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A model for the evolution of the *Vicia faba* chloroplast genome

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Summary. The *Vicia faba* chloroplast genome lacks inverted repeat sequences and contains only one set of ribosomal RNA genes. The genetic organization has been altered by inversions, relative to the typical arrangement of most higher plant chloroplast genomes. The *Vicia faba* plastid genome thus represents one of the more interesting results of chloroplast genomic evolution. The present study employs small DNA probes and Southern blot hybridizations to investigate the steps involved in the evolution of the *Vicia faba* chloroplast genome. The data from heterologous hybridizations between chloroplast DNA of *Brassica napus* (a conserved genome) and of *Vicia faba* led to three observations: 1) The inverted repeat segment closest to the *psbA* gene was deleted prior to the rearrangements. 2) A quarter of the ancestral small single copy region was lost during the deletion. 3) The genetic organization observed in *Vicia faba* resulted from three inversions after the deletion event. Our findings, combined with previous observations, helped devise a stepwise model for the evolution of the *Vicia faba* chloroplast genome. The area of the small single copy region absent from the *Vicia faba* chloroplast chromosome lacks in vivo transcription activity in *Brassica napus*.

Key words: *Vicia faba* – Chloroplast genomic evolution – Rearrangements – Recombination sites – Deletions

Introduction

Chromosomal rearrangements are one of the more interesting phenomena that occur in the organellar genomes during evolution. In plant mitochondrial genomes, site-specific recombinations have resulted in a complex array of rearrangements and a wide spectrum of molecular configurations (Palmer and Shields 1984; Lonsdale et al. 1984). The chloroplast genome, although more stable than the mitochondrial genome in terms of evolution, has accumulated a number of rearrangements that characterize particular plants.

The chloroplast chromosomal arrangement, typified by the spinach plastid genome, is highly conserved among higher plants. The chromosome contains two inverted repeat sequences that separate the small and large single copy region. A majority of the rearrangements found in typical chloroplast chromosomes are simple inversions (see review by Palmer 1985). All of these inversions have been relatively stable and resulted in the reversal of the gene order involved, without altering the remaining chromosomal arrangement. Typical chloroplast genomes also exist as two orientation isomers, probably arising from site-specific recombination within the inverted repeat sequences (Palmer et al. 1984). The circular DNA chromosomes differ only by the directional arrangement of the small single copy sequence relative to the large unique region. Orientation heterogeneity has been demonstrated for *Phaseolus vulgaris* (Palmer 1983), *Cyanophora paradoxa* (Bohnert and Loffelhardt 1982), *Chlamydomonas reinhardii* (Aldrich et al. 1985), *Glycine max* (Palmer et al. 1984) and *Osmunda cinnamonea* (Palmer et al. 1984).

All site-specific recombination activity in the chloroplast has been postulated to occur between two strong recombination sites located in the inverted repeat (Palmer et al. 1984). These sites pair intramolecularly at high frequency. There may also be a number of alternate sites scattered throughout the chromosome which serve as substrates for the site-specific recombination reaction, at considerably reduced frequency. Recombination events occurring at the alternate sites are likely to create both lethal and nonlethal changes to the genome. Rearrangements resulting in stable, nonlethal changes are sustained and may become part of the evolutionary pathway leading to a particular chloroplast genome. These evolutionary rearrangements are detected in comparative studies of chloroplast genome organization (Palmer and Thompson 1982).

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The most prominent rearrangement in chloroplast genomic evolution is the deletion of one entire section of the inverted repeat in a small group of legumes. Genomes of this type include *Vicia faba* (Koller and Delius 1980; Ko et al. 1983, 1984), alfalfa (Palmer et al. 1984) and pea (Palmer and Thompson 1981; Chu et al. 1981). These legumes have also sustained a number of further rearrangements. DNA cross-hybridization studies (Palmer and Thompson 1982; Shinozaki et al. 1984) have demonstrated that the surviving small single copy and inverted repeat regions are divided into two different areas of the *V. faba* chloroplast genome. Since these experiments utilized relatively large DNA probes, the nature of the rearrangements and the location of the recombination sites could not be determined accurately.

In this report, small DNA probes representing the small single copy region and the inverted repeat of *Brassica napus* were used to investigate the events leading to the organization observed in the *V. faba* plastid genome. The small DNA probes permitted the assignment of directions to the regions in question, which were then used as markers to determine the sequential rearrangements. The combination of data from this and other studies permitted the construction of a stepwise model for the evolution of the *V. faba* chloroplast genome.

Materials and methods

Isolation of nucleic acids

Chloroplast DNAs from *Vicia faba* and *Pisum sativum* were isolated as described in Ko et al. (1983). *Brassica napus* (cv. 'Triton') and *Medicago sativa* chloroplast DNAs were prepared by a modification of the method described by Bookjans et al. (1984). After lysis the mixtures were adjusted to a final volume of 6.0 ml with 50 mM Tris-HCl, pH 8.0, 20 mM ethylenediaminetetra-acetic acid (EDTA) and purified by CsCl gradients. The DNA samples were further purified by extraction with phenol and chloroform. – Nuclear DNA was prepared from *Vicia faba* roots according to the procedure of Scott and Timmis (1984). – Mitochondrial DNA was isolated from etiolated *Vicia faba* embryos by a slight modification of the method (Bookjans et al. 1984) described for chloroplast DNA isolation. After pelleting the chloroplasts at 1,500 × g for 5 min, mitochondria were collected from the supernatant by further centrifugation at 10,000 × g for 20 min. The mitochondria pellets were processed in the same fashion as chloroplasts. – Total plant cell RNA was isolated as described by Cashmore (1982) from *Vicia faba* leaves of plants grown for three weeks in the greenhouse.

Construction of recombinant plasmids

The 14.5 and 10.0 kbp Pst I *Vicia faba* chloroplast DNA fragments (designated P4 and P6, respectively) were inserted into the plasmid vector pBR322 (Bolivar et al. 1977) as previously reported (Ko et al. 1983). Subfragments of the cloned P4 and P6 region, generated by the restriction enzymes Pst I, Hind III and Bam HI, were inserted into appropriate sites of pBR322 and pDPL13 (Gendel et al. 1983).

Probes representing different sections of the small single copy region of the *Brassica napus* chloroplast genome were constructed by inserting different DNA fragments into pBR322 or pDPL13. The DNA fragments were generated by the restriction enzymes Pst I, Bam HI, Sac I and Hind III. The recombinant plasmids were transformed into *E. coli* HB101 and isolated as described in Ko et al. (1983).

Blot hybridization analysis

Chloroplast DNA and cloned DNA fragments were digested by various restriction endonucleases according to the suppliers' instructions. DNA fragments were separated in 0.7% horizontally submerged agarose gels for 18–20 h at 1.5 V/cm of gel. The electrophoresis buffer was 80 mM Tris-HCl, pH 8.0, 10 mM sodium acetate, 2 mM EDTA. The gels were stained with ethidium bromide and visualized on an UV transilluminator. DNA fragments were transferred onto nitrocellulose sheets (Schleicher and Schuell-BA85) according to Southern (1975). The filters were hybridized with ^{32}P probes according to Fluhr and Edelman (1981). RNA blot analysis was carried out as described by Thomas (1983). The resulting blots were exposed to Kodak XAR X-ray film with intensifying screens at –70 °C for various durations. All radioactive probes were prepared by nick translation according to Rigby et al. (1977) using a kit purchased from Bethesda Research Laboratories Inc.

Results and discussion

Cross-hybridization analysis of the small single copy region

DNA probes constructed from the small single copy (SSC) region of *Brassica napus*, representing a conserved genome, were used to search for corresponding DNA sequences in the *V. faba* chloroplast genome (Table 1). A physical map of the *B. napus* SSC region

Table 1. Cross-hybridization analysis of the small single copy region

SSC probes Designations	*Vicia faba* chloroplast DNA fragments			
	Pst I	Kpn I	Xho I	Sal I
Bam12	P5	K5, K7a	X1	S1b
Bam4	P4	K3	X4, X9	S1b
Bam13	n.r.	n.r.	n.r.	n.r.
Pst7	P4	K3	X4	S1b
Sac11	P3	K3	X4	S3b
Hind14	P3, P5	K3, K5	X1, X4	S1b, S3b
pBP4	P4	K3	X4	S1b
pBH4	P4	K3	X4	S1b
pBH5	n.r.	n.r.	n.r.	n.r.
pHS17	P3	K3	X4	S3b
pBH10	P3	K2	X10a	S3b
pBS5	P3	K2	K4	S3b

The restriction fragments are designated according to Ko et al. (1983). The location of the probes is illustrated in Fig. 1. The probes designated pBH10 and pBS5 are not depicted in Fig. 1. The plasmid pBH10 was constructed from the DNA region between the Hind III junction site of 23/12 and the Bam HI junction site of 5/23. The plasmid pBS5 was constructed using the DNA fragment between the Sac I junction site of 11/10 and the Bam HI junction site of 5/23 (see Xiao et al. 1986). The abbreviation n.r. means no reaction

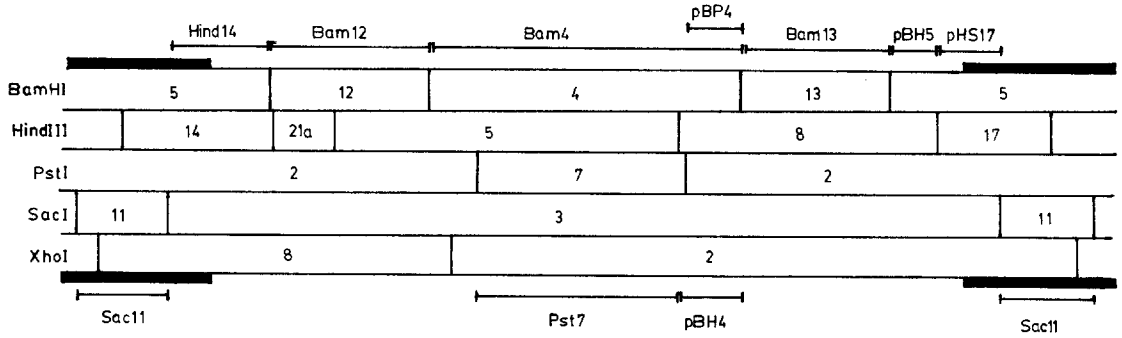

Fig. 1. A physical map of the small single copy region of the *Brassica napus* chloroplast genome. Restriction sites for Bam HI, Hind III, Pst I, Sac I and Xho I are indicated (Palmer et al. 1983; Vedel and Mathieu 1983; Xiao et al. 1986). The restriction fragments are numbered according to Xiao et al. (1986). The sections related to the inverted repeat sequences are represented by the bold area. The extent of the subclones is indicated above and below the map. The DNA probe designations are marked

and the positions of the different DNA probes are represented in Fig. 1. The DNA probes were radiolabelled and hybridized to nitrocellulose blots of separated PstI, KpnI, XhoI and SalI fragments of *V. faba* chloroplast DNA.

The first set of DNA hybridization experiments involved six DNA fragments spanning the SSC region from left to right (Fig. 1). The DNA probes represent the BamHI fragments 4, 12, 13; HindIII fragment 14; PstI fragment 7 and SacI fragment 11 of *B. napus* (Figs. 2, 3; Table 1). The results confirm that the surviving SSC region is divided into two areas of the *V. faba* chloroplast DNA. One section, represented by Hind14 and Bam12 probes, is present in the P5/S1b area of *V. faba*. The Hind14 probe from the extreme left side of the SSC region is composed of DNA sequences originating from within the inverted repeat (IR) boundaries and the SSC region. The DNA segment from the IR hybridizes to P3 near the *V. faba* ribosomal RNA cistron. The adjacent region of the IR represented by the SacI fragment 11 (the Sac11 probe) reacts to the same area of *V. faba*. The DNA segment of the Hind14 probe unique to the SSC region is assumed to be present in the overlapping part of P5 and K5 fragments of *V. faba*. The Bam12 probe, which represents the section adjacent to the Hind14 probe, is homologous to a region transversing the Kpn I junction site of K5 and K7a (Fig. 4).

This set of hybridization data led to several observations. In *V. faba*, the left section of the SSC region is separated from the ribosomal RNA cistron (*rrn*) by approximately 27–30 kbp in the P5 fragment. DNA sequences corresponding to the extreme left side of the SSC region (right half of the Hind14 probe) are closer to the *V. faba rrn* than DNA sequences represented by the Bam12 probe. This configuration allowed us to set an arbitrary direction for the left side of the SSC region. The assigned direction in *B. napus* proceeds from Hind14 to Bam12, which is in the same direction as the left-hand *rrn* of *B. napus* (Fig. 4). In *V. faba*, the same orientation has been maintained relative to the direction of the *V. faba rrn*. With the exception of the separation, DNA sequences representing the left side of the SSC region in *V. faba* is co-linear, relative to the direction of the *rrn*, with *B. napus* (Fig. 4).

The middle of the SSC region is separated from the Hind14/Bam12 section and placed in the P4 area of the *V. faba* plastid DNA. Both the Bam4 and Pst7 probes hybridized to a section transversing the XhoI junction site of fragments X9 and X4 (Figs. 2 and 3; Table 1). The DNA probes from the extreme right end of Bam4, pBP4 and pBH4, are homologous to the X4 region of *V. faba* (Figs. 2 and 3). The results indicate that DNA sequences corresponding to the middle section are located approximately 18 kbp from the Hind14/Bam12 sequences. The reaction of pBP4 and pBH4 to the X4 fragment of *V. faba* indicates that the right side of Bam4 is closer to the *V. faba rrn* than the left side of Bam4. The asymmetric location of the Bam4 probe in *V. faba* provides the opportunity to assign an arbitrary direction to the middle section of the SSC region. The assigned orientation of the Bam4 region in *B. napus* is shown in Fig. 4. The direction of the corresponding DNA sequence in *V. faba* is reversed relative to the orientation of the *V. faba rrn* and the DNA sequences related to the Hind14/Bam12 probes. The direction proceeds from X9 to X4 towards the *V. faba* 23S rRNA genes as illustrated in Fig. 4.

The Bam13 probe from the right side of the SSC region did not hybridize to any *V. faba* chloroplast DNA fragment. The hybridization was repeated four separate times, obtaining the same results. It appears that the entire Bam13 section has been deleted from the *V. faba* chloroplast DNA. To ensure that the probe was representative of the BamHI fragment 13, control hybridizations were performed simultaneously with adjacent lanes of separated XhoI, PstI and SacI *B. napus* chloro-

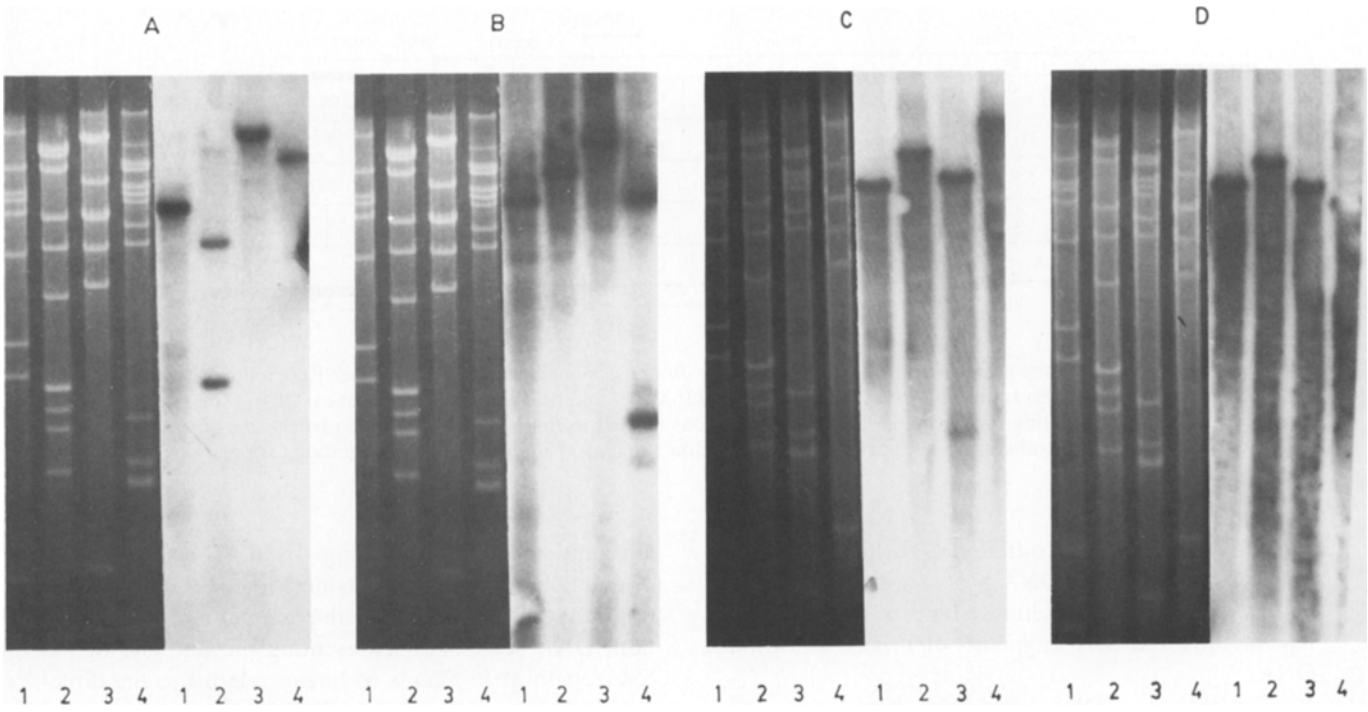

Fig. 2. Hybridization of *Brassica napus* chloroplast DNA probes to filter-bound *Vicia faba* chloroplast DNA. Results are shown for the DNA probes Bam12 (panel *A*), Bam4 (panel *B*), Pst7 (panel *C*), Sac11 (panel *D*). The probes are designated as shown in Fig. 1. The agarose gels are shown on the left and the corresponding autoradiograms are shown on the right of each panel. The order of restriction patterns is Pst I (lane 1), Kpn I (lane 2), Sal I (lane 3) and Xho I (lane 4) for panels *A* and *B*. In panels *C–D*, the order of restriction patterns is Pst I (lane 1), Kpn I (lane 2), Xho I (lane 3) and Sal I (lane 4). The data are summarized in Table 1 and discussed in the text

plast DNA fragments. Reactions occurred with XhoI fragment 2, SacI fragment 3 and the PstI fragment 2 thus confirming that the Bam13 probe originated from the intended position.

The extent of the deleted Bam13 section was determined using adjacent DNA fragments. Two subclones immediately to the left of BamHI fragment 13 were used as DNA probes to set the left limit. The pBP4 and pBH4 probes representing the extreme right side of Bam4 reacted to the P4 region indicating that these sections have been retained in *V. faba* (Fig. 4). At this level of resolution, the left limit of the deleted section is placed at the BamHI junction site at BamHI fragments 4 and 13 of *B. napus*. To determine the extent of loss at the right end of BamHI fragment 13, two DNA probes were constructed from the neighboring right side (see Fig. 1). The pBH5 probe did not react with any *V. faba* chloroplast DNA fragments and pHS17 hybridized to the P3 region (Fig. 3). The positive reaction of the pHS17 probe is attributed to the small segment of the IR contained within this subfragment. At this level of resolution, the right limit of the deleted section is placed at the right SSC-IR boundary. The deleted DNA sequence stretches from the BamHI junction site between 4/13 to the right SSC-IR boundary of *B. napus*, a length of approximately 5,000 base pairs (Fig. 4).

Table 2. Cross-hybridization analysis in the P4 region of the *Vicia faba* chloroplast genome

Probes	*Brassica napus* chloroplast DNA fragments			
	PstI	BamHI	HindIII	XhoI
pVFP4	2, 3, 7	4, 16, 22b, 24	5, 8, 9, 15, 25	1, 2
pVFHind1	2, 3, 7	4, 16, 22b	5, 15, 25	1, 2
pVFHind2	3	16, 22b, 24	9	1
pVFHind3	2	4	8	2
pVFBam3	3	16	5, 15	1

The DNA probes were radiolabelled and hybridized to nitrocellulose blots of separated PstI, BamHI, HindIII and XhoI chloroplast DNA fragments of *Brassica napus*. The location of the DNA probes is illustrated in Fig. 5. The restriction fragments are designated according to Xiao et al. (1986)

The loss of a 5.0 kbp DNA fragment from the right side of the SSC region suggests that the right segment of the inverted repeat region (the side closest to the 32 kd herbicide-binding protein gene [*psbA*]) has been entirely deleted. The deletion encompasses approximately 22.0 kbp of the IR and 5.0 kbp of the SSC region. The deletion of the right IR segment was also proposed from a DNA cross-hybridization study between mung bean and alfalfa (Palmer et al. 1984; Palmer 1985; Palmer, pers. commun.). Further evidence that deletion oc-

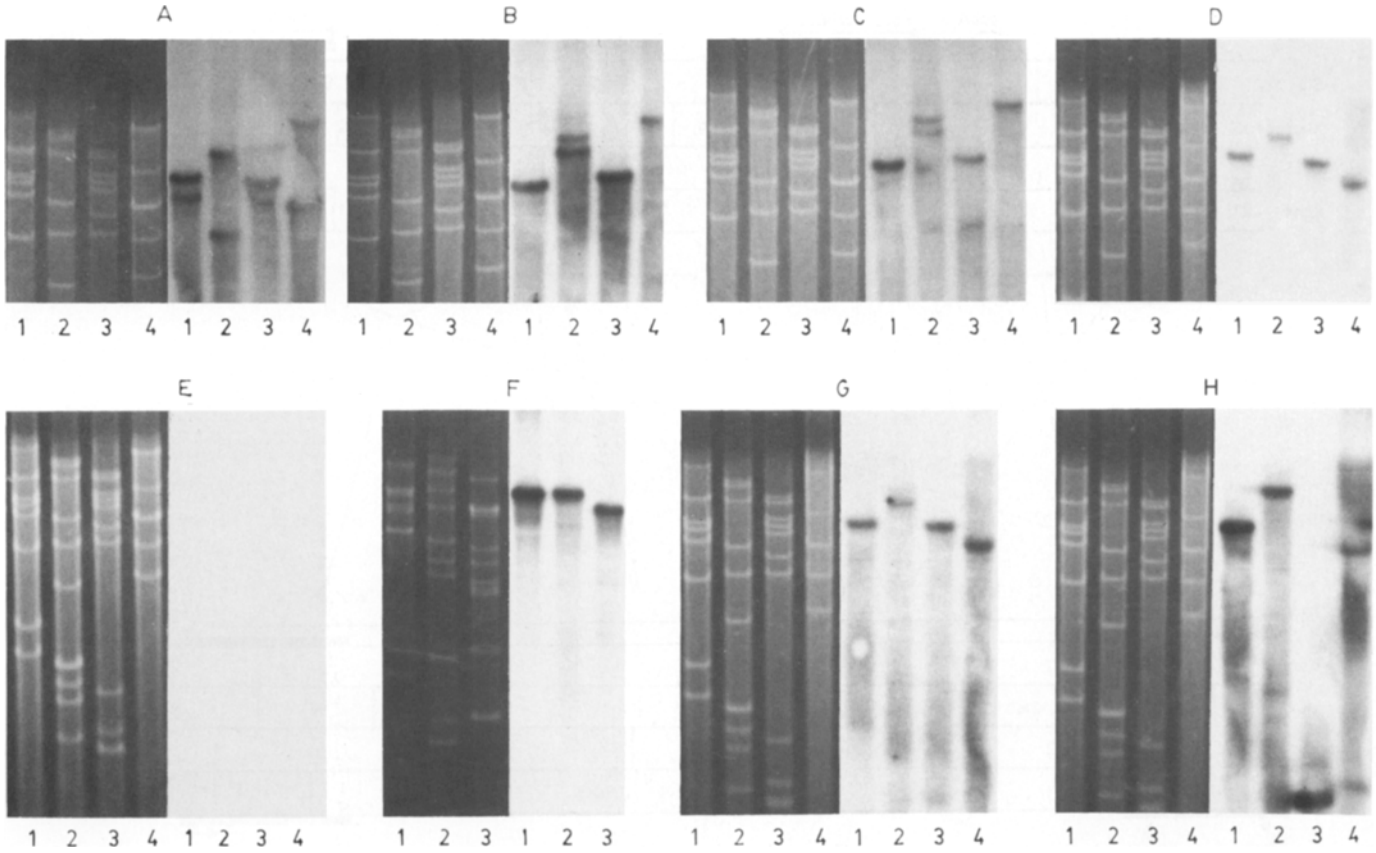

Fig. 3. Hybridization of *Brassica napus* SSC DNA probes to filter-bound *Vicia faba* chloroplast DNA. Results are presented for Hind14 (panel *A*), pBP4 (panel *B*), pBH4 (panel *C*), pHS17 (panel *D*), pBH5 (panels *E–F*), pBS5 (panel *G*) and pBH10 (panel *H*). The agarose gels are shown on the left and the corresponding autoradiograms are shown on the right side of each panel. The order of restriction patterns is Pst I (lane 1), Kpn I (lane 2), Xho I (lane 3) and Sal I (lane 4) for all panels except F. In the control experiment (panel F) *Brassica napus* chloroplast DNA was digested with Pst I (lane 1), Sac I (lane 2) and Xho I (lane 3). The data are summarized in Table 1 and discussed in the text

curred with the right IR segment can be seen in tRNA mapping studies. The gene for the $tRNA^{Leu}_{UAG}$ has been located towards one side of the SSC region in maize (Selden et al. 1983), *Phaseolus vulgaris* (Mubumbila et al. 1983) and tobacco (Bergmann et al. 1984; Sugiura, pers. commun.). This tRNA gene, which represents the opposite side to the deleted section (the left side), is present in the K7a fragment of *V. faba* (Mubumbila et al. 1984). The loss of the 5.0 kbp section also correlates with the molecular size difference observed between the chloroplast genomes of *V. faba* and *B. napus*. The *B. napus* genome is approximately 150,000 base pairs in total length. The deletion of one segment of the inverted repeat region (22.0 kbp) reduces the genome to about 128,000 base pairs. A further loss of 5.0 kbp from the SSC region brings the molecular size to 123,000 base pairs, the length observed for *V. faba*.

The Bam4 DNA sequence of *B. napus* is the largest portion of the SSC region (7.4 kbp) maintained in *V. faba*. It is located entirely within the P4 fragment. The placement of Bam4 within P4 was determined by restriction and cross-hybridization analysis of the cloned P4 fragment and the related subclones (Table 2). Restriction sites for Bam HI and Hind III were mapped for the P4 region (Fig. 5). The minimum boundaries of the Bam4 DNA sequence were determined by hybridizing P4 and each of the subclones to blots of separated Pst I, Bam HI, Hind III and Xho I fragments of *B. napus* chloroplast DNA (Fig. 5, Table 2). The data confirmed that part of the P4 DNA fragment originated from the SSC region. Both pVFHind1 and pVFHind3 hybridized to Bam HI fragment 4 of *B. napus* indicating that the Bam4 related sequence lies within these two sections of *V. faba*. The pVFBam3 probe did not react with the SSC region of *B. napus*. Therefore, the maximum length of the *V. faba* DNA segment that contains Bam4 related sequences is 9,400 base pairs (Fig. 5).

The loss of the Bam13 fragment in other legumes

Vicia faba is only one member of the group of legumes that has lost one segment of the IR. The loss has been documented in two other members, pea (Palmer and Thompson 1981; Chu et

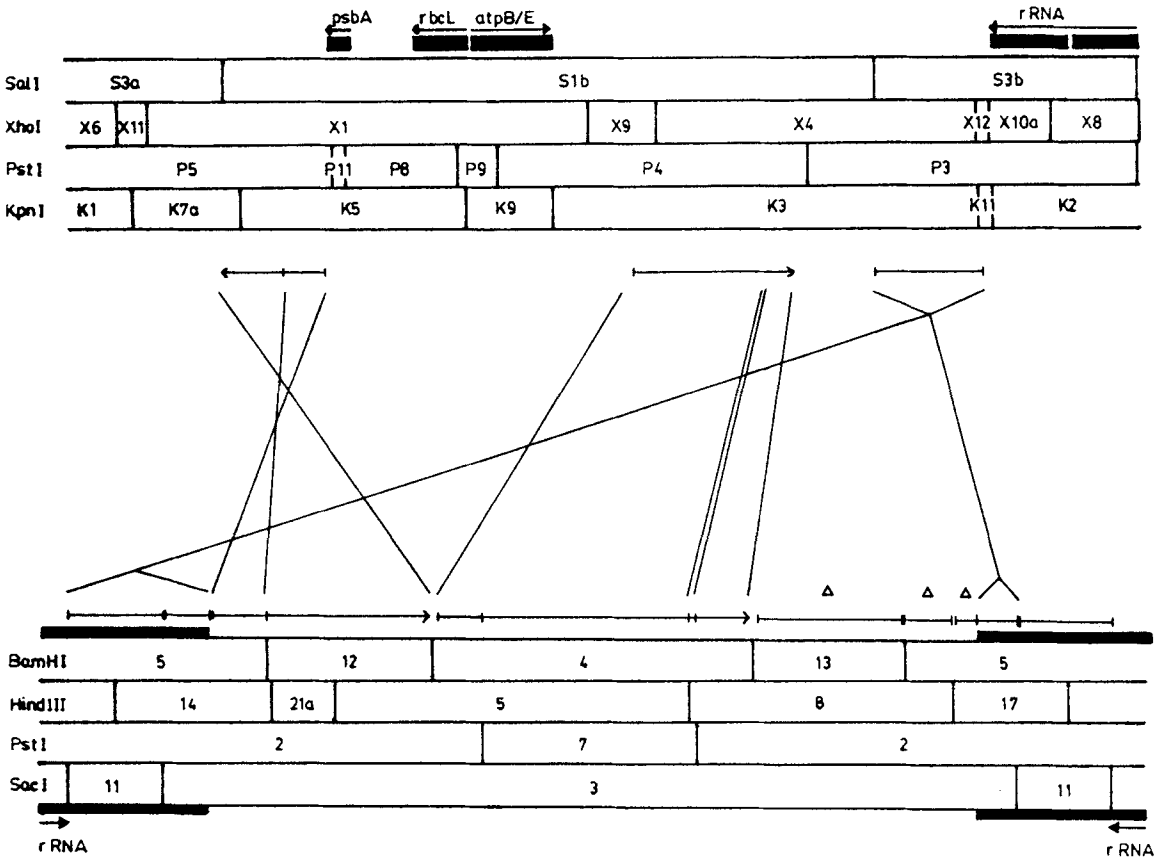

Fig. 4. Rearrangement of small single copy sequences between the *Brassica napus* (lower map) and *Vicia faba* (upper map) chloroplast genomes. The hybridization data summarized in Table 1 are diagrammatically represented in terms of the *Brassica napus* and *Vicia faba* physical maps. The extent and assigned direction of the SSC region DNA probes are indicated by the arrows above the lower map. The *Vicia faba* DNA fragments to which the DNA probes hybridize are indicated by the lines. The extent and changes in direction of the corresponding *Vicia faba* DNA fragments are shown by the arrows in the upper map. The segment that has been deleted in *Vicia faba* is marked by the hollow triangles (lower map). Gene designations: *rbcL* – the large subunit of ribulose-1,5-bisphosphate carboxylase; *psbA* – the 32 kd herbicide binding protein of photosystem II; *atpB, atpE* – the beta and epsilon subunits, respectively, of the ATP synthase; rRNA – the ribosomal RNA operon. The extent of the genes and inverted repeat region (in *Brassica napus*) are represented by the bold areas. The directions of transcription are indicated by the arrows above the bold area. Restriction sites for Pst I, Kpn I, Xho I and SalI are indicated in the upper map of *Vicia faba.* In the lower map of *Brassica napus,* Bam HI, Hind III, PstI and SacI sites are shown. The maps were constructed from published data (*Vicia faba:* Ko et al. 1983, 1984; Koller and Delius 1980; *Brassica napus:* Palmer et al. 1983; Vedel and Mathieu 1983; Xiao et al. 1986). The DNA fragments are designated according to Ko et al. (1984) for *Vicia faba* and Xiao et al. (1986) for *Brassica napus*

al. 1981) and alfalfa (Palmer et al. 1984; Palmer, pers. commun.) and physical maps of their chloroplast DNAs have been determined. The Bam13 probe was used to determine if the loss of the 5.0 kbp segment from the SSC region is a common occurrence in these plants. The results showed that the Bam13 probe does not react with either pea or alfalfa chloroplast DNA while the *B. napus* chloroplast DNA did react. This suggests that the loss of the 5.0 kbp fragment from the SSC region probably occurred simultaneously with the deletion of the IR region in this group of legumes.

Chloroplast DNA fragments are known to cross-hybridize to sections of DNA in the mitochondrial and nuclear genomes (Stern and Palmer 1984; Scott and Timmis 1984; Whisson and Scott 1984; Stern and Lonsdale 1982). The significance of these so-called "promiscuous" DNAs (Ellis 1982; Timmis and Scott 1984) is unknown.

Preliminary studies were conducted to determine if the 5.0 kbp deleted DNA fragment belongs to the "promiscuous" type of DNA. The Bam13 probe was hybridized to PstI and Bam HI digested *V. faba* nuclear and mitochondrial DNA (Fig. 6). The blots contained adjacent lanes of similarly digested *V. faba* chloroplast DNA to help differentiate reactions with contaminating chloroplast DNA fragments. Reaction occurred with one PstI fragment (approximately 26.0 kbp) and two Bam HI fragments (2.2 and 1.5 kbp) from the mitochondrial DNA. Weak hybridization signals were observed with nuclear DNA, consisting of two PstI fragments approximately 5.5 and 4.5 kbp long and a 2.4 kbp Bam HI

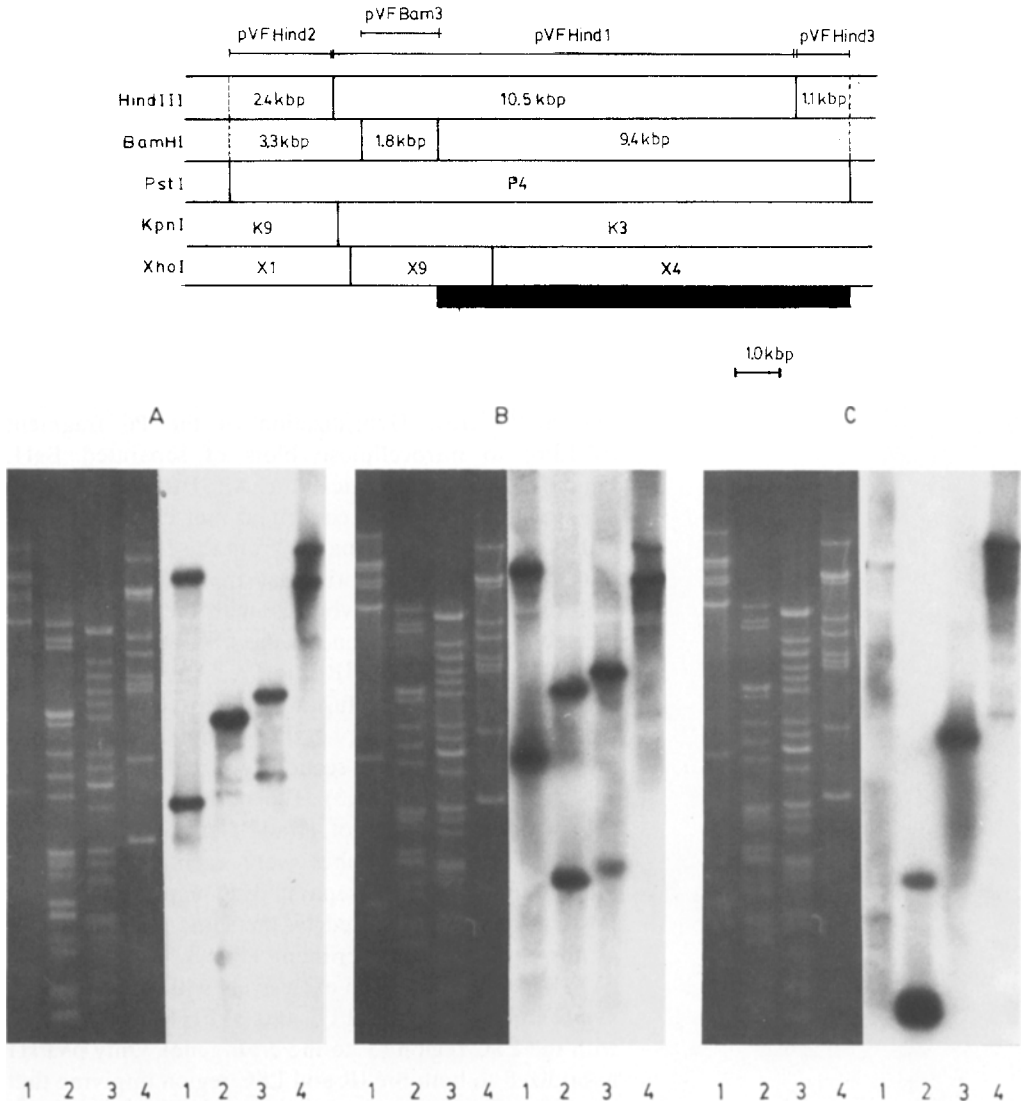

Fig. 5. Hybridization of *Vicia faba* chloroplast DNA probes to filter-bound *Brassica napus* chloroplast DNA. A physical map and subcloning strategy for the 14.5 kbp P4 fragment of the *Vicia faba* chloroplast genome is given at the top of the figure. Restriction sites are shown for Bam HI, Hind III, Pst I, Kpn I and Xho I. The DNA fragments are designated according to Ko et al. (1983). The Bam HI and Hind III DNA fragments are indicated in kilobase pairs. The extent of the subclones is indicated by the bars above the map. The dashed vertical lines represent the extent of the subclones, i.e. the Pst I sites. The DNA probe designations are marked. Hybridization results are presented for pVFP4, pVFHind1 and pVFHind2 in panels *A–C*, respectively. The agarose gels are shown on the left and the corresponding autoradiograms are to the immediate right side of each panel. The order of restriction patterns is the same in each panel Pst I (lane 1), Xho I (lane 2), Bam HI (lane 3) and Hind III (lane 4). The data are summarized in Table 2 and discussed in the text. The maximum limit of the Bam4 related sequence is indicated by the bold area at the bottom of the map

fragment. The possibility of an equivalent DNA sequence in the mitochondrial and nuclear genomes suggests that the Bam13 fragment may be of the "promiscuous" DNA type and is transferred from the chloroplast to the other compartments. This aspect of the DNA segment merits further research.

Cross-hybridization analysis of the P6 region

The surviving IR segment is divided into two different areas of the *V. faba* chloroplast DNA (Palmer and Thompson 1982; Shinozaki et al. 1984), which are approximately 35 kbp apart. The section stretching from the large single copy (LSC) region to the middle of the IR is located in the S3a region (Fig. 4). The ends of this section are marked by two different sets of genes. In conserved plastid genomes such as spinach, the LSC-IR junction is generally marked by the two putative ribosomal protein genes *rps19* and *rp12* (Zurawski et al. 1984). In *V. faba*, the two ribosomal protein genes are in the P1/S3a region, thus marking one end of this IR section (Ko and Straus, in press). The other end of this IR section is marked by the $tRNA^{Leu}_{CAA}$ gene in K7a (Bonnard et al. 1985; Mubumbila et al. 1984). This tRNA gene is normally found in the middle region of the typi-

cal IR. In *V. faba* the $tRNA^{Leu}_{CAA}$ gene is located close to the $tRNA^{Leu}_{UAA}$ and $tRNA^{Phe}$ genes that are usually positioned within the LSC region of the typical chloroplast DNA (Bonnard et al. 1985).

The remaining section, representing the stretch from the middle of the IR to the SSC-IR junction, is located in the P6/P3 region of *V. faba*. The end representing the SSC-IR junction was characterized in the previous section. Both the Hind14 and Hind17 DNA probes, which represent the left and right SSC-IR junctions, respectively, hybridized to the overlapping region of P3/X4/K3/S3b (Figs. 1, 4). The Sac11 and pBS5 probes originating from within the IR of *B. napus* (Fig. 1), were also homologous to the P3/X4/K3/S3b area. A DNA probe from a region further into the middle of the IR (pBH10) is homologous to the X10a area (Fig. 3, Table 1). These results indicate that the boundary related to the SSC-IR junction is located in the overlapping region of P3/X4/K3/S3b which is about 3.0 kbp. The opposite end of this IR section is located in the general vicinity of the P6/P3 area. Hybridization of the P6 fragment (pVFP6) to nitrocellulose blots of separated BglI, HindIII, PstI, PvuII, SacI, and XhoI fragments of *B. napus* chloroplast DNA confirmed that P6 is composed of DNA sequences originating from the IR and LSC region (Fig. 8). The two principal areas of the *B. napus* chloroplast DNA that hybridize with the P6 region are located 3′ to the *atpE* gene in the LSC region and 5′ to the rRNA operon in the IR (Fig. 7).

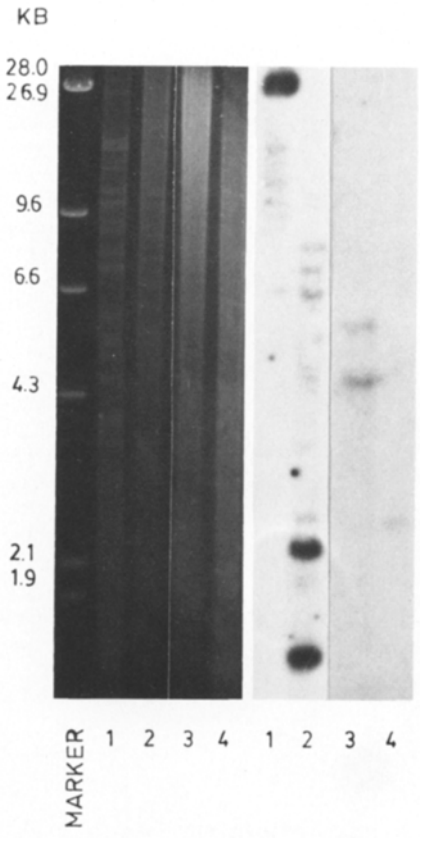

Fig. 6. Hybridization of the Bam13 probe to *Vicia faba* mitochondrial and nuclear DNA. The agarose gel is presented on the left and the corresponding autoradiogram is on the right of the panel. The mitochondrial DNA is digested with Pst I (lane 1) and Bam HI (lane 2). The nuclear DNA is similarly digested (Pst I in lane 3 and Bam HI in lane 4). A Hind III digest of lambda DNA is present in the left lane titled marker. The molecular sizes are indicated in kilobase pairs

The location of the fusion site between the IR and the LSC DNA sequence, i.e. the location of the remaining end of the split IR section, was determined using subclones of P6 (Table 3). The positions of the DNA probes and placement of HindIII sites are shown in Fig. 8A. The DNA probes were each hybridized to nitrocellulose blots of separated *B. napus* chloroplast DNA fragments generated by the same set of enzymes as for the pVFP6 experiment (Fig. 8, Table 3). The pVFH2 probe hybridized exclusively within the IR of *B. napus*, in contrast to pVFH3 and pVFH4 which reacted with the LSC region (3′ to the *atpE* gene). Only pVFH1 hybridized to both the IR and LSC region implying that the fusion site lies within the 6,300 bp HindIII fragment (Fig. 7). Due to the conservation of the HindIII site (the one between the 6,300 and 1,600 bp fragments, Fig. 7) between the right side of P6 and the related area of the IR, the location of the fusion site was placed within the 6,300 bp HindIII fragment at 2–5 kbp away from the PstI junction site of P6/P3. The location takes into ac-

Table 3. Cross-hybridization studies in P6 region of the *Vicia faba* chloroplast genome

Probes	*Brassica napus* chloroplast DNA fragments					
	Bgl I	Hind III	Pst I	Pvu II	Sac I	Xho I
pVFP6	1, 5 (2×)	2, 4, 10 (2×), 15	3, 6 (2×)	1, 3, 4, 6	1, 2, 5	1, 3 (2×)
pVFH1	1, 5 (2×)	2, 15	3, 6 (2×)	1, 3, 4, 6	1, 2, 5	1, 3 (2×)
pVFH2	5 (2×)	10 (2×)	6 (2×)	1, 3	1, 2	3 (2×)
pVFH3	1	4	3	4	5	1
pVFH4	1	4	3	4	5	1

The restriction fragments are designated according to Xiao et al. (1986). The numbers inside the brackets depict stoichiometry. The location of the DNA probes is illustrated in Fig. 7

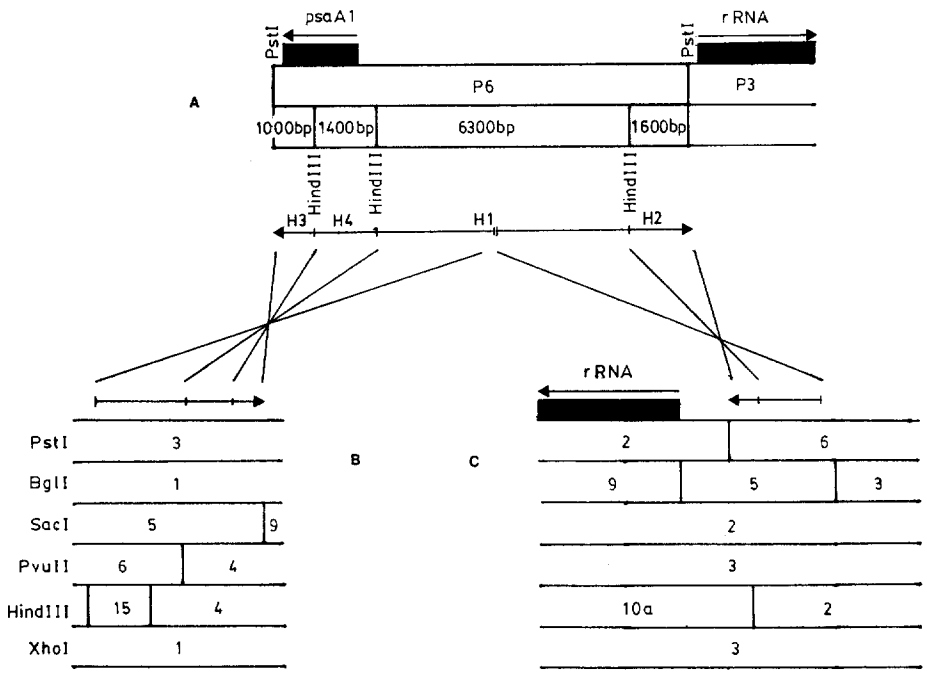

Fig. 7. A diagrammatic illustration of the rearrangement of the DNA sequences related to the P6 region. Diagram *A* represents a map of the *Vicia faba* P6 region. Restriction sites mapped for Hind III are indicated and the molecular sizes are in base pairs. The location and direction of transcription for the P700 chlorophyll *a* apoprotein gene (*psaA1*) and the ribosomal RNA genes (rRNA) are marked by the bold area and arrow, respectively (Ko et al. 1983; Koller and Delius 1980). The four DNA probes pVFH3 (H3), pVFH4 (H4), pVFH1 (H1) and pVFH2 (H2) are shown at the bottom of diagram *A*. The extent and assigned arbitrary directions of each probe is indicated. The two cross-hybridizing areas of the *Brassica napus* chloroplast genome are connected by the lines to diagrams *B* and *C*. The extent and changes in direction of the related areas in *Brassica napus* are indicated by arrows above diagrams *B* and *C*. The *Brassica napus* maps were constructed from published data (Palmer et al. 1983; Vedel and Mathieu 1983; Xiao et al. 1986). The DNA fragments are designated according to Xiao et al. (1986)

count the 1,600 bp DNA segment between the conserved Hind III site and the Pst I site of P6/P3 of *V. faba* (Fig. 7), and the maximum distance between the Hind III site of 10a/2 and the Bgl I site of 5/3 in *B. napus* (approximately 3.6 kbp) (Fig. 7).

The assignment of directions to P6 DNA sequences was also possible. Since pVFH3 and pVFH4 hybridized to a region distal to the *atpE* gene, and pVFH1 is proximal to the *atpE* of *B. napus,* an arbitrary direction can be assigned to this DNA segment. The assigned direction proceeds from the 6,300 bp Hind III fragment (pVFH1) to the 1,000 base pair Pst I-Hind III (pVFH3) DNA fragment (Fig. 7) which is in the same direction as the *V. faba psaA1* gene. In *B. napus,* the direction of the related region was the same relative to the orientation of the *atpB,E* genes. The direction of *atpB,E* is the same polarity as the *psaA1* gene. Although the *psaA1* gene has yet to be mapped on *B. napus,* the colinearity of the chromosome with *Petunia* and spinach suggests that there were no major changes to this region (Palmer et al. 1983; De Heij et al. 1983).

The remaining section of P6 can also be assigned a direction. Since pVFH2 reacted to a region closer to the *B. napus rrn* than pVFH1, an arbitrary direction can be given to this DNA segment (Fig. 7). The direction proceeds from the 6,300 bp Hind III to the 1,600 bp Hind III-Pst I fragment, which is the same direction as the *V. faba rrn.* The orientation relative to the direction of the *rrn* is the same in *B. napus* (Fig. 7). These results indicate that there were no major alterations within the P6 region following the inversion that divided the surviving IR.

A model for the evolution of the Vicia faba chloroplast genome

"Global" hybridization studies (Palmer and Thompson 1982; Palmer 1985; Palmer et al. 1984; Palmer, pers. commun.) revealed that the *V. faba* chloroplast DNA probably evolved from an alfalfa-like ancestral genome by two major inversions. In this report, detailed studies into the rearrangement of the SSC region and IR revealed that a third inversion is involved. The stepwise model encompasses data from this study as well as data from previous global-hybridization (Palmer and Thompson 1982; Palmer, pers. commun.) and tRNA mapping studies (Bonnard et al. 1985; Mubumbila et al. 1984).

The model begins at an ancestral chloroplast genome (Fig. 9) typified by *B. napus* and spinach. The predecessor to the alfalfa-like chloroplast DNA is represented by the mung bean chloroplast DNA, a legume that possesses the IR. The mung bean chloroplast DNA differs from the typical spinach genome by a 50.0 kbp simple inversion. The reversal of the segment between the *psbA* and *petA* genes places *rbcL-atpB,E* next to *psbA* and *atpH/atpA* next to the *petA* gene as shown in Fig. 9 B (Palmer and Thompson 1982; Palmer 1985; Palmer, pers. commun.). The alfalfa chloroplast genome differs from conserved genomes with IR by a simple deletion of one entire segment of the IR. Despite the deletion, the gene arrangement is co-linear with mung

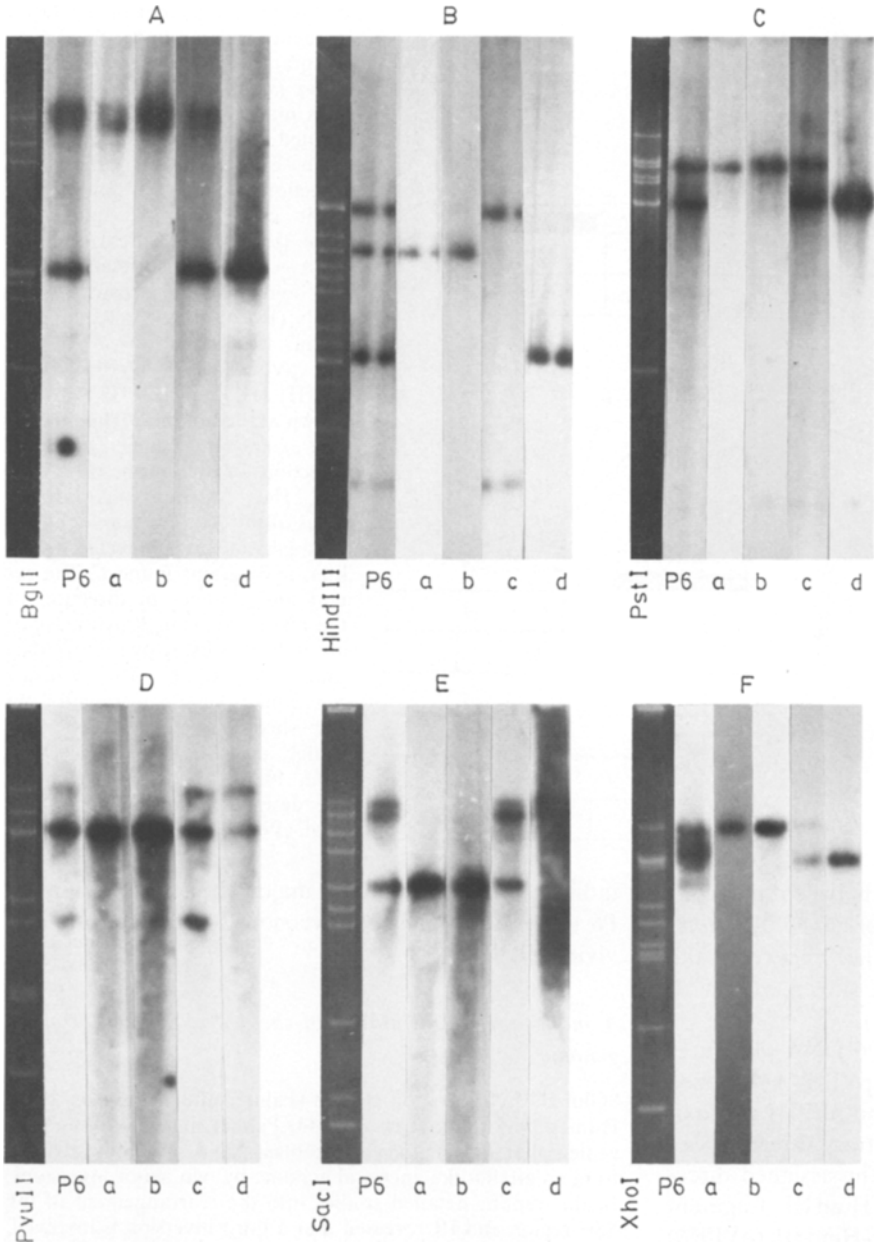

Fig. 8. Cross-hybridization analysis of the P6 region. The DNA probes pVFP6 (lane marked P6), pVFH3 (lane a), pVFH4 (lane b), pVFH1 (lane c) and pVFH2 (lane d) were hybridized to *Brassica napus* chloroplast DNA digested by Bgl I (panel *A*), Hind III (panel *B*), Pst I (panel *C*), Pvu II (panel *D*), Sal I (panel *E*) and Xho I (panel *F*). The restriction patterns are shown in the marked lanes. The data are summarized in Table 3, Fig. 7 and discussed in the text

bean (Palmer et al. 1984; Palmer, pers. commun.). The sequences representing the SSC region are assigned directions according to those set in the previous section. The directional arrangement of SSC sequences can be extrapolated for both mung bean and spinach (Palmer and Thompson 1982), since global-hybridization studies have not revealed any rearrangements in this region. The directions have no significance in terms of gene sequences, but differentiate the specific ends of the section. The arrangement of sequences for the alfalfa-like chloroplast genome is shown in Fig. 9C.

Three simple inversions, all occurring within a stretch of 50 kbp, are probably responsible for the arrangement of sequences present in *V. faba*. One inversion involves the entire 50 kbp section from the middle of the surviving IR 5′ to the 16S rRNA gene to the 3′ side of the *atpB/E* genes (Fig. 9D). The ends of the region are marked by genes for $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{UAA}$. One endpoint is located just right of the $tRNA^{Leu}_{CAA}$ gene and the other is situated just right of $tRNA^{Leu}_{UAA}$ between the *atpB/E* and *psaA1* genes. In addition to reversing the order of the sequences within the 50.0 kbp segment, the inversion separates the surviving IR into two different regions, breaking up the surviving IR at a sequence to the right of the $tRNA^{Leu}_{CAA}$ gene. The $tRNA^{Leu}_{CAA}$ gene which normally occurs in the middle of the IR re-

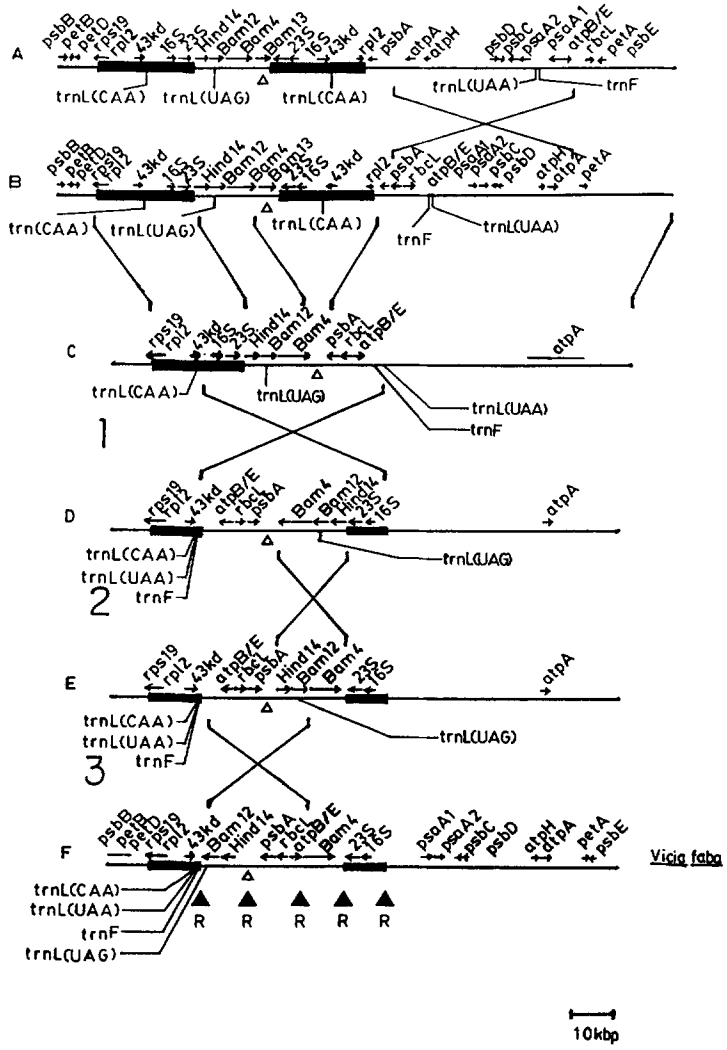

Fig. 9. A stepwise model for the evolution of the *Vicia faba* chloroplast genome. The model illustrates the five steps leading to the organization observed in *Vicia faba*. Molecule *A* represents the ancestral chloroplast genome typified by spinach (Herrmann et al. 1984). A 50 kbp inversion within the large single copy region of molecule *A* resulted in the arrangement found in the mung bean-type chromosome (molecule *B*) (Palmer 1985). Molecule *C* represents the loss of one of the inverted repeated sequences from a mung bean-like ancestor yielding the alfalfa-type genome (Palmer et al. 1984; Palmer, pers. commun.). Three subsequent inversions (numbered steps 1–3) resulted in the organization observed in the present-day *Vicia faba* chloroplast chromosome. Four transfer RNA genes have also been included. They are designated: trnL(CAA)–$tRNA^{Leu}_{CAA}$; trnL(UAG)–$tRNA^{Leu}_{UAG}$; trnL(UAA)–$tRNA^{Leu}_{UAA}$ and trnF–$tRNA^{Phe}$ (Mubumbila et al. 1984; Bonnard et al. 1985). The arrows marked by Hind14, Bam12, Bam4 and Bam13 are not gene designations. They indicate position and relative orientation of blocks of sequences. The hollow triangle points to the position of the Bam13 block (in molecules *A* and *B*) and, upon its subsequent deletion, it points to the fusion site (in molecules *C*–*F*). The solid triangles in molecule *F* indicate the location of recombination/inversion sites. Gene designations: *rbcL* – the large subunit of ribulose-1,5-bisphosphate carboxylase; *atpA, atpB, atpE, atpH* – the alpha, beta, epsilon and proton-translocating subunits, respectively, of the ATP synthase; *psaA1* and *psaA2* – the two P700 chlorophyll *a* apoproteins of photosystem I; *psbA, psbB, psbC, psbD* and *psbE* – genes for the 32 kd herbicide- binding, 51 kd chlorophyll *a*-binding, 44 kd chlorophyll *a*-binding, "D2" and cytochrome b_{559} proteins, respectively, of photosystem II; *petA, petB* and *petD* – cytochrome *f*, cytochrome b_6 and subunit 4 components, respectively, of the cytochrome b6/f complex; *rpl2* and *rps19* – putative chloroplast ribosomal protein genes homologous to *E. coli* ribosomal proteins L2 and S19, respectively

mains in the same location, thus marking the end of the unaltered segment of the surviving IR. An unidentified chloroplast polypeptide gene (named 43 kd) that is located in the middle of the IR is found as one copy within the K7a fragment immediately to the left of the $tRNA^{Leu}_{CAA}$ gene. The other side of the breakpoint that is yet unmarked by genes is inverted and subsequently placed 50 kbp away in the P6/P3 region. The two genes for $tRNA^{Leu}_{UAA}$ and $tRNA^{Phe}$, which were originally located between *atpB/E* and *psaA1* of the LSC region, are inverted and placed next to the $tRNA^{Leu}_{CAA}$. The resulting order of the tRNA genes is $tRNA^{Leu}_{CAA}$ – $tRNA^{Leu}_{UAA}$ – $tRNA^{Phe}$. This arrangement agrees with the tRNA mapping data reported by Bonnard et al. (1985).

A second smaller inversion within the major one reverses the directional arrangement of the surviving SSC region (Fig. 9E). The direction of the SSC region is reversed relative to the *V. faba rrn*. The $tRNA^{Leu}_{UAG}$ normally found close to *rrn* becomes distant. The segment involved is approximately 12.0–14.0 kbp.

To arrive at the arrangement observed in *V. faba*, a third inversion is required. This inversion reverses the arrangement between *atpB/E* and the SSC segment, Bam12 (see Fig. 9F). The reversal places the *atpB/E* closer to the 23S/16S rRNA cistron and the Hind14/Bam12 section next to the unrearranged half of the surviving IR. As a result, the $tRNA^{Leu}_{UAG}$ gene is placed closer to the $tRNA^{Leu}_{CAA}$, $tRNA^{Leu}_{UAA}$ and $tRNA^{Phe}$ gene cluster. This arrangement agrees with the placement of the tRNA genes in other mapping studies (Mubumbila et al. 1984; Bonnard et al. 1985). The resulting inversion also divides the surviving SSC region into two different areas. The Bam4 region remains close to the *rrn* in the same directional arrangement as in the step before. The

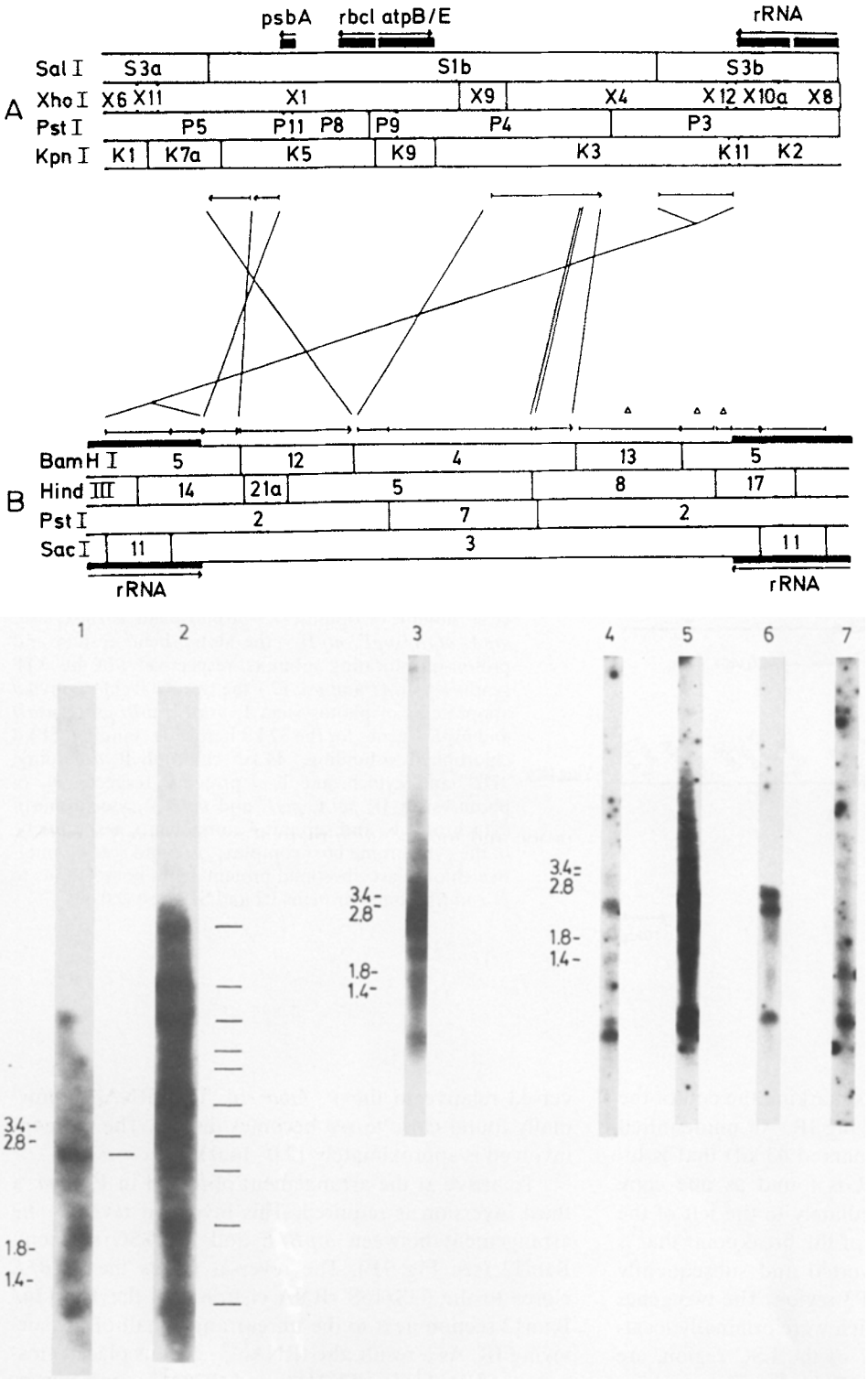

Fig. 10. In vivo transcription patterns are shown for the *Brassica napus* and *Vicia faba* chloroplast genomes. Patterns produced by the hybridization of Bam12 and Bam4 to *Brassica napus* total RNA are shown in lanes 1–2, respectively. The markings on the right of lanes 1 and 2 indicate positions of mRNA bands. Patterns generated by the hybridization of the DNA probes pVFP4, Bam4, pVFHind1, pVFHind2, pVFHind 3 to *Vicia faba* total RNA are shown in lanes 3–7, respectively. Information concerning the DNA probes are found in Figs. 1 and 5. The nucleotide size markers are indicated in kilobases. A map of the related regions in *Vicia faba* and *Brassica napus* is shown in diagrams *A* and *B*, respectively (see Fig. 4 for details)

remaining segment of the SSC region is reversed and placed adjacent to the unrearranged half of the IR segment.

The three inversions divide both the surviving IR and SSC region into two different areas of the *V. faba* chloroplast chromosome. The inversions also change the directional arrangement of the divided segments relative to their original order. The number of recombination steps required to arrive at the final arrangement agrees with "global" hybridization data (Palmer and Thompson 1982). The regions corresponding to the 3′ end of the Bam12 segment and the middle of the surviving IR are each homologous to two different zones of the chromosome. Inversions occurring once at each of these sites would result in two homologous regions. This observation correlates well with one inversion event predicted for each of these sites (steps 1 and 3). The region at the 3′ end of the *atpB/E* genes shows homology to three different areas of the *V. faba* chloroplast genome. This indicates that the site has been involved in two inversion events resulting in homologous sequences present in three different parts of the genome. This also agrees with the model where two inversions (steps 1 and 3) involve the region at the 3′ end of the *atpB/E* gene. The three inversion steps can occur in different temporal order from that shown in Fig. 9, but step 2 must always precede step 3.

The presence of multiple homologous sites may suggest that the inversion process involves duplication events. Nucleotide sequencing of the extreme left region of the 50 kbp inversion has been recently reported (Bonnard et al. 1985). The intergenic region between the genes for $tRNA^{Leu}_{CAA}$ and $tRNA^{Leu}_{UAA}$ possesses three sets of direct repeats. One repeat involves a partial duplication of the $tRNA^{Leu}_{UAA}$ gene in its immediate upstream region. These repeats may have been involved in the inversion events. The two sets of extensive direct repeats (177 and 19 bp) may correspond to the two inversional events that occurred at this site.

The model has helped in the identification of at least five potential recombination sites in the chloroplast chromosome of *V. faba*. The sequences may be analogous to the sites postulated by Palmer et al. (1984) in their general chloroplast genomic evolution model. The one located in the middle of the IR may be a strong recombination site, normally involved in creating orientation heterogeneity in the typical chloroplast genome (Palmer 1983; Palmer et al. 1984). The loss of one of these strong sites in *V. faba* may force recombination between the remaining strong site and alternate sites present throughout the chromosome. Finally, the maintenance of certain sections as a unit is apparent and may indicate that recombination within these units is lethal. The importance of maintaining these expression units can be concluded from studies into the rearrangement of other highly restructured chloroplast genomes such as pea and clover.

Analysis of in vivo transcriptional products

The transcriptional organization of DNA sequences related to the SSC region of *B. napus* and *V. faba* were investigated and compared by Northern blot analysis. In vivo transcription products were detected using the same DNA probes. The three main sections of the *B. napus* SSC region represented by the Bam12, Bam4 and Bam13 probes were hybridized to total *B. napus* RNA (Fig. 10). The Bam12 probe hybridized to one transcript. The adjacent region (Bam4) displayed a high level of in vivo transcription activity with a mRNA pattern consisting of at least 10 mRNAs. The Bam13 probe, the section that has been lost during the evolution of the *V. faba* plastid genome, did not reveal any in vivo transcripts. It appears that Bam13 is not actively transcribed in *B. napus*.

The DNA region corresponding to the Bam4 probe of *B. napus* is located in P4 of *V. faba*. The mRNA pattern revealed by hybridization of the P4 probe to total *V. faba* RNA is complex and contains a minimum of ten in vivo transcripts (Fig. 10). In the first section, cross hybridization studies mapped the Bam4 related DNA sequence to within a 9.4 kbp segment of *V. faba* (Fig. 5). The pVFHind1 probe (the 10.5 kbp HindIII fragment) reacted with a minimum of nine mRNAs. The pattern obtained with pVFHind1 is similar to pVFP4 (Fig. 10). The *V. faba* transcripts exhibited by both pVFP4 and pVFHind1 are comparable to the pattern produced by the Bam4 probe with *B. napus* RNA. The two remaining clones, pVFHind2 and pVFHind3, hybridized to four transcripts (Fig. 10).

Cross-hybridization between the *B. napus* Bam4 probe and total *V. faba* plant RNA displayed a mRNA pattern, although not as strong, identical to the patterns displayed by *V. faba* probes pVFP4 and pVFHind1 (Fig. 10). The Bam4 probe cross-hybridized to at least six *V. faba* in vivo mRNAs. This confirms that the gene sequences present in the Bam4 region of the *B. napus* SSC region are homologous to the gene sequences in the *V. faba* P4 region.

Rearrangement of the SSC region into different areas of the *V. faba* chloroplast chromosome appears to have no major effect on the transcriptional organization and activity. The major area of transcriptional activity in *B. napus* (Bam4) is maintained in the P4 region of *V. faba*. The rearrangement characteristics exhibited by Bam4 and Bam12 indicate that each section is self-contained and functional in different directions. The surrounding environment appears to have no major effect on their activity. The Bam13 region appears to be the only dispensible segment, since the *V. faba* chloroplast survives without it. The lack of in vivo transcriptional activity suggests that DNA sequences within the Bam13 region are probably not involved in the function of the chloroplast.

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