Theor Appl Genet (1997) 94 : 583–591

J. A. Scheffler · A. G. Sharpe · H. Schmidt · P. Sperling
I. A. P. Parkin · W. Lühs · D. J. Lydiate · E. Heinz

Desaturase multigene families of *Brassica napus* arose through genome duplication

Received: 14 August 1996 / Accepted: 18 October 1996

Abstract This paper reports the estimated gene copy number and restriction fragment length polymorphism (RFLP) map locations of five different desaturase cDNA clones from *Brassica napus* (oilseed rape). The desaturase enzymes encoded by four of these genes catalyze successive reactions that insert double bonds into lipid-linked fatty acid residues. Delta-12 (*e2*) and delta-15 (*e3*) desaturases are active in the endoplasmic reticulum, while omega-6 (*p2*) and omega-3 (*p3*) desaturases catalyze analogous desaturation reactions via a parallel pathway located in plastids. The fifth cDNA clone (*b5*) contains a desaturase-like domain bound to a cytochrome b_5 segment. Estimates of gene copy number based on Southern blot analysis of 16 oilseed rape varieties and three different resynthesized *Brassica napus* lines indicated that *e2* had 4–6 gene copies and *e3*, *p2*, *p3* and *b5* each had 6–8 gene copies per haploid genome. Estimates of the gene copy number for the two progenitor species, *Brassica oleracea* and *Brassica rapa*, supported the premise that all these genes were at least duplicated or triplicated in the two progenitor species before they combined to form *B. napus*. RFLP mapping results showed that the *e2* probe detected 4 distinct loci, the *e3* probe 6 loci and *p2*, *p3* and *b5* each detected 8 loci, with pairs of loci often mapping to homoeologous regions on 2 different linkage groups. The 28 mappable loci were distributed across 12 linkage groups of the *B. napus* map (Parkin et al. 1995) and were usually represented by single RFLP fragments. A collinear segment containing the *e2* and *p3* loci was positioned on *B. napus* linkage groups N1, N11, N3, N13, N5 and N15. This segment was collinear with a 30-cM region of *Arabidopsis thaliana* chromosome 3 that contains the homologous *fad2* (*e2*) and *fad7*(*p3*) genes. This suggests that the desaturase multigene families arose as the result of duplication of large chromosome segments rather than duplication of individual genes.

Key words *Brassica napus* · Collinearity · Desaturation · Fatty acid · Genetic mapping

Communicated by F. Salamini

J. A. Scheffler (✉) · H. Schmidt[1] · P. Sperling · E. Heinz
Institut für Allgemeine Botanik, Universität Hamburg,
Ohnhorststr. 18, 22609 Hamburg, Germany

A. G. Sharpe · I. A. P. Parkin · D. J. Lydiate
John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

W. Lühs
Institut für Pflanzenbau und Pflanzenzüchtung I, Ludwigstr. 23,
35390 Giessen, Germany

Present address:
[1] Bundesanstalt für Züchtungsforschung an Kulturpflanzen,
Institut für Züchtung Landwirtschaftlicher Kulturpflanzen,
Institutsplatz, 18190 Gross Lüsewitz, Germany

Introduction

Oilseed rape (*Brassica napus* L.) is the most productive oilseed crop grown in temperate climates. The fatty acid profile of its triacylglycerols is dominated by oleic acid (60–70%) with the remaining 30–40% composed of linoleic acid, linolenic acid and palmitic acid. Erucic acid is naturally present in the seed oil of *B. napus*, but it has been essentially eliminated in oilseed rape varieties destined for human consumption. This modification was possible because low erucic acid genotypes already existed within *B. napus*, and plant breeders needed only to transfer the trait to a suitable agronomic background (Stefansson and Hougen 1964). Additional alterations of the fatty acid profile in these varieties has long been the goal of plant breeders, and intensive efforts have been made to find naturally occurring genotypes or generate new genotypes via chemical mutagenesis (Lühs and Friedt 1994a). A low linolenic acid variety was produced using chemical

mutagenesis and selection (Röbbelen and Nitsch 1975; Scarth et al. 1988), but most attempts have achieved only partial success, as levels of many fatty acids, including oleic acid, have remained resistant to change. Other methods may be required to successfully alter these fatty acids.

The cloning of a complete set of plant acyl group desaturases and progress in understanding the biochemical mechanisms of lipid biosynthesis, including the desaturation and intercompartmental exchange of acyl groups, have provided information useful for developing alternative biotechnological strategies by which to modify the desaturation profile of acyl groups in seed triacylglycerols (Ohlrogge 1994). One such biotechnological approach is based on seed-specific expression of sense or antisense mRNA which inhibits the production of selected desaturases. The efficiency of this method depends on several factors, one of which is the number of copies of the endogenous target gene. Therefore, the copy number of the different desaturase genes, and in particular those expressed during seed development, is of interest.

Arabidopsis thaliana is the only Brassicaceae for which desaturase gene copy numbers have been systematically estimated. In this plant, the various desaturase genes are usually single copy, and to date only one possible exception (omega-3 desaturase) has been reported (Gibson et al. 1994). The situation for *B. napus* is expected to be different for several reasons. *Brassica napus* is an amphidiploid species probably originating from the hybridization of the two diploid progenitor species *Brassica oleracea* (C genome) and *Brassica rapa* (A genome) (U 1935). The multiple gene copies reported for the stearoyl-ACP desaturase gene of *B. napus* (Slocombe et al. 1994) probably result from this polyploidy rather than from selective duplication of the individual gene. Relatedness of the A and C genomes has been investigated cytogenetically by a number of researchers, including Attia and Röbbelen (1986). The two genomes are considered to be partially homologous and derived from a common ancestral genome (Prakash and Hinata 1980). Although the two progenitor species are classified as diploids, a high proportion of their genomes consists of duplicated regions (Slocum et al. 1990; Song et al. 1991).

Genetic analysis of a cross between resynthesized *B. napus* (an interspecific hybrid between *B. rapa* and *B. oleracea*) and normal winter oilseed rape demonstrated that the ten chromosomes of *B. rapa* origin each paired with specific chromosomes of the *B. napus* A genome and that the nine chromosomes of *B. oleracea* origin each paired with specific chromosomes of the *B. napus* C genome (Parkin et al. 1995). The same 19 linkage groups have been identified in genetic maps of *B. napus* derived from more conventional crosses (Sharpe et al. 1995; Howell et al. 1996), and the nine linkage groups of the *B. napus* C genome are almost completely collinear with the corresponding linkage groups of *B. oleracea* (Bohoun et al. 1996). Loci homologous to gene-specific probes can now be easily positioned on these integrated RFLP maps. Mapping these known genes not only contributes new information towards understanding the relationships among the species in the *Brassica* genus but may also, in combination with other molecular techniques, assist plant breeders in altering previously recalcitrant plant characters.

This study reports the estimated gene copy number and restriction fragment length polymorphism (RFLP) map locations of four different fatty acid desaturase genes in *B. napus*. The desaturase enzymes encoded by these genes are involved in the successive conversion of oleic via linoleic to linolenic acid residues. Parallel fatty acid desaturation pathways, and their corresponding enzymes, exist in the membranes of the endoplasmic reticulum (microsomes) and in plastids (Fig. 1). Although these four enzymes are related functionally and structurally, they are not isozymes and their corresponding genes are non-allelic (Ohlrogge and Browse 1995; Somerville and Browse 1996). In this paper, the four desaturases are designated *e2*, *p2*, *e3* and *p3*. The abbreviation indicates the subcellular localization (*e* for endoplasmic reticulum and *p* for plastid) and the number of the desaturation step catalyzed (Fig. 1). Therefore, *2* designates the second step which produces an additional delta-12 (omega-6) *cis*-double bond, and *3* the third step producing an additional delta-15 (omega-3) *cis*-double bond, when monoenoic *cis*-delta-9 oleic acid is the predominate substrate for either desaturase pair (*e2*, *e3* or *p2*, *p3*). Biochemical evidence

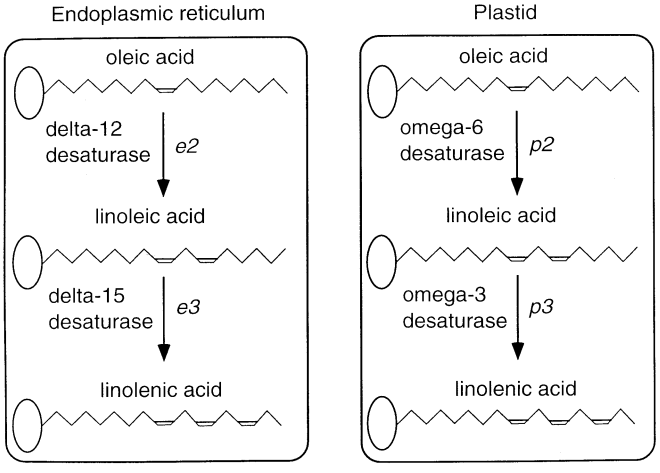

Fig. 1 Simplified diagram illustrating the two biosynthetic pathways for the desaturation of fatty acids when oleic acid (18:1) is the substrate. The endoplasmic reticular delta-12 (*e2*) or the plastidial omega-6 (*p2*) desaturase catalyzes insertion of a second double bond into lipid-bound oleic acid. The endoplasmic reticular delta-15 (*e3*) or the plastidial omega-3 (*p3*) desaturase catalyzes insertion of a third double bond into lipid-bound linoleic acid. In plastids, delta-7 hexadecenoic acid (16:1) is another possible substrate for the omega-6 desaturase, with the resulting fatty acids having 16 carbons instead of 18

suggests that microsomal and plastidial enzymes may differ with respect to the mechanism used to locate the prospective desaturation site within the acyl chain (Heinz 1993), but our abbreviated terminology for the desaturases does not take into account these differences. Also included in this study was a *B. napus* cDNA clone (*b5*) with a desaturase-like domain fused to a cytochrome b_5 fragment.

Materials and methods

cDNA clones

cDNA clones of genes encoding the four different desaturase enzymes (*e2*, *e3*, *p2* and *p3*) were isolated from cDNA of *B. napus* leaf or root tissue using degenerate oligonucleotide primers and polymerase chain reaction PCR-based cloning. The first set of degenerate primers was constructed based on conserved 'histidine boxes' found in membrane-bound desaturases isolated from *Synechocystis* (Wada et al. 1990), *Spinacia oleracea* (Schmidt et al. 1994) and *Arabidopsis thaliana* (Arondel et al. 1992). Multiple fragments were amplified and sequenced. On the basis of this sequence data, new primers were synthesized for a second round of PCR-based cloning. Larger clones were later obtained using rapid amplification of cDNA ends RACE-PCR techniques. Several different cDNA clones were isolated for each of the desaturase genes, with the nucleotide sequences of the cDNA clones for a given desaturase showing greater than 90% homology within their coding regions. The *b5* clone was also isolated by PCR-based cloning using cDNA of *B. napus* immature seed tissue.

The identities of the *p2*, *e3* and *p3* cDNA fragments were confirmed by comparisons with known sequences from *B. napus* found in the GenBank (accession numbers L29214, L01418 and L22963, respectively). The identity of the *e2* clone was verified by comparing it to the *A. thaliana fad2* gene (accession number L26296). The exact function of the *B. napus b5* clone is unknown, but it shows 67% amino acid identity to a cDNA clone previously isolated from *Helianthus annuus* (Sperling et al. 1995) and putatively encodes a fusion protein linking a cytochrome b_5 segment with a desaturase-like domain. Pairwise comparisons were also made between the four known *B. napus* desaturases and their *A. thaliana* homologues *fad2* (*e2*), *fad3* (*e3*), *fad6* (*p2*) and *fad7* (*p3*) (Genbank accession numbers L26296, L22931, U09503 and L22961). Percentage identities were calculated based on amino acid sequences aligned using the MacVector software program (International Biotechnologies, New Haven, Conn.).

Gene copy-number analysis

Southern blotting and hybridization were used to estimate the number of gene copies present for each of the cDNA clones in 8 spring and 8 winter oilseed rape (*B. napus*) varieties, plus 5 *B. rapa* subspecies and 6 *B. oleracea* subspecies. Using the four identified desaturases, we similarly analyzed three resynthesized lines of *B. napus* and their respective parents to determine the possible origin of the multiple bands detected in *B. napus* (Table 1). Genomic DNA was extracted from 1.5 g of leaf tissue using the urea extraction method of Chen and Dellaporta (1993). DNA was digested using either *Bam*HI or *Eco*RI, electrophoresed on a 0.7% gel for 20 h at 38 V and blotted onto Hybond N^+ (Amersham, Little Chalfont, UK) according to manufacturer's directions. The filters were then probed with a selected segment from one of the five cDNAs which was labeled with [^{32}P] using random-primed oligonucleotide labeling (Feinberg and Vogelstein 1983). Hybridizations were carried out overnight at 64°C followed by two low-stringency washes (2 × SSPE, 1% SDS) and two 30-min high-stringency washes (0.2 × SSPE, 0.1% SDS) at 64°C. Because of difficulty in resolving some of the higher-weight-molecular bands and the chance of partial digestion

Table 1 Estimated gene copy number in the three resynthesized lines

Resynthesized line[a]	Number of bands found in[b]	cDNA clone							
		e2		*p2*		*e3*		*p3*	
		*Bam*HI	*Eco*RI	*Bam*HI	*Eco*RI	*Bam*HI	*Eco*RI	*Bam*HI	*Eco*RI
Resyn 239	B.r. + B.n.	2	1[c]	4	2	2	1	2	2
	B.r + B.n. + B.o.	1	0	1	2	1	3	1	1
	B.o. + B.n.	2	3	4	2	3	1	2	3
	Total B.n. bands	5	5[d]	9	6	6	5	5	6
Rc50 × A12DHd	B.r. + B.n.		2		2		3		2
	B.r + B.n. + B.o.		0		2		1		3
	B.o. + B.n.		2		2		2		1
	Total B.n. bands		4		6		6		6
Rm29 × A12DHd	B.r. + B.n.		1		3		3		2
	B.r + B.n. + B.o.		0		1		0		3
	B.o. + B.n.		2		2		3		1
	Total B.n. bands		3		6		6		6

[a] Parents of Resyn 239 were *B. rapa* ssp. *trilocularis* ('yellow sarson YSPb-24') and *B. oleracea* convar '*botrytis*' var '*botrytis*' (cauliflower K2287) (Lühs and Friedt 1994b). Parents of the other two resynthesized *B. napus* were R-c-50, an inbred line of *B. rapa* ssp. *chinensis*, or Rm29, an inbred line of *B. rapa* ssp. *oleifera* (C Bowman and DJ Lydiate unpublished), and A12DHd, a doubled haploid line of *B. oleracea* ssp. *alboglabra* (Bohoun et al. 1996).

[b] Number of bands of the same size observed in *B. rapa* and *B. napus* only (B.r. + B.n.), or in all three *B. rapa*, *B. napus* and *B. oleracea* (B.r. + B.n. + B. o.) or in *B. oleracea* and *B. napus* only (B.o. + B.n.)

[c] One additional band was observed in the *B. rapa* parent that was not present in the resynthesized line 239

[d] One additional band was observed in the resynthesized line 239 that was not present in either parent

producing extraneous bands, all of the filters were prepared and examined independently at least twice.

A single cDNA species of each desaturase gene was used as the labeled probe. These cDNA fragments were selected to maximize discrimination between the five cDNAs while keeping the length of the probe between 650 and 1,200 bp. Each of the five probes was generated by PCR amplification of the cDNA clone using oligonucleotide primers to specifically amplify the desired segment. The PCR products were purified using the Quickspin purification kit (Qiagen GmbH, Hilden, Germany). The *e2* probe was amplified using forward primer 5′-AAAGCAAT[CA]CCACCGCA[TC]TG-3′ and reverse primer 5′-GGGTGAGTGTGTTGAA[GA]TA-3′. The *p2* probe was amplified with forward primer 5′-GCTCATAA[GA]TCCTTTTCAAAG-3′ and reverse primer 5′-TCTTCATCAA[CA]CGCCAATTC-3′. Both the *e3* (forward 5′-TT[TC]GTi[GCAT]TiGGiCACGATTGT-3′ and reverse 5′-AGGTTTCACGGGTACATTTTTAACT-3′) and *p3* probes (forward 5′-TT[CT]GTi[GCAT]TiGGiCATGATTGT-3′ and reverse 5′-ACAAAGTTGGGGTTTCCATCTGA-3′) were amplified using a degenerate forward primer and a specific reverse primer. The *b5* probe, including the b_5 domain and the desaturase-like segment up to the third histidine box, was amplified with the forward primer 5′-AACCATCTCTGTTTCAAC-3′ and reverse primer 5′-GG[GA]AA[CATG]A[GA][GA]TG[GA]TG[TC]TC-3′.

RFLP mapping

Loci homologous to the five cDNA clones were mapped using two established and aligned *B. napus* maps. One map with 277 loci was based on a population of 92 doubled haploid (DH) lines derived from a cross between a winter and a spring oilseed rape (N-o-72-8; Sharpe et al. 1995). The other map, with 399 loci, was based on a population of 50 DH lines derived from a cross between winter oilseed rape and a resynthesized *B. napus* (N-fo-61–9; Parkin et al. 1995). Southern hybridization and genetic linkage analysis were carried out as described by Sharpe et al. (1995) using the previously described PCR-amplified segments of *e2*, *p2*, *e3*, *p3* and *b5* as probes and filters carrying genomic DNA derived from DH lines of the N-o-72-8 and N-fo-61-9 mapping populations. A number of *Brassica Pst*I clones, which had been used in the generation of the two *B. napus* maps, were hybridized to a population of *A. thaliana* recombinant inbred (RI) lines (Lister and Dean 1993) and their homologous loci mapped. In addition, loci detected by four *Arabidopsis* probes previously mapped to *A. thaliana* chromosome 3 were mapped in the two *B. napus* populations: RAC1 and RAC3, both *RPM1*-associated cDNAs (Grant et al. 1995); mi74, a random *Pst*I clone and m560, a cosmid subclone (Arabidopsis Biological Resource Center, Columbus, Ohio). Southern hybridization and genetic linkage analysis were as described by Sharpe et al. (1995) except that the washes were carried out at low stringency (2 × SSC, 0.1% SDS).

Results

Sequence relationships between the different desaturases

The percentage protein identity between the four desaturases (*e2*, *p2*, *e3* and *p3*) and their respective homologues in *A. thaliana* ranged from 88% to 94%, indicating a high degree of sequence conservation between the two species for these genes. Pairwise comparisons among the deduced amino acid sequences of the *B. napus* cDNA clones (*e2*, *p2*, *e3* and *p3*) confirmed that while they all shared regions of similarity, they represented distinct protein products. The *e3* and *p3* clones shared the highest percentage identity (72%), with all the other pairwise comparisons giving identities less than 50%. Not suprisingly, comparisons among the *fad2*, *fad3*, *fad6* and *fad7 A. thaliana* genes showed similar results. The *fad3* and *fad7* sequences had the highest percentage identity (73%) with all other comparisons ranging from 30% to 50%. These estimates are similar to those reported by Yadav et al. (1993). The observed close relationship between the endoplasmic reticular omega-3 desaturase (*e3*, *fad3*) and the plastidial delta-15 desaturase (*p3*, *fad7*) may indicate that the two genes have only recently diverged or, as proposed by Schmidt et al. (1994), that a formerly prokaryotic enzyme, after slight modification, could be expressed in eukaryotic membranes. The prokaryotic gene and the new eukaryotic gene are both expressed, but each is part of a different pathway.

Copy-number estimates for desaturase genes in *B. napus*

As expected from the degree of sequence divergence, probes derived from the five cDNAs gave distinct banding patterns when used as probes for Southern hybridization analysis. However, in contrast to *A. thaliana* where most of the desaturases are represented by a single-copy gene, in *B. napus* all of the desaturases evaluated appeared to be represented by multigene families. Gene copy-number estimation in *B. napus*, based on Southern blot analysis of the oilseed rape varieties, detected at least four homologous genomic DNA fragments for each of the cDNA clones. Based on the number of bands observed, we estimated that *e2* was present in four to six copies per haploid genome, while *e3*, *p2*, *p3* and *b5* had six to eight copies. There

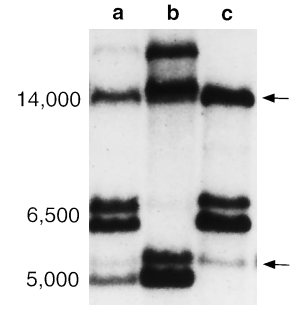

Fig. 2 Autoradiograph of the banding patterns observed for the *a* resynthesized line 239 (Lühs and Friedt 1994b) and its two parents, *b B. oleracea* convar '*botrytis*' var '*botrytis*' (cauliflower K2287) and *c B. rapa* ssp. *trilocularis* ('yellow sarson YsPb-24'), when the genomic DNA was cut with *Eco*RI and probed with *p2*. Numbers along the *left side* denote the approximate size of the bands in number of base pairs. *Arrows* indicate the two bands in the resynthesized line that were present in both parents

Table 2 Location of the desaturase genes in the *B. napus* genome

cDNA	Locus[c]	Linkage group	*Brassica napus* mapping population			
			N-fo-61-9[a]		N-o-72-8[b]	
			Flanking marker 1	Flanking marker 2	Flanking marker 1	Flanking marker 2
e2	a	N1	pO12e	pW136bNM		
	d	N11	pO12c/fNM	pW136cNM		
	c	N5	pN215aNP	pO123cNP		
	b	N15	pW197dNP	pN215c		
p2	e	N1	pW225c	pW145eNM		
	c	N11	pW145a	pN52eNM		
	b	N5	pO46b	pN53dNM		
	a	N17	pW120aNP	pW225a		
	d*	N8	pN168b	pC2c		
	f*	N13	flower	pR54a		
		Two monomorphic bands				
e3	b	N3	pW102c	pN22a	pW102c	pN22a
	a	N5	PN66a	pW115e	pO46bNM	pN215a
	e	N14	pN66b	pW218cNP		
	c	N14	pN151dNM	pN59cNP	pN173b	pN151b
	d	N4	pN151c	pN59f	pN151c	pN44c
		One monomorphic band				
p3	d	N1	pN148a	pO12e	pN148a	pO12e
	f	N11			pW108c	pO12c
	a	N5	pN215aNP	pO123cNP		
	e	N15	pN215cNP	pO123aNP		
	g	N3			pO12b	pN215b
	c	N19			pW203b	top of group
	b	N9			pO147a	pW122a
		One monomorphic band				
b5	e	N3	pW181b	pN215b		
	a	N13	pO12a	pW181a		
	c	N5	pN53dNM	pN215a		
	b	N5	pN174a	pN120b	pN174a	pN194b
	d	N14	pN174b	pN194a	pN174b	pN194a
	f	N11	pW108c	pO12cNM		
	h		unlinked			
		One monomorphic band				

[a] For the complete linkage map showing position of flanking markers and map distances see Parkin et al. 1995

[b] For the complete linkage map showing position of flanking markers and map distances see Sharpe et al. 1995

[c] *denotes co-segregating bands at a locus

was only a small degree of restriction fragment length polymorphism among the different varieties, with most of the winter varieties having one type of banding pattern and the spring varieties having a similar, but slightly different pattern. Gene copy-number estimates for *B. rapa* (*e2* with 2–4 copies; *p2*, *e3*, *p3* and *b5* with 4–6 copies) and *B. oleracea* (*e2* with 2–4 copies; *p2*, *e3*, *p3* and *b5* with 3–6 copies) were consistently lower than those for *B. napus*. The level of RFLP between subspecies of both *B. rapa* and *B. oleracea* was greater than between the *B. napus* oilseed rape varieties, and it was difficult to correlate the bands observed in either progenitor species with those observed in *B. napus*.

In newly resynthesized *B. napus* lines, the pattern of bands homologous to each of the four different desaturases usually matched the combined pattern observed for the two diploid parental lines, and showed which bands originated from each parent. Further examination of the banding patterns of the parents revealed that most of the cDNA clones were at least in triplicate in both of the parents (Table 1). The sum of the number of bands observed for the two parents was often higher than the total number of bands present in the resynthesized line. In most of these cases, the two parental species contributed coincidental bands. Figure 2 shows the banding patterns for the resynthesized line 239 and its two parents when genomic DNA was hybridized to the *p2* probe. The alignment of the bands is evident as are the two bands common to both parents.

Copy number estimates based solely on Southern hybridization analysis of varieties or resynthesized lines have limitations because the co-migration of bands can cause an underestimation of gene copy number, while restriction sites located within genomic sequences homologous to the probe cause an overestimation of copy number. Copy-number analysis also provides little information concerning the distribution of duplicated gene copies within the genome. Therefore, the cDNA clones were further analyzed using RFLP mapping.

Mapping the duplicated desaturase genes of *B. napus*

Genetic analysis of the loci homologous to *e2*, *e3*, *p2*, *p3* and *b5* located 4–8 distinct loci for each cDNA probe (Table 2). Analysis of the loci homologous to *e2* in the N-o-61–9 population identified 4 distinct loci (Table 2). These 4 loci were localized to 4 different linkage groups (Fig. 3), with 2 loci on the A-genome linkage groups (N1 and N5) and 2 loci on the C-genome linkage groups (N11 and N15). Linkage groups N1 and N11 represent a homoeologous pair of chromosomes, and N5 and N15 share an extensive homoeologous chromosome segment (Parkin et al. 1995; Sharpe et al. 1995) suggesting that duplicate copies of *e2* were present in the common ancestor of *B. rapa* and *B. oleracea*. Furthermore, each of the *e2* loci was represented by an allelic pair of DNA fragments in the N-fo-61–9 population, and no DNA fragments homologous to *e2* were unmapped. The fact that each RFLP allele was represented by a single DNA fragment suggested that the *e2* genes were present in the genome as isolated copies and not as duplicated clusters of copies. Analysis of the loci homologous to *e3*, *p2*, *p3* and *b5* revealed that while the number of distinct loci detected for each cDNA was greater (5–8 loci) than that observed for *e2*, these loci were similarly distributed throughout the genome, usually as pairs of related loci mapping to homoeologous regions (Fig. 3). This pattern of gene duplication is consistent with the duplication of large chromosomal segments rather than gene-specific duplication events. The one exception was *p2*, which had loci on linkage groups N8 and N13 represented by two pairs of co-segregating bands and might represent multiple-linked copies of the gene.

The *e2*, *p3* and *b5* loci were often localized to the same regions of the *B. napus* genome, i.e. linkage groups N1, N11, N3, N13, N5 and N15 (Fig. 3). In *A. thaliana*, the same grouping of the homologous *fad2* (*e2*) and *fad7* (*p3*) genes was observed on chromosome 3 (Fig. 4) (Koornneef 1994). *A. thaliana* loci homologous to a selection of *Brassica* RFLP probes that detect loci in the regions of N1, N11, N3, N13, N5 and N15 (containing the *e2* and *p3* loci) all mapped to a 30-cM segment of chromosome 3 that was collinear with the six segments of the *B. napus* genome. Conversely, the four *A. thaliana* probes detecting loci within the 30-cM region of chromosome 3 mapped to the six collinear segments of the *B. napus* genome (Fig. 4). This analysis confirmed that the desaturase multigene families of *B. napus* were produced by large-scale genome duplication events rather than gene-specific processes.

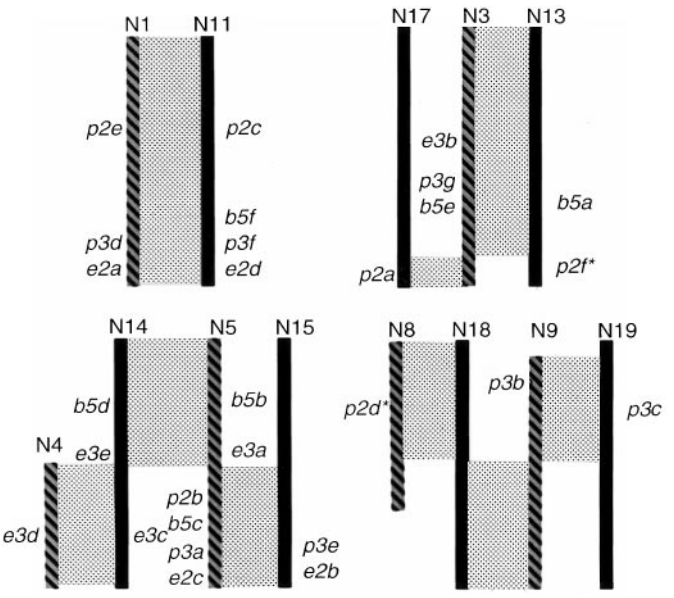

Fig. 3 Diagrammatic representation of the distribution of the five desaturase loci across 12 linkage groups corresponding to the *B. napus* RFLP map. *Striped vertical lines* designate linkage groups of A-genome (*B. rapa*) origin, and *solid vertical lines* designate linkage groups of C-genome (*B. oleracea*) origin. The *shaded areas between lines* indicate homoeologous regions between the A and C genomes. The *asterisk* denotes a locus with co-segregating bands. The placement of the loci on the linkage map is given in Table 2

Discussion

Desaturase gene conservation and copy number

It has been observed that there is often a high degree of sequence conservation between *A. thaliana* genes and their counterparts in *B. napus* (Arondel et al. 1992), and this was also the case for the four identified desaturase genes described in this study. Because of this conservation, it has been possible to isolate *B. napus* cDNA clones using *A. thaliana* cDNAs as low-stringency hybridization probes (Arondel et al. 1992; Yadav et al. 1993; Hitz et al. 1994) and vice versa (Iba et al. 1993). However, while the homology between the genes of *A. thaliana* and *B. napus* has been conserved, the number of copies of individual genes has clearly increased in the *B. napus* genome. In total, the five *B. napus* cDNA clones (*e2*, *p2*, *e3*, *p3* and *b5*), each encoding a distinct desaturase enzyme, detected at least 28 loci containing homologous sequences. These loci were positioned on 12 different linkage groups. Each cDNA clone detected

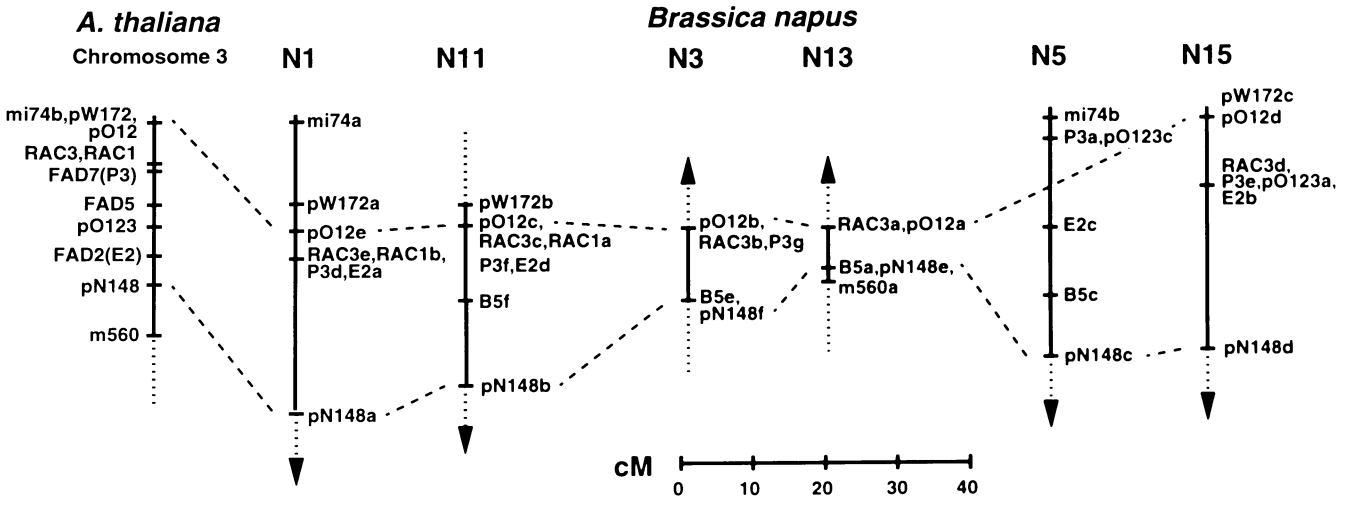

Fig. 4 Schematic representation of the six regions of the *B. napus* genome that are collinear with a 30 cM segment of *A. thaliana* chromosome 3 carrying *fad2* (*e2*), *fad5* and *fad7*(*p3*). *Vertical lines* denote segments of linkage groups, and *arrows* indicate the orientation of linkage groups with respect to those used by Parkin et al. (1995); i.e. N1, N11, N5 and N15 are inverted compared to their conventional orientations. *Dotted lines* join the duplicated loci of the *B. napus* genome homologous to pO12 and pN148

at least 4 related loci (gene copies), with *e2* having an estimated four copies, *e3* six copies and *p2*, *p3* and *b5* each having an estimated eight copies (Table 2). Other *B. napus* genes coding for enzymes involved in lipid metabolism are present in similarly high-copy numbers; for example stearoyl-acyl-carrier protein desaturase (4 copies, Slocombe et al. 1994), acyl-CoA-binding protein (6 copies, Hills et al. 1994) and the acyl-ACP thioesterase (6–8 copies, Loader et al. 1993).

Classical genetic analysis of genes controlling linolenic acid or oleic acid concentration, carried out using progeny testing (Brunklaus-Jung and Röbbelen 1987), diallel analysis (Pleines and Friedt 1989) or segregation ratios in microspore-derived populations (Chen and Beversdorf 1990) suggested the involvement of two to four genes with additional modifying genes present and significant environmental effects. The presence of four to eight copies of each desaturase gene per haploid genome could also create this complex segregation pattern.

The high copy number of the various desaturase genes may be one reason for the lack of progress in modifying the levels of oleic and linolenic acid using mutagenesis and selection. Unfortunately, a large number of gene copies might also hinder attempts to modify desaturase activities using molecular techniques. While the desaturase genes of *B. napus* are clearly highly duplicated, the number of functional gene copies is not known and may be smaller than the number of homologous loci because some of these loci may represent silenced copies or pseudogenes. As the next step it would be useful to identify and analyze the loci of each desaturase gene to determine the degree of homology between the loci and which produce functional enzymes during seed development. For modification of the fatty acid profile using biotechnological strategies such as antisense inhibition, this information may be useful, as previous research has indicated that these factors could play a role in the ability of the antisense RNA to inhibit desaturation (Kohno-Murase et al. 1995).

Duplication of chromosome segments

Each desaturase probe frequently detected pairs of loci assigned to homoeologous regions of 2 different linkage groups (Fig. 3). Other loci may also have had corresponding homoeologous counterparts that were monomorphic and therefore could not be mapped. On the basis of the distribution of loci in the *B. napus* genome, it appears that all of the desaturase genes were at least duplicated or triplicated in *B. rapa* (A genome) and *B. oleracea* (C genome) before the formation of *B. napus* (Fig. 3). For example, *p3* maps to the A-genome linkage groups N1, N3, N5 and N9 and the C-genome linkage groups N11, N15 and N19. It has been suggested that the *Brassica* species *B. nigra* is a polyploid which has evolved through the triplication of a simple ancestral genome (Lagercrantz et al. 1996). In this study, the pattern of gene duplication observed for the five cDNA clones suggests that the two diploid progenitors of *B. napus* (*B. rapa* and *B. oleracea*) are also ancient polyploids.

Collinearity

It has been shown previously that there is clear collinearity between the linkage groups of *B. rapa* and *B. oleracea* (Lydiate et al. 1995; Parkin et al. 1995). These

observations have been corroborated using the desaturase loci evaluated in this study (Fig. 3). There is also evidence that collinear segments exist in the genomes of *A. thaliana* and *Brassica* species (Lagercrantz et al. 1996; Sadowski et al. 1996). In this study, six regions of the *B. napus* genome were shown to be collinear with a segment of *A. thaliana* chromosome 3 which contained the *fad2*, *fad5* and *fad7* loci of *A. thaliana* (Fig. 4). A similar pattern of collinearity has been observed for the *fad6* (*p2*) locus of *A. thaliana* located on chromosome 4 close to the *fca* locus. The homologous *p2* loci in *B. napus* map to regions of linkage groups N1, N11, N17 and N8 (Table 2) which have previously been shown to be collinear with this region of *A. thaliana* chromosome 4 (A. Cavell, unpublished). This indicates that the multiple copies of the desaturase genes within the *B. napus* genome are the result of the duplication of large chromosomal segments rather than individual gene duplication.

Acknowledgments The authors thank M. Ford and C. Bowman for providing valuable seeds and technical advice. This work was supported by Mittel für Frauenförderung HSPII, the Bundesministerium für Forschung und Technologie (grants BEO 22/0310 528 F and 0311156, BEO 22/0319412B and 0310528B), Hoechst Schering AgrEvo GmbH, Deutsche Saatveredelung Lippstadt-Bremen GmbH, KWS Kleinwanzlebener Saatzucht AG vorm. Rabbethge & Giesecke and Norddeutsche Pflanzenzucht Hans-Georg Lembke KG.

References

Arondel V, Lemieux B, Inhwan H, Gibson S, Goodman HM, Somerville CR (1992) Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. Science 258: 1353–1355

Attia T, Röbbelen G (1986) Cytogenetic relationship within cultivated *Brassica* analyzed in amphihaploids from the three diploid ancestors. Can J Genet Cytol 28: 323–329

Bohoun EJR, Keith DJ, Parkin IAP, Sharpe AG, Lydiate DJ (1996) Alignment of the conserved C genomes of *Brassica oleracea* and *Brassica napus*. Theor Appl Genet 93: 833–839

Brunklaus-Jung E, Röbbelen G (1987) Genetical and physiological investigations on mutants for polyenoic fatty acids in rapeseed (*Brassica napus* L.). Plant Breed 98: 9–16

Chen JL, Beversdorf WD (1990) Fatty acid inheritance in microspore-derived populations of spring rapeseed (*Brassica napus* L.). Theor Appl Genet 80: 465–469

Chen J, Dellaporta S (1993) Urea based plant DNA minipreparation. In: Freeling M, Walbot V (eds) The maize handbook. Springer, Berlin Heidelberg New York, pp 526–527

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to a high specific activity. Anal Biochem 132: 6–13

Gibson S, Arondel V, Iba K, Somerville C (1994) Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from *Arabidopsis thaliana*. Plant Physiol 106: 1615–1621

Grant MR, Godiard L, Straube E, Ashfield T, Lewald J, Sattler A, Innes RW, Dangl JL (1995) Structure of the *Arabidopsis RPM1* gene enabling dual specificity disease resistance. Science 269: 843–846

Heinz E (1993) Biosynthesis of polyunsaturated fatty acids. In: Moore TS Jr (ed) Lipid metabolism in plants. CRC Press, Boca Raton, Fla., pp 34–89

Hills MJ, Dann R, Lydiate DJ, Sharpe A (1994) Molecular cloning of a cDNA from *Brassica napus* L. for a homologue of acyl-CoA-binding protein. Plant Mol Biol 25: 917–920

Hitz WD, Carlson TJ, Booth JR Jr, Kinney AJ, Stecca KL, Yadav NS (1994) Cloning of a higher-plant plastid omega-6 fatty acid desaturase cDNA and its expression in a cyanobacterium. Plant Physiol 105: 635–641

Howell PM, Marshall DF, Lydiate DJ (1996) Towards developing intervarietal substitution lines in *Brassica napus* using marker-assisted selection. Genome 39: 348–358

Iba K, Gibson S, Nishiuchi T, Fuse T, Nishimura M, Arondel V, Hugly S, Somerville C (1993) A gene encoding a chloroplast omega-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the *fad7* mutant of *Arabidopsis thaliana*. J Biol Chem 268: 24099–24105

Kohno-Murase J, Murase M, Ichikawa H, Imamura J (1995) Improvement in the quality of seed storage protein by transformation of *Brassica napus* with an antisense gene for cruciferin. Theor Appl Genet 91: 627–631

Koornneef M (1994) *Arabidopsis* genetics. In: Meyerowitz E, Somerville C (eds) Arabidopsis. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

Lagercrantz U, Putterill J, Coupland G, Lydiate DJ (1996) Comparative mapping in *Arabidopsis* and *Brassica*, fine-scale genome collinearity and congruence of genes controlling flowering time. Plant J 9: 13–20

Lister C, Dean C (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *A. thaliana*. Plant J 4: 745–750

Loader NM, Woolner EM, Hellyer A, Slabas AR, Safford R (1993) Isolation and characterization of two *Brassica napus* embryo acyl-ACP thioesterase cDNA clones. Plant Mol Biol 23: 769–778

Lühs W, Friedt W (1994a) The major oil crops. In: Murphy DJ (ed) Designer oil crops breeding, processing and biotechnology. VCH, Weinheim New York, pp 27–36

Lühs W, Friedt W (1994b) Stand und Perspektiven der Züchtung von Raps (*Brassica napus*) mit hohem Erucasäure-Gehalt im Öl für industrielle Nutzungszwecke. Fat Sci Technol 96: 137–146

Lydiate D, Dale P, Lagercrantz U, Parkin I, Howell P (1995) Selecting the optimum genetic background for transgenic varieties, with examples from *Brassica*. Euphytica 85: 351–358

Ohlrogge JB (1994) Design of new plant products: engineering of fatty acid metabolism. Plant Physiol 104: 821–826

Ohlrogge J, Browse J (1995) Lipid biosynthesis. Plant Cell 7: 957–970

Parkin IAP, Sharpe AG, Keith DJ, Lydiate DJ (1995) Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). Genome 38: 1122–1131

Pleines S, Friedt W (1989) Genetic control of linolenic acid concentration in seed oil of rapeseed (*Brassica napus* L.). Theor Appl Genet 78: 793–797

Prakash S, Hinata K (1980) Taxonomy, cytogenetics and origin of crop Brassicas, a review. Opera Bot 55: 1–57

Röbbelen G, Nitsch A (1975) Genetical and physiological investigations on mutants for polyenoic fatty acids in rapeseed, *Brassica napus* L. Zeitschrift Pflanzenzuechtg 75: 93–105

Sadowski J, Gaubier P, Delseny M, Quiros CF (1996) Genetic and physical mapping in *Brassica* diploid species of a gene cluster defined in *Arabidopsis thaliana*. Mol Gen Genet 251: 298–306

Scarth R, McVetty PBE, Rimmer SR, Stefansson BR (1988) Stellar low linolenic-high linoleic acid summer rape. Can J Plant Sci 68: 509–511

Schmidt H, Dresselhaus T, Buck F, Heinz E (1994) Purification and PCR-based cDNA cloning of a plastidial n-6 desaturase. Plant Mol Biol 26: 631–642

Sharpe AG, Parkin IAP, Keith DJ, Lydiate DJ (1995) Frequent nonreciprocal translocations in the amphidiploid genome of oilseed rape (*Brassica napus*). Genome 38 : 1112–1121

Slocum MK, Figdore SS, Kennard WC, Suzuki JY, Osborn TC (1990) Linkage arrangement of restriction fragment length polymorphism loci in *Brassica oleracea*. Theor Appl Genet 80 : 57–64

Slocombe SP, Piffanelli P, Fairbairn D, Bowra S, Hatzopoulos P, Tsiantis M, Murphy DJ (1994) Temporal and tissue-specific regulation of a *Brassica napus* stearoyl-acyl carrier protein desaturase gene. Plant Physiol 104 : 1167–1176

Somerville C, Browse J (1996) Dissecting desaturation: plants prove advantageous. Trend Cell Biol 6 : 148–153

Song KM, Suzuki JY, Slocum MK, Williams PH, Osborn TC (1991) A linkage map of *Brassica rapa* (syn. *campestris*) based on nuclear restriction fragment length polymorphism loci. Theor Appl Genet 82 : 296–304

Sperling P, Schmidt H, Heinz E (1995) A cytochrome-*b5*-containing fusion protein similar to plant acyl lipid desaturases. Eur J Biochem 232 : 798–805

Stefansson BR, Hougen FW (1964) Selection of rape plants (*Brassica napus*) with seed oil practically free from erucic acid. Can J Plant Sci 44 : 359–364

U N (1935) Genome-analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7 : 389–452

Wada H, Gombos Z, Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature 347 : 200–203

Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Perez-Grau L, Kinney AJ, Hitz WD, Booth JR Jr, Schweiger B, Stecca KL, Allen SM, Blackwell M, Reiter RS, Carlson TJ, Russell SH, Feldmann KA, Pierce J, Browse J (1993) Cloning of higher plant omega-3 fatty acid desaturases. Plant Physiol 103 : 467–476