolation, this fragment could not be cloned, even though nearby fragments (eg. B8 and B10) were recovered during these attempts. It appears that this fragment, which contains the triazine resistant *psbA* gene, is lethal in

E. coli. Three PstI fragments (P4, P6, P8), which span the B9 region of the chromosome, were cloned without difficulty and used as probes.

The map was constructed by determining which fragments from a particular restriction enzyme were adjacent to one another because they reacted to the same Bam HI fragment. This mapping strategy readily gives the positions of the larger fragments for all the restriction enzymes used. However, the clustering of many small Bam HI fragments in certain regions of the genome (P3, P6/P2) creates difficulties in accurately ordering these fragments. This problem was overcome by two methods. Where possible, data from the hybridization of gene probes was used. For example, the positioning of B22, B24, H24 and H26 were facilitated by data from the *atpB, atpE* and *atpA* gene probes. Additionally, double and triple digestions of the Pst I clone bank along with hybridization data using Pst probes were used to confirm the order of the small Bam HI fragments in these regions.

The final map is presented in Fig. 2. As has been found for nearly all higher plant ctDNAs mapped, the chloroplast genome of *B. napus* (cv 'Triton') contains two inverted repeat regions of approximately 24 kb. These repeats separate the remaining single copy portions of the chromosome into two regions of approximately 20 and 84 kb.

Gene mapping

Specific genes were placed on the 'Triton' ctDNA map by heterologous hybridization using the gene probes defined in Table 1. The *cytF* containing 3.3 kb BglII fragment from pea ctDNA bound to Pv1, Pv12, X1 and X4 but not to Bg7 and H10 (Fig. 3 a). From these data, the position of *cytF* can be localized to an area of about 3.5 kb. The *atpB* probe from spinach hybridized with B24, B27, S4 and S14 (Fig. 3 b) and thus has been localized to a 2.1 kb region. The *atpE* probe reacted with B16, B22, B24, S5, S14, H9 and H25 (Fig. 3 c). This gene has been placed on the map within 2.5 kb. Similarly, the *atpA* gene has been localized to a 3 kb area about 6 kb away from the *psbA* gene by the reaction of its probe to H3, H24 and H26 (Fig. 3 d). The 1.6 kb Eco RI fragment of pSOP3 that contains *atpH* bound to Pv5, Pv8 and Pv12 (Fig. 3 e); therefore, *atpH* is situated about 3 kb from *atpA*. Heterologous gene probes for

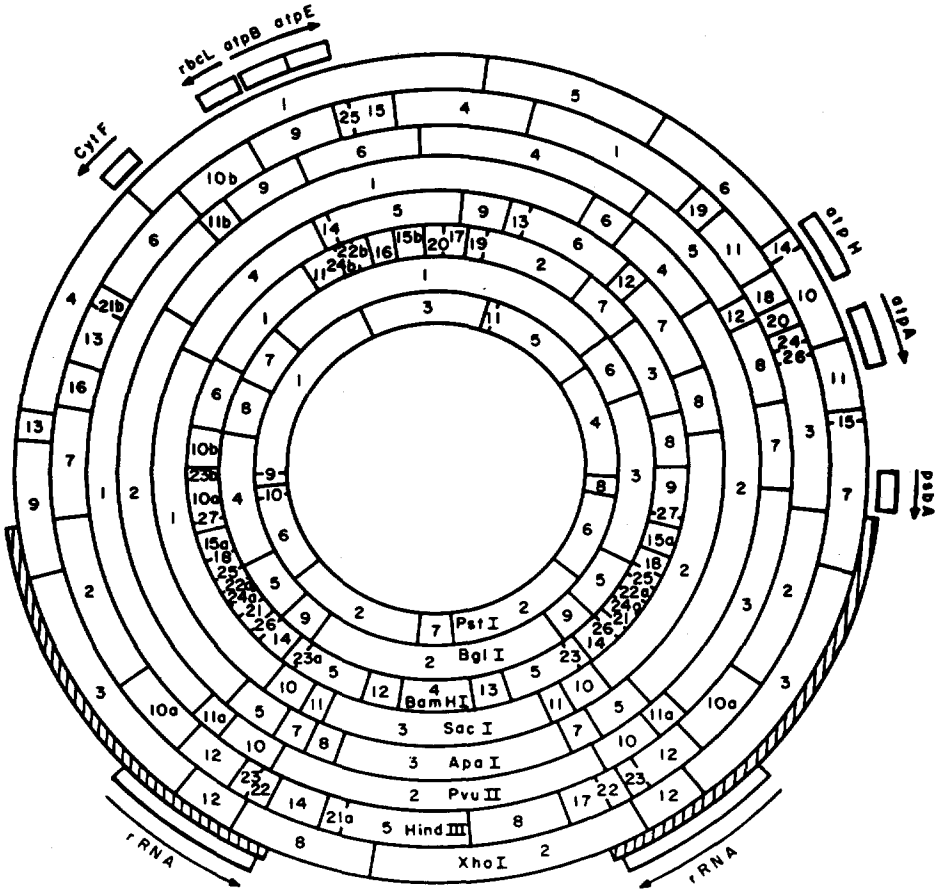

Fig. 2. Physical map of the *Brassica napus* (cv 'Triton') chloroplast chromosome. Restriction sites for PstI, BglI, Bam HI, SacI, ApaI, PvuII, HindIII and XhoI are shown. The positions of the genes for *rRNA, rbcL, cytF, psbA, atpA, atpB, atpE* and *atpH* are indicated and, where known, the direction of transcription is also shown

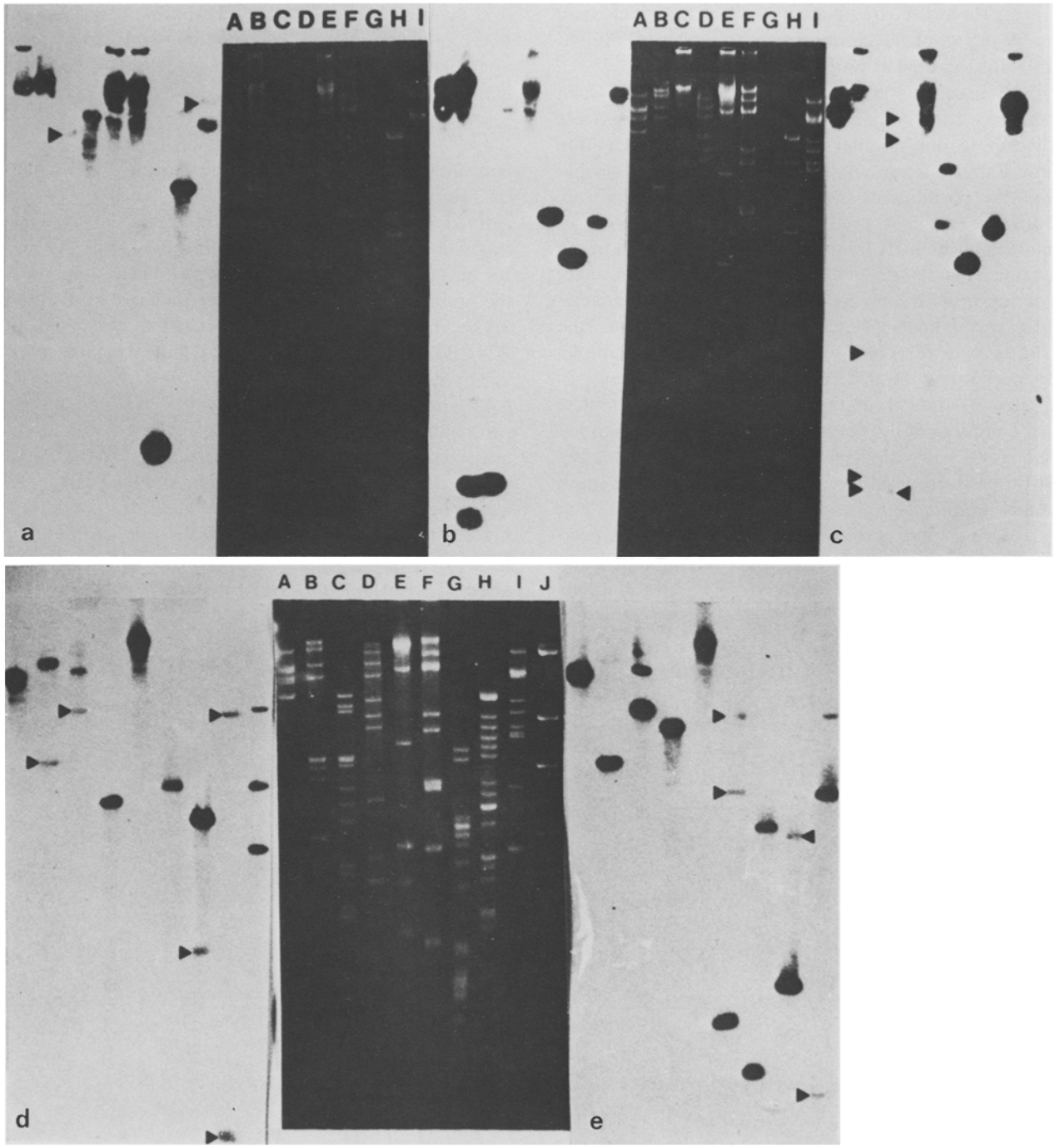

Fig. 3a–e. Heterologous hybridization of various gene probes to *B. napus* (cv 'Triton') chloroplast DNA. Capital letters indicate *B. napus* ctDNA restriction digests as described in Fig. 1. Gene containing fragments (Table 1) were isolated from the appropriate recombinant plasmid for use as probes. Hybridization patterns are shown following hybridization to probes for: **a)** *cytF*, **b)** *atpB*, **c)** *atpE*, **d)** *atpA*, and **e)** *atpH*

psbA, *rbcL* and *rRNA* were also used to locate the positions of these genes on the *B. napus* (cv 'Triton') chloroplast chromosome. The positions of these genes are consistant with the reports of related *Brassica* species (Vedel and Mathieu 1983; Palmer et al. 1983) and are indicated on the *B. napus* ctDNA map (Fig. 2).

Gene orientation

The direction of transcription of a number of genes has been determined by reverse heterologous hybridization. In this technique, nick translated 'Triton' ctDNA fragments are hybridized to restriction enzyme digested

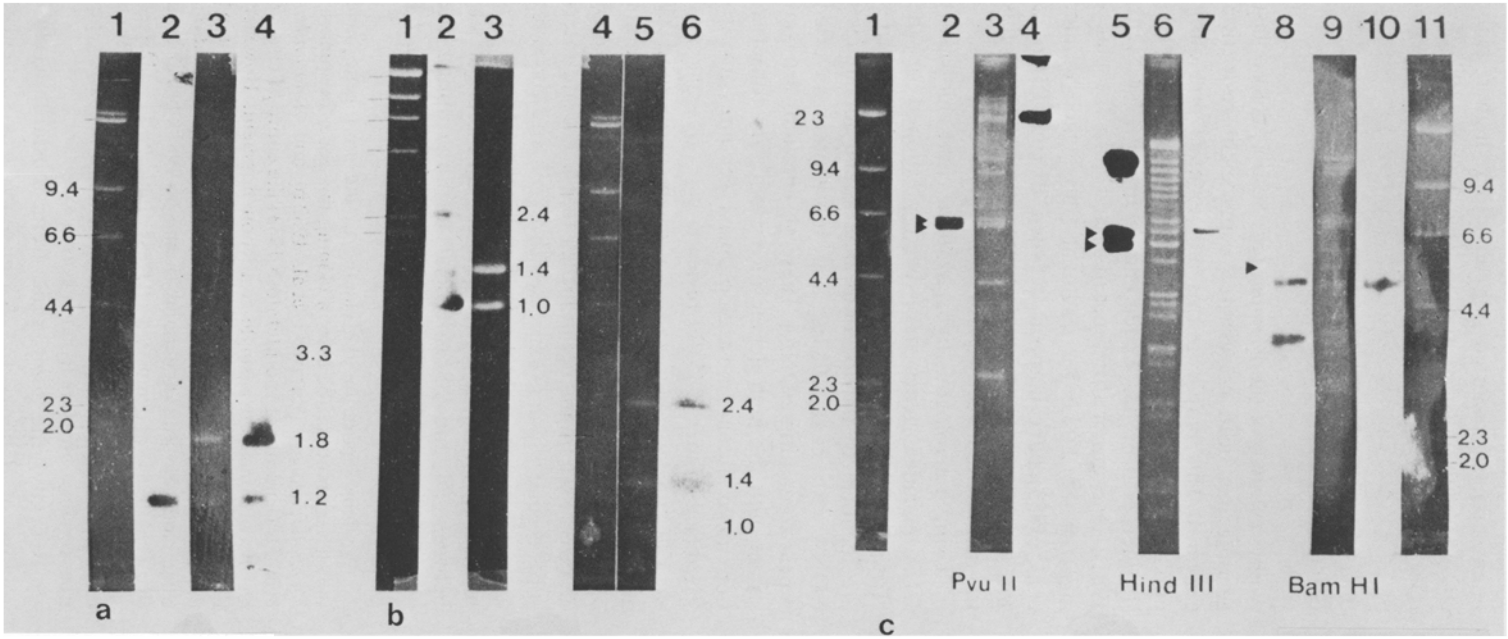

Fig. 4a–c. Heterologous hybridization to determine gene orientation. **a** Orientation of *cytF*. The 3.3 kb Bg1 II fragment from pPSP2 was digested with Bam HI (*lane 3*) and hybridized with X4 (*lane 2*) and X1 (*lane 4*). **b** Orientation of *atpA*. The 2.4 kb Sal I *atpA* containing fragment from pSOP3, digested by Hind III (*lanes 3* and *5*), was hybridized with X11 (*lane 2*) and X10 (*lane 6*). **c** Orientation of *psbA* and *rbsL*. *Lanes 3, 6* and *9* contain *B. napus* ctDNA restriction digests as indicated in each panel. *Lanes 2, 5* and *8* are hybridization patterns obtained with probe pVFP8. *Lanes 4* and *10* show the reactions with pVFP11 while *lane 7* is the result of hybridization to pVFP9. *Lanes a1, b1, b4, c1* and *c11* contain Hind III digested lambda DNA

probe DNA. The procedure can only be used where the cloned gene probe can be assymetrically cleaved by a restriction enzyme into distinct 5′ end and 3′ end fragments. Due to the absence of such a restriction site, the orientation of the *atpH* gene can not be determined in this way.

Xho I digested 'Triton' ctDNA fragments were isolated from low melting temperature gels and nick translated to determine the orientations of *cytF* and *atpA*. Bam HI cleaves the *cytF* containing 3.3 kb Bgl II fragment from pPSP2 into three fragments which can be ordered 5′ end, 1.8 kb, 1.2 kb, 0.3 kb, 3′ end (Willey et al. 1984). Soft gel isolated, nick translated X4 reacted with the 1.2 kb band (the 0.3 kb fragment was run off the end of the gel in this experiment); X1 reacted with both the 1.8 kb and the 1.2 kb fragments, but more strongly with the 1.8 kb band (Fig. 4 a). The direction of transcription of *cytF* thus goes from X1 to X4. Similarly, the *atpA* containing 2.4 kb Sal I fragment from pSOP3 is digested into a 5′ 1.4 kb fragment and a 3′ 1.0 kb fragment by Hind III (Alt et al. 1983 b). X10 hybridizes to only the 1.4 kb fragment while X11 reacts with both fragments but more strongly with the smaller one. Therefore, the direction of *atpA* transcription is from X10 to X11 (Fig. 4 b).

The orientations of *psbA* and *rbcL* were determined by a modification of this technique, using *Vicia faba* ctDNA clones containing incomplete genes as probes. It was reported that the plasmid pVFP8 contains the 5′ end of *psbA* and 3′ end of *rbcL*; pVFP9 contains the 5′ end of *rbcL* and pVFP11 contains the 3′ end of *psbA* (Ko et al. 1984). Nick translated pVFP8 reacts with B9, B11, Pv7, Pv9, H3, H9 and H10; pVFP9 hybridized with H9 while pVFP11 bound to B9 and Pv3 (Fig. 4 c). Of the fragments hybridized by pVFP8, Pv7 and H3 are fragments which contain the *psbA* gene. The hybridization of pVFP8 to Pv7 while pVFP11 reacts with Pv3 indicates that the *Brassica psbA* gene is transcribed in the direction Pv7 to Pv3. Thus, *psbA* is transcribed toward the adjacent inverted repeat region. The hybridization of pVFP8 with H9 and H10 and of pVFP9 with H9 only demonstrate that *rbcL* is transcribed from H9 to H10, the same direction as *cytF*.

The direction of *atpB* and *atpE* transcription can be determined by assuming structural conservation with other reported species. Zurawski et al. (1982 a) and Krebbers et al. (1982) reported that *atpB* and *atpE* are cotranscribed into a dicistronic mRNA with the *atpB* stop codon overlapping the *atpE* start codon. Thus, once the relative location of these two genes has been determined on the ctDNA map, their polarity can be easily determined. In 'Triton' these two genes are located on the opposite strand from the adjacent *rbcL* gene (Fig. 2). The orientation of the *rRNA* genes can also be determined in this way by assuming the same orientation as reported for all other higher plant ctDNAs with inverted repeats (see Palmer 1985 for review).

Discussion

A physical map of the *B. napus* (cv 'Triton') chloroplast genome has been constructed using eight restriction enzymes. This map confirms and extends previously published restriction maps of related *Brassica* species (Palmer et al. 1983; Vedel and Mathieu 1983; Link 1981). One minor difference detected is in the placement of the 2.0 kb Pst fragment (P9), which Palmer et al. (1983) have mapped between P1 and P3 in 13 *Brassica* cultivars while our results indicate that this fragment lies between P1 and P10.

Using heterologous hybridization, eight chloroplast DNA encoded genes have been positioned on the 'Triton' ctDNA map. The positions of all these genes are identical to the locations determined for spinach ctDNA (Alt et al. 1983 a) and most other inverted repeat containing ctDNAs. These observations are consistent with the high degree of evolutionary conservation noted among most chloroplast genomes investigated (see Palmer 1985, for review).

The "uncloneable" nature of fragment B9 (and X7 and H3 as well) was an interesting, though frustrating observation. The fact that this region could be cloned as three Pst I fragments indicates that a Pst site interrupts a gene which is expressed in *E. coli* and codes for a protein lethal to the bacterium. Further cloning and reconstruction experiments indicate that the Pst site between P4 and P8 is crucial for interrupting the putative lethal gene. This site falls within the *psbA* gene.

Previous reports on the cloning of the *psbA* gene have shown variability in the case of cloning this gene. In spinach, *Nicotiana debneyi* (Zurawski et al. 1982 b) and *Amaranthus hybridus* (Hirschberg and McIntosh 1983) the entire *psbA* gene has been cloned on a single restriction fragment. However, Zhu et al. (1982) failed to clone a Bam HI fragment containing the entire *psbA* gene from *Nicotiana tabacum*. In addition, clone banks for pea (Palmer and Thompson 1981), broad bean (Ko et al. 1984) and mustard ctDNAs (Link 1981) contained split *psbA* genes cloned in two restriction fragments. Thus, these reports are consistent with the possibility that *psbA* genes from certain sources are lethal to *E. coli*.

Recently, the *psbA* gene from mustard, a close relative of *B. napus* and *B. campestris* was sequenced (Link and Langridge 1984). This sequence was obtained from two separate Pst clones. While the coding sequence of this gene is nearly identical to that of *N. debneyi* and spinach, there is a fairly large (70 bp) insertion just upstream of the −35 region of the *psbA*

promotor. We are presently investigating whether this region, which shows a high degree of secondary structure, influences the strength of the *psbA* promotor in *E. coli*, resulting in overproduction of this membrane protein and subsequent cell death. The sequence of the *psbA* gene from *B. napus* (cv 'Triton') and its upstream regions are also currently under investigation.

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