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Temporal and Tissue-Specific Regulation of a *Brassica napus* Stearoyl-Acyl Carrier Protein Desaturase Gene[1]

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The nucleotide sequence of a *Brassica napus* stearoyl-acyl carrier protein desaturase gene (Bn10) is presented. This gene is one member of a family of four closely related genes expressed in oilseed rape. The expression of the promoter of this gene in transgenic tobacco was found to be temporally regulated in the developing seed tissues. However, the promoter was also particularly active in other oleogenic tissues such as the tapetum and pollen grains. This raises the interesting question of whether seed-expressed lipid synthesis genes are regulated by separate tissue-specific determinants or by a single factor common to all oleogenic tissues. Parts of the plants undergoing rapid development such as the components of immature flowers and seedlings also exhibited high levels of promoter activity. These tissues are likely to have an elevated requirement for membrane lipid synthesis. Stearoyl-acyl carrier protein desaturase transcript levels have previously been shown to be temporally regulated in the *B. napus* embryo (S.P. Slocombe, I. Cummins, R.P. Jarvis, D.J. Murphy [1992] Plant Mol Biol 20: 151–155). Evidence is presented demonstrating the induction of desaturase mRNA by abscisic acid in the embryo.

Lipid synthesis in plants fulfills a requirement for both structural lipids and storage TAG. The accumulation of TAG occurs in a number of tissues such as seeds, the tapetal layer of anthers, and pollen grains. TAG synthesis is also inducible in leaves in response to osmotic stress or ozone exposure (Ohlrogge et al., 1991; Evans et al., 1992).

To understand further the role of gene regulation in plant lipid synthesis, the expression of the Δ9 stearoyl-ACP desaturase was studied in oilseed crop *Brassica napus*. The stearoyl-ACP desaturase carries out the first step in C_{18}-fatty acid desaturation and contributes to both structural and storage lipid synthesis. The enzyme is a soluble dimer of 75 kD dependent on reduced Fd and molecular oxygen for activity (McKeon and Stumpf, 1982). Originally purified from avocado mesocarp and safflower embryos, cDNA sequences from the following plants have been obtained: safflower (Thompson et al., 1991), cucumber, castor (Shanklin and Somerville, 1991), potato (Taylor et al., 1992), spinach (Nishida et al., 1992), *Brassica rapa* (Knutzon et al., 1992), jojoba (Sato et al., 1992), and a *B. napus* cDNA clone (Bn9) (Slocombe et al., 1992).

The storage products of the rapeseed embryo accumulate at different points during the course of development. The onset of oil synthesis occurs during the first half of embryo development and precedes that of the storage proteins cruciferin and napin. Accumulation of oleosin follows after storage proteins. Insertion of oleosin into the oil bodies is believed to prevent coalescence during desiccation of the seed (Murphy and Cummins, 1989; Cummins et al., 1993). The activities of some of the oil synthesis enzymes have been studied during embryo development in rape. Those of the diacylglycerol acyltransferase (Weselake et al., 1993), the NADH-dependent enoyl-ACP reductase (Slabas et al., 1986), β-ketoacyl-ACP synthase I and II (MacKintosh et al., 1989), and ACP (Safford et al., 1988) precede the accumulation of lipid. Transcript levels of the *B. napus* stearoyl-ACP desaturase in the embryo peaked at 45 DAF, whereas the oleosin transcript peaked much later at 70 DAF as seed desiccation began (Slocombe et al., 1992). Early expression of transcript levels in rape embryos has also been observed in the case of ACP and enoyl-ACP reductase (Kater et al., 1991). Maximum stearoyl-ACP desaturase transcript levels in the embryo were 100-fold higher than in the leaf and this was reflected in the protein levels determined by western blotting (Slocombe et al., 1993).

Many processes occurring at different stages of embryo development are influenced by ABA. ABA concentration in soybean seeds has been linked to seed size, seed growth rate, and in vitro Suc uptake (Schussler et al., 1984). Production of linoleic acid metabolites in very young maize embryos is inducible by ABA (Abián et al., 1991). Transcription of cruciferin and napin message is also increased by ABA and this effect is greatest during the middle stage of development, when these proteins appear in situ (DeLisle and Crouch,

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Abbreviations: ABRE, ABA-responsive element; ACP, acyl carrier protein; GUS, β-glucuronidase; 1× SSC (150 mM NaCl, 15 mM Na citrate); TAG, triacylglycerol; X-gluc, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid.

1989). Microspore-derived haploid embryoids from *B. napus* increase fatty acid production, especially of long-chain forms, when exposed to ABA (Holbrook et al., 1992). Therefore, it is possible that ABA regulates the expression of genes encoding enzymes of oil synthesis.

The aim of this work was to study the expression of an oil synthesis gene in detail in different plant tissues. A further aim was to establish the role of the gene promoter in temporal regulation, especially in the seed and in the response of oil synthesis to hormonal factors such as ABA.

MATERIALS AND METHODS

Isolation of a Genomic Clone

The genomic stearoyl-ACP desaturase clone (Bn10) was isolated from *Brassica napus* cv Jet Neuf library constructed in λEMBL3 from a *Sau3A* partial digest (Ryan et al., 1989). Approximately 10^5 plaques generated in *Escherichia coli* strain LE392 (Sambrook et al., 1989) were screened using an *Eco*RI/*Bgl*II fragment of the previously reported cDNA clone Bn9 as a probe (Slocombe et al., 1992). The fragment consisted of the first 105 bp of the cDNA insert and was selected to facilitate isolation of clones carrying 5′ noncoding regions. Plaque screening was performed using Hybond N^+ (Amersham) according to the manufacturer's instructions. Hybridization with the ^{32}P-oligonucleotide probe was carried out under stringent conditions using the method of Domoney and Casey (1985), with final wash conditions of 0.1 × SSC, 0.1% (v/v) SDS at 65°C. Hexanucleotide-primed labeling of the probe with [^{32}P]dCTP (New England Nuclear) was done with an oligolabeling kit (Pharmacia). λDNA was obtained from the genomic clone using a CsCl step-gradient procedure (Sambrook et al., 1989). The genomic clone was mapped by restriction digestion followed by Southern hybridization under the conditions described above. DNA fragments of 1 to 2 kb were subcloned into the Bluescript II KS^- vector (Stratagene) for sequencing (see Fig. 1A). Standard DNA manipulations were performed according to Sambrook et al. (1989). Unilateral deletions were generated from these subclones by exonuclease III digestion of insert DNA followed by double-stranded DNA sequencing (Kavanagh and Murphy, 1988). DNA sequencing was performed using the dideoxy chain-termination method with the Sequenase Version 2.0 kit (United States Biochemical). Sequence data were analyzed using Genetics Computer Group Inc. programs (Sequence Analysis Software Version 7.2, 1992) on a Vax system. Intron/exon boundaries were confirmed by sequencing a portion of cDNA corresponding to Bn10. This fragment was isolated by PCR from an amplified cDNA library from which the probe cDNA (Bn9) was originally cloned (Slocombe et al., 1992). Primer extension was performed on RNA extracted from embryo tissue at 40 DAF and from young leaf tissue extracted according to Prescott and Martin (1987). Extension was performed with the oligonucleotide PX-3 (5′-TTGTAAGGCTGAGATGCC-3′), which was complementary to the stearoyl-ACP desaturase gene between nucleotides 136 and 153, as described by Bensi et al. (1985).

Determination of Gene Copy Number

DNA was isolated from the Jet Neuf and Topas cultivars of *B. napus* by the method of Covey and Hull (1981). Samples were also taken from a synthetic *B. napus* hybrid and the progenitor parent species *Brassica rapa* and *Brassica oleracea*. DNA was restricted with *Eco*RV and electrophoresis was carried out on a large-format gel apparatus (240 × 200 mm, IBI, Kodak) at 1.5 V/cm for 20 h in 1% (w/v) agarose using the Tris-borate buffer system (Sambrook et al., 1989). Southern blotting was carried out as described above. A 400-bp *Nco*I/*Bam*HI fragment of Bn9 was used as a probe.

ABA Induction Experiments

Staged zygotic embryos were harvested from *B. napus* cv Topas grown under greenhouse conditions and placed in sterile Petri dishes containing Murashige minimum organic medium (Imperial) at 4.7 g/L and 30 mM Suc. These were incubated up to 24 h in the light at 25°C. ABA as a 10-mM stock solution in 0.1 M NaOH was applied to the culture medium up to a maximum concentration of 100 μM. Samples were removed into liquid nitrogen for grinding and RNA was extracted (Prescott and Martin, 1987). RNA samples thus purified were separated on formaldehyde gels and blotted onto nitrocellulose (Sambrook et al., 1989). Hybridization was carried out under stringent conditions at 65°C using the entire Bn9 cDNA probe (Sambrook et al., 1989). Final wash was with 0.2× SSC, 0.1% (w/v) SDS at 65°C.

Promoter-GUS Plasmid Construction

Three plasmids (see Fig. 4) were used to examine the expression of the stearoyl-ACP desaturase promoter: pSSGD9 (desaturase promoter-GUS fusion), pSSGC14 (35S promoter-GUS fusion), and pBI221.8 (truncated −90 35S promoter-GUS fusion). Plasmid pSSGD9 contained a 2164-bp fragment of the desaturase promoter (−2051 to 113, see Fig. 1) driving the GUS reporter gene and was created as follows. A 2179-bp *Pst*I/*Hin*dIII fragment of the gene was subcloned into Bluescript II KS^- (pSSD9). An *Nco*I site was engineered at the translational start ATG codon by PCR using the primer MUT-2 (5′-CGATAAGCTTCAATGCCATGGTC TTT-3′, mismatch in bold). This was complementary to the stearoyl-ACP desaturase gene between bases 106 and 128 and included the *Hin*dIII site 9-bp downstream of the ATG (see Fig. 1). PCR was carried out on the promoter subclone using the T7 primer and MUT-2. From this PCR product a 154-bp *Aat*II/*Hin*dIII fragment containing the engineered *Nco*I site was subcloned back into the promoter clone forming pSSD9M. The sequence of the small PCR fragment was checked at this stage for possible errors introduced by PCR. A *Bam*HI/*Nco*I fragment containing the promoter was then subcloned into pBI221.8 (a pUC19-derived vector) in front of the GUS reporter gene forming pSSGD9. The entire promoter-GUS construct was transferred into the binary vector Bin 400 as an *Eco*RI/*Hin*dIII fragment. The 35S promoter-GUS construct (pSSGC14) contained 1.4 kb of the 35S promoter. This was derived as an *Eco*RI/*Nco*I fragment from another transformation vector (pJJ3792) and cloned into pSSGD9 between the *Sma*I and *Nco*I sites (replacing the

desaturase promoter) forming pSSGC14. Promoter-GUS constructs from pSSGC14 and pBI221.8 were also transferred into Bin 400 as *Eco*RI/*Hin*dIII fragments.

Tobacco Transformation

Binary vectors were transformed into *Agrobacterium tumefaciens* strain T37SE by a freeze-thawing protocol (P.R. Ebert, unpublished data) using DNA prepared with Qiagen columns (Hybaid, Teddington, UK). An *Agrobacterium* culture obtained by inoculating 20 mL of YEP (10 g/L peptone, 10 g/L yeast extract [Difco] and 86 mM NaCl) with 0.8 mL of an overnight culture was grown for 4 h at 29°C. Cells were pelleted at 4000*g* for 7 min, resuspended in 0.2 mL of YEP, and stored on ice for 5 min. DNA was dispersed into the cell suspension as 10 μL of a 0.1 to 0.2 g/L solution. The cells were immediately immersed in liquid nitrogen for 15 s and placed in a 37°C water bath for 5 min. Treated cells were incubated at 29°C for 1 to 2 h after the addition of 1 mL of YEP. Transformants were selected on 100 mg/L spectinomycin and plasmid content was checked by DNA isolation and restriction analysis (Stanton et al., 1988). Leaf discs of *Nicotiana tabacum* cv Petit Havana were transformed with *Agrobacterium* carrying the binaries according to Horsch et al. (1985). Transformed tobacco cells were selected with 100 mg/L kanamycin and tested for neomycin phosphotransferase II activity using an ELISA-based kit (5 Prime → 3 Prime, Inc.). Positive transformants were transferred to soil and grown in glasshouse conditions with 16 h of supplementary light. Untransformed plants were also maintained for use as negative controls.

Fluorometric GUS Assays

Flowers from 5 to 8 transformant lines per construct were tagged and seeds were harvested from 14 to 40 DAF at 2-d intervals. The seeds were homogenized in a combined leaf and germ (bud) juice press (Erich Pollahne, Wennigsen, Germany) adding extraction buffer at 250 μL per 100 seeds. For each tobacco pod three separate samples were extracted for fluorometric assay. Leaves of 100 mm length were also taken from T1 transformants and extracted in a similar fashion. T1 transformant seeds were geminated on solid Murashige-Skoog media containing 30 mM Glc and 100 mg/L kanamycin sulfate (Sigma) for 10 d. Roots, cotyledons, and stems were homogenized separately. Extraction and assay procedure for GUS activity were carried out according to Jefferson (1987). Reactions were carried out at 37°C in a volume of 200 μL containing 20 μL of plant extract. AT 1, 10, 30, 60, and 120-min intervals, 20-μL samples were removed into 180 μL of stop solution in a microtiter plate well. Fluorescence was measured using a Titertek Fluoroscan II (Flow Laboratories, Ltd.). Protein concentration was determined using the Bradford assay (Bradford, 1976). A_{595} nm was measured in microtiter plates using a Dynatech MR7000 spectrophotometer.

Histochemical Assays

GUS activity was localized in transgenic tobacco seeds and reproductive organs by histochemical staining with X-gluc (Biosynth AG, Zurich, Switzerland). Sections of tobacco seed of 30-μm thickness were generated using a cryostat after embedding in O.C.T. (Optimum Cutting Temperature) compound (BDH, Poole, UK) and transferred directly onto microscope slides coated in 3-aminopropyltriethoxysilane (Sigma). Sections of floral parts were cut by hand with a razor blade. Staining and fixing of sections were carried out according to Jefferson (1987). All sections were incubated for 2 h at 37°C in the presence of X-gluc unless stated otherwise.

RESULTS

Analysis of a Stearoyl-ACP Desaturase Genomic Clone

The nucleotide sequence of a *B. napus* stearoyl-ACP desaturase cDNA termed Bn9 has previously been reported (Slocombe et al., 1992). A genomic clone (Bn10) with a nucleotide sequence identity of 95.3% to Bn9 has now been characterized (Fig. 1). A 2.16-kb section of nucleotide sequence 5′ from the predicted ATG start site has been determined along with the sequence of three exons and two introns that constitute the entire gene.

The sequence surrounding the putative ATG start has 78% identity to the consensus for plant translation initiation proposed by Lütcke et al. (1987). It is also the first in-frame Met codon following the transcriptional start site. The transcriptional start site was identified by primer extension of RNA extracted from both leaf and embryo *B. napus* tissues (data not shown). A putative TATA motif (TAGA) is located 35 bp upstream from the start site. Incomplete homology to the TATA motif has been observed in a minority of plant enzyme genes (Joshi, 1987).

The Stearoyl-ACP Desaturase Gene Family

Gene copy number was assessed by probing a Southern blot at high stringency with a 400-bp fragment of the Bn9 cDNA clone. In Figure 2 the banding patterns obtained with a resynthesized *B. napus* hybrid are compared with those of the parent species (*B. rapa* and *B. oleracea*). Four, strong bands were obtained with the resynthesized line. Of these, two corresponded to bands observed with *B. rapa* and two were from *B. oleracea*. Additional high mol wt bands were observed with the *B. oleracea* DNA, which did not correspond to any bands in the resynthesized line. These bands could be attributed to partial digestion. Therefore, these data are consistent with the existence of four closely related genes in *B. napus*, two genes originating from each of the parent species. Four bands were also observed with the *B. napus* cv Topas (spring variety) and three with Jet Neuf (winter variety). In the latter case, one of these bands was double the intensity of the others and probably contained more than one gene. These findings are consistent with restriction fragment-length polymorphism mapping data indicating the existence of four genes mapping to three linkage groups in *B. napus* (D. Lydiate, personal communication).

The sequences of three of these genes (Bn1, Bn9, and Bn10) from *B. napus* cv Jet Neuf have been examined. The exon sequences of Bn10 are 95.3% identical to the Bn9 cDNA and 88.0% identical to the Bn1 cDNA. Bn1 is a partial cDNA clone very similar (97.7% identical) to a published *B. rapa*

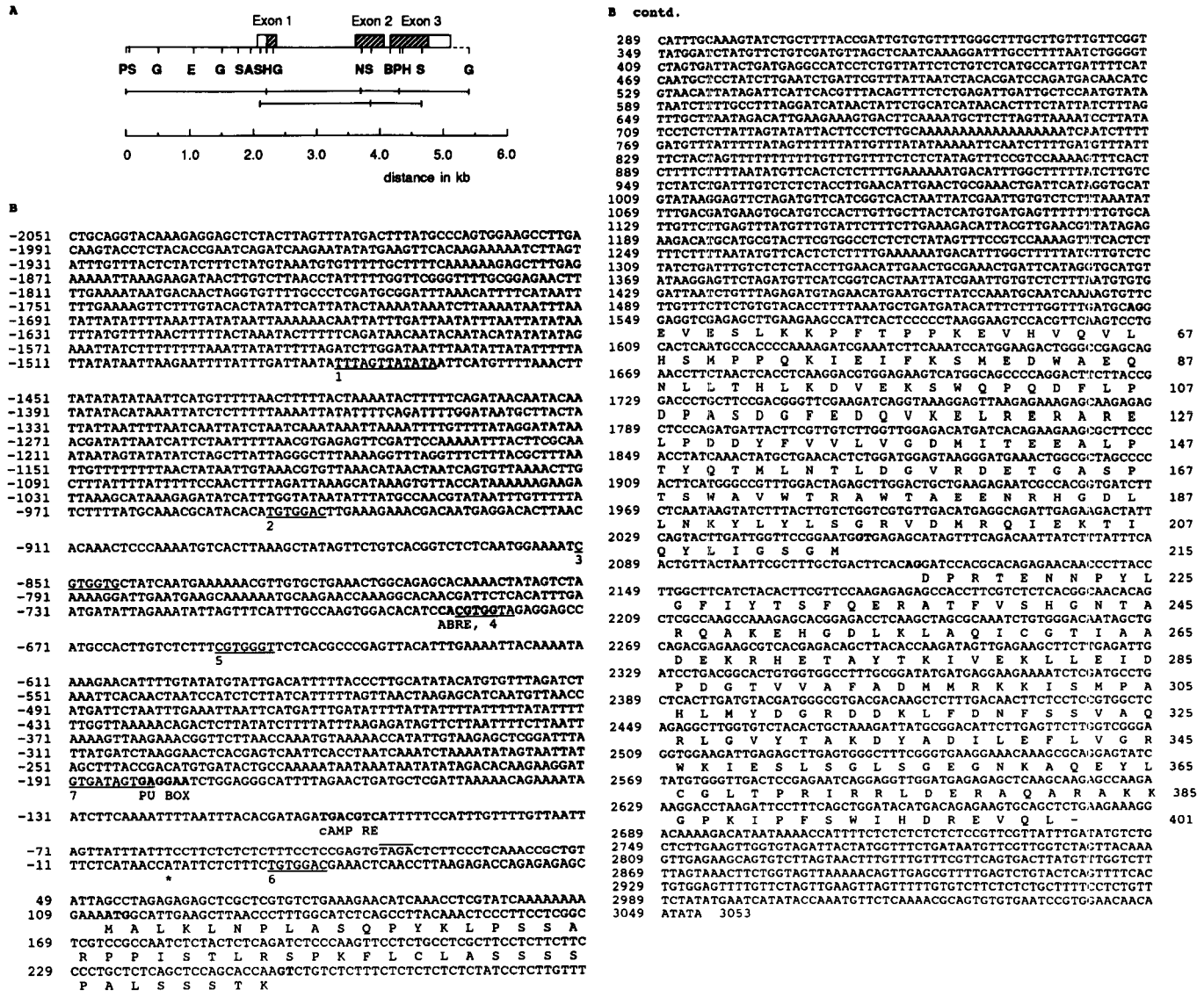

Figure 1. Structure and sequence of the stearoyl-ACP desaturase gene Bn10. A, Exons are shown as boxes with the coding regions shaded. Fragments subcloned for sequencing are indicated by bars. Restriction sites are abbreviated as follows: *Aat*II (A), *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Hin*dIII (H), *Nco*I (N), *Pst*I (P), and *Sst*I (S). The transcriptional start site is marked with an asterisk and a putative TATA-like box is overlined. Homologies to pollen-specific promoter DNA motifs are underlined and the sequences are compared in Figure 7. A potential ABRE, cAMP RE (Roesler et al., 1988), and PU box (Pettersson and Schaffner, 1987) are shown in bold type. GT/AG nucleotides corresponding to the intron boundaries are also shown in bold. The derived amino acid sequences are presented in single-letter code.

cDNA clone (Knutzon et al., 1992). Apart from the missing N terminus of about 50 residues, Bn1 encodes for an identical polypeptide. The amino acid sequences derived from the *B. rapa* clone and from Bn9 and Bn10 are all 96% identical and encode for 398 to 401 residues.

ABA Induction of Desaturase Message

Given that ABA can influence lipid synthesis in rape embryos, it was significant that the stearoyl-ACP desaturase promoter (Fig. 1B) contains a putative ABRE (Williams et al., 1992). To investigate the effect of ABA on desaturase transcript levels, excised rape embryos of 30 DAF were placed in liquid culture medium for 24 h in the presence of a range of ABA concentrations (Fig. 3A). Transcript levels of the stearoyl-ACP desaturase were elevated in embryos incubated with ABA over a concentration range of 1 to 100 μM. The induction response was concentration dependent up to 5 μM ABA. Induction of stearoyl-ACP desaturase transcript was monitored with respect to time at a concentration of 10 μM ABA (Fig. 3B). An increase in desaturase transcript levels was apparent within 10 min but continued up to 3 h, suggesting regulation at the level of transcription.

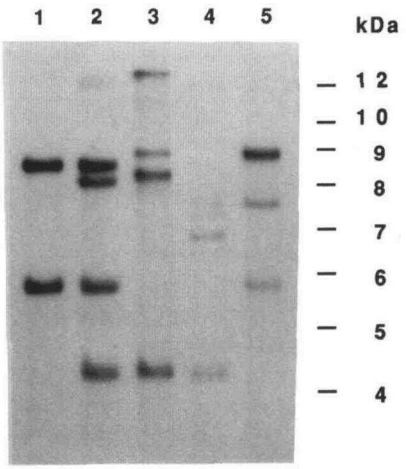

Figure 2. Determination of stearoyl-ACP desaturase gene copy number in *Brassica*. Southern blotting of genomic DNA was carried out after restriction with *Eco*RV at high stringency using Bn9 cDNA as a probe. DNA samples were: lane 1, *B. rapa*; lane 2, resynthesized *B. napus* line; lane 3, *B. oleracea*; lane 4, *B. napus* cv Topas; and lane 5, *B. napus* cv Jet Neuf.

Expression of a Desaturase Gene Promoter in Transgenic Tobacco

To study the regulatory properties of one of the rape stearoyl-ACP desaturase promoters in the embryo and other tissues, a chimeric gene was constructed containing 2.17 kb of the desaturase promoter region from Bn10 fused to GUS. As a control the constitutive 35S cauliflower mosaic virus promoter was also fused to GUS as a 1.4-kb fragment directing high levels of expression. In a further construct a truncated −90 fragment that directed expression at low levels was fused to GUS (Fig. 4).

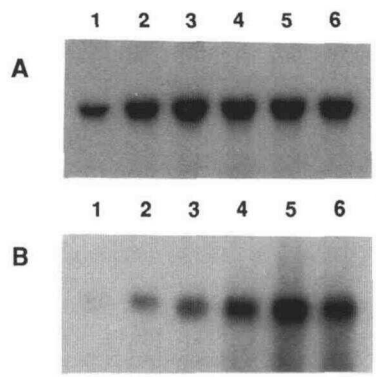

Figure 3. Effect of exposure to ABA on stearoyl-ACP desaturase transcript levels in excised rape zygotic embryos 30 DAF. Total RNA loadings of 10 μg were probed with the Bn9 cDNA by northern blotting. A, Excised rape embryos of 30 DAF were cultured for 24 h in the presence of the following ABA concentrations: lane 1, zero; lane 2, 1 μM; lane 3, 5 μM; lane 4, 10 μM; lane 5, 50 μM; and lane 6, 100 μM. B, Time course of stearoyl-ACP desaturase transcript induction in the presence 10 μM ABA. Samples were analyzed after the following time intervals: lane 1, zero; lane 2, 10 min; lane 3, 30 min; lane 4, 1 h; lane 5, 3 h; and lane 6, 12 h.

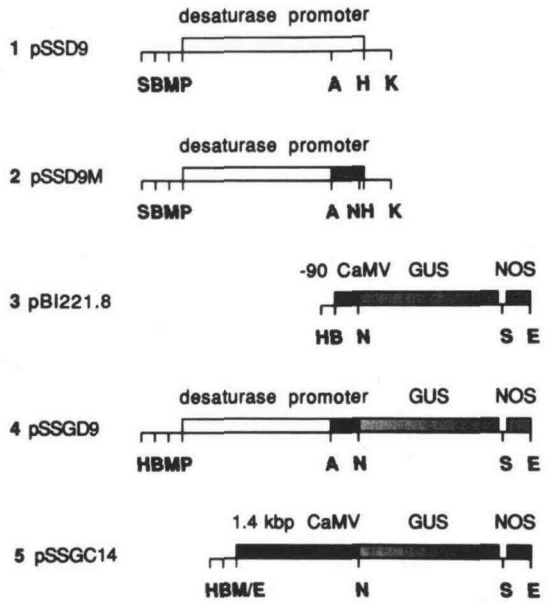

Figure 4. Diagram showing the promoter-GUS constructs used in tobacco transformation and plasmid intermediates involved in their assembly. Restriction sites are abbreviated as follows: *Aat*II (A), *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N), *Pst*I (P), *Sma*I (M), and *Sst*I (S).

Tobacco seeds were harvested from three sets of independent transformants each containing one of the three constructs. Seeds were taken at approximately 2-d intervals from 14 to 34 DAF for fluorometric assay of GUS activity. Lipid was evident in protein extracts taken for fluorometric assay by 18 to 20 DAF and the seeds were desiccated within 30 to 34 DAF. In Figure 5, best-fit curves relating GUS activity to seed development from four 35S-GUS transformants, eight desaturase promoter-GUS transformants, and five −90-GUS transformants were averaged and presented on a semilog plot. These data show that a large increase in GUS activity occurs in the desaturase-GUS transformant seeds during development (90-fold from 14–30 d) compared with the two sets of constitutive promoter-GUS plants (3-fold from 14–30 d). This demonstrated that the desaturase promoter was subject to temporal regulation in tobacco seeds.

In Table I, specific GUS activities in leaves and seeds are compared from eight independent desaturase promoter-GUS transformants. The GUS activities presented for seeds were the maximum values attained during seed development and were taken at approximately 30 DAF. Leaf GUS activities were derived from young samples of 100-mm length. These data indicate that in tobacco plants this rape desaturase promoter expresses in seeds on average at a 2.5-fold higher level, on a protein basis, than in young leaves. In Table II, the maximum levels of GUS activity observed in the seed are compared with those from leaves (i.e. one leaf pair and cotyledon pair), stem, and root of 10-d-old germinated tobacco seedlings. Seedlings were examined from five independent desaturase promoter-GUS T1 transformants. These

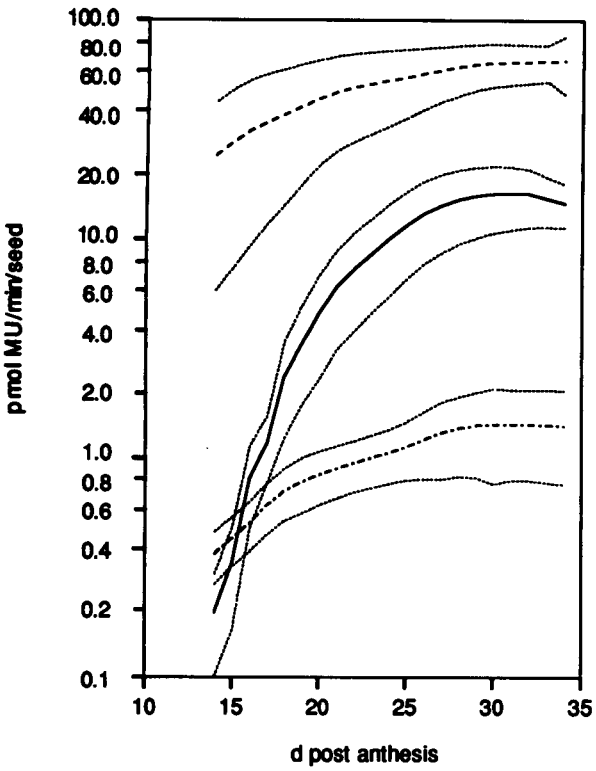

Figure 5. Comparison of GUS activity during seed development in transgenic tobacco plants containing the desaturase promoter-GUS construct (—), the 35S promoter-GUS construct (----), and the truncated −90 35S promoter-GUS construct (·-·-). The curves are averages from several independent transformants with SE values indicated by the dotted curves.

data indicate that on average there were similar levels of desaturase promoter activity throughout the seedling. The promoter was operating in the seedlings at about half the maximal level observed in maturing tobacco seeds.

The expression of the desaturase promoter was examined in developing seeds and flowers by histochemical staining for GUS activity (Fig. 6). In this figure, representative sections of 30-DAF seeds are shown from tobacco containing the desaturase promoter-GUS construct (Fig. 6, A and B). These results demonstrate strong levels of expression in the embryo and endosperm. Prominent staining in the cotyledons and poor expression in the axis of the embryo were consistently observed in different independent desaturase promoter-GUS transformants. By comparison, embryo sections from the 35S-GUS transformants showed a uniform distribution of GUS activity through the embryo and endosperm (Fig. 6, C and D). GUS activity was not observed in untransformed tobacco seeds.

Anther sections from a developing flower (bud length 20 mm) indicated that the desaturase promoter was most active in the tapetal layer (Fig. 6, E and F). The longitudinal section was incubated for 10 h in GUS substrate, whereas the anther cross-section was incubated for 2 h. When anther sections were taken from flowers close to opening (length 40 mm), staining was very strong in the pollen grains (Fig. 6G). In comparative sections from 35S-GUS transformants that were

Table I. *Comparison of GUS activity in young leaves and seeds of desaturase promoter-GUS tobacco transformants*

Transformant Line	GUS activity (SE)		Relative GUS Activity	
	Young leaf	Seed	Young leaf	Seed
	nmol min^{-1} mg^{-1} protein		%	
D9.1	6.75 (1.3)	34.3 (1.1)	16.5	83.5
D9.2	7.70 (2.3)	8.02 (0.3)	49.0	51.0
D9.3	3.59 (0.3)	32.5 (4.1)	10.0	90.0
D9.4	0.76 (0.2)	9.36 (1.5)	7.50	92.5
D9.5	5.89 (0.7)	11.6 (2.7)	33.6	66.4
D9.6	2.73 (0.9)	9.77 (1.9)	21.8	78.2
D9.7	21.4 (6.8)	9.95 (1.0)	68.3	31.7
D9.8	3.13 (0.4)	11.4 (1.0)	21.6	78.4
Average (SE)			28.5 (7.4)	71.5 (7.4)

Table II. *Comparison of GUS activity in organs of germinated seedlings with maximal levels in the seeds of desaturase promoter-GUS tobacco transformants*

Transformant Line	GUS Activity (SE)			
	Leaf	Root	Stem	Seed
	nmol min^{-1} mg^{-1} protein			
D9.1	45.6 (17.6)	28.6 (4.1)	45.2 (3.8)	34.3 (1.1)
D9.2	2.71 (0.3)	3.16 (1.3)	4.71 (0.5)	8.02 (0.3)
D9.6	0.27 (0.1)	2.43 (0.2)	1.63 (0.1)	9.77 (1.9)
D9.7	3.93 (0.8)	6.25 (0.2)	5.52 (0.2)	9.95 (1.0)
D9.9	14.9 (5.4)	5.07 (1.6)	17.1 (5.3)	6.54 (2.9)
	Relative GUS Activity			
	%			
D9.1	29.7	18.6	29.4	22.3
D9.2	14.6	17.0	25.3	43.1
D9.6	1.90	17.2	11.6	69.3
D9.7	15.3	24.4	21.5	38.8
D9.9	34.2	11.6	39.2	15.0
Average (SE)	19.1 (5.8)	17.8 (2.0)	25.4 (4.5)	37.7 (9.4)

Figure 6. (on facing page). Histochemical localization of GUS activity in sections of seeds and floral parts of tobacco plants containing desaturase (Δ9-GUS) or 35S-GUS promoter constructs. Patterns of expression were revealed by incubation with X-gluc. A, Longitudinal section through 30 DAF seed (Δ9-GUS); B, cross-section through 30 DAF seed (Δ9-GUS); C, longitudinal section through 30 DAF seed (35S-GUS); D, cross-section through 30 DAF seed (35S-GUS); E, longitudinal section through anther from 20-mm flower bud (Δ9-GUS); F, cross-section through anther from 20-mm flower bud (Δ9-GUS); G, cross-section through anther from 40-mm flower bud (Δ9-GUS); H, cross-section through anther from 40-mm flower bud (35S-GUS); I, section through entire 15-mm flower bud (Δ9-GUS); J, section through entire 20-mm flower bud (Δ9-GUS); K, cross-section through ovary from 20-mm flower bud (Δ9-GUS). Abbreviations: en, endosperm; em, embryo; ax, axis; ts, testa; lo, locule; tp, tapetum; et, endothecium; sm, stomium; po, pollen; sp, sepal; pt, petal; an, anther; ov, ovary; st, stamen; ow, ovary wall; ou, ovule; and pl, placenta.

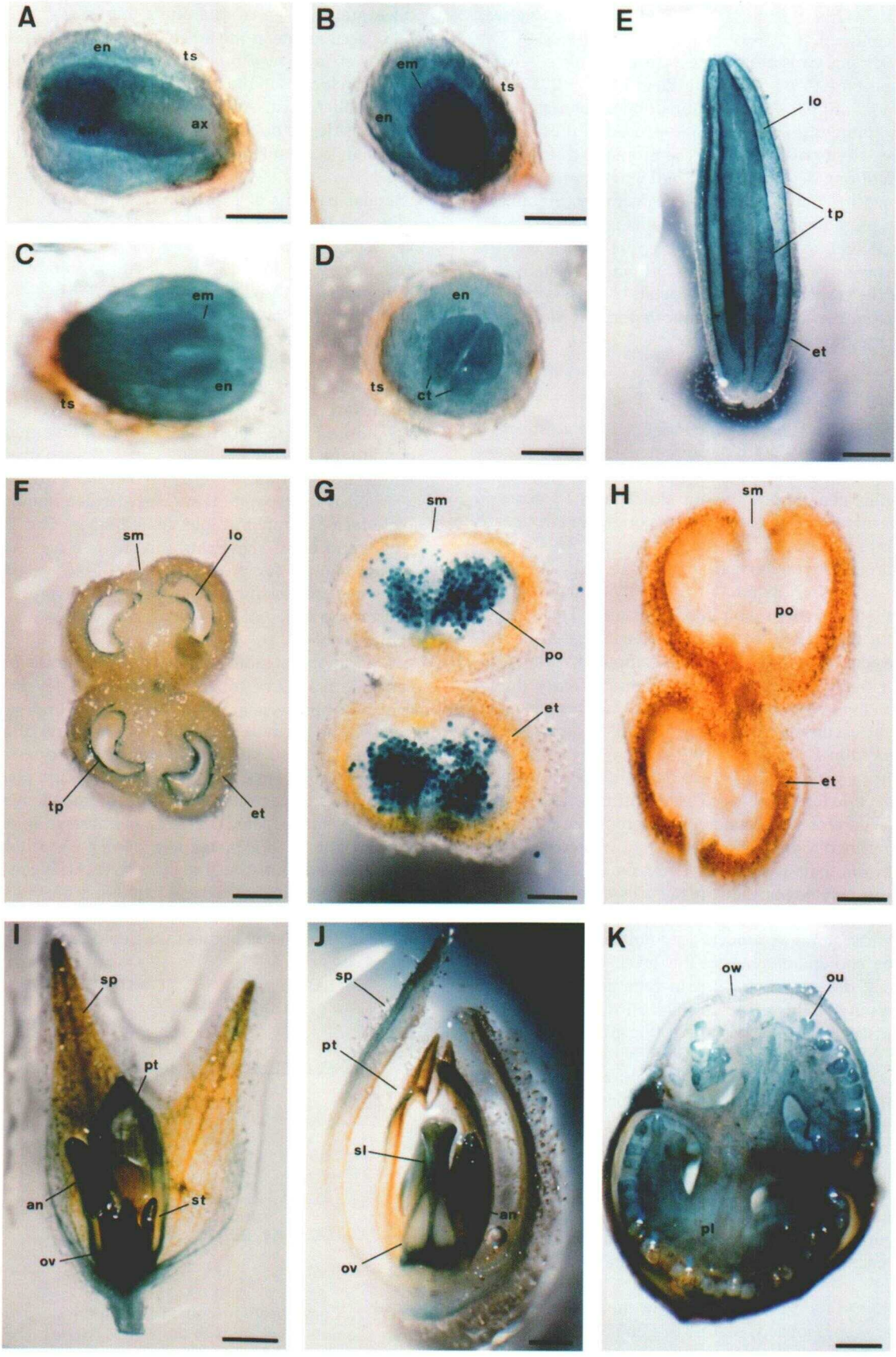

Figure 6. Legend appears on opposite page.

incubated in X-Gluc for the same period (2 h), staining was poor and confined to a few pollen grains (Fig. 6H). The cauliflower mosaic virus promoter expresses relatively weakly in this tissue type (Van der Leede-Plegt et al., 1992). Furthermore, no floral parts from untransformed tobacco exhibited GUS staining under the conditions used in these experiments. The activity of the desaturase promoter in the entire floral organ during development at bud lengths of 15 and 20 mm is shown in Figure 6, I and J, respectively. Staining is particularly prominent in the components undergoing rapid development such as the anther, the entire carpel, and the tips of the petals. Vascular tissue is prominently stained at the base of the carpel and in young petals. A cross-section of the ovary from the 20-mm bud indicated strong staining in the placenta and ovules.

DISCUSSION

In this paper the nucleotide sequence of a *B. napus* stearoyl-ACP desaturase gene (Bn10) is presented. Analysis of gene copy number by Southern blotting at high stringency indicated that this gene is one of four closely related genes in oilseed rape and that two of these genes originated from each of the parent species *B. rapa* and *B. oleracea*.

A 2-kb promoter fragment from stearoyl-ACP desaturase gene Bn10 was found to drive GUS activity at high levels in oleogenic tissues in transgenic tobacco plants. During seed development the activity of the promoter increased considerably between 15 and 25 DAF when compared with constitutive promoters. Activation of the promoter early during embryo development in tobacco suggests that this gene contributes to the accumulation of stearoyl-ACP desaturase transcript observed in *B. napus* embryos (Slocombe et al., 1992). This desaturase gene promoter was active in both the endosperm and cotyledons of tobacco seeds but to a lesser extent in the axis. Therefore, expression of the promoter in the seed is largely confined to the regions of storage oil accumulation. It is significant that in soybean embryos, ABA levels are higher in the cotyledons than in the axis (Schussler et al., 1984), which suggests that this hormone may be involved in dictating where storage products accumulate in the seed.

ABA was shown to increase the levels of message for stearoyl-ACP desaturase in vitro in rape zygotic embryos, and the rapidity of this effect suggested involvement at the level of transcription. This was interesting given the presence of a single ABRE (Williams et al., 1992) within the promoter sequence of one of the stearoyl-ACP desaturase genes (Bn10). To establish the effect of ABA on the regulation of this gene promoter, transgenic tobacco embryos containing the desaturase promoter-GUS construct were exposed to ABA in culture but showed no significant effect (data not shown). This problem may be circumvented by using microspore-derived haploid embryos. These have lower levels of endogenous ABA compared with zygotic embryos and could therefore be more responsive to exogenous ABA (Yamaguchi-Shinozaki et al., 1990).

In addition to its expected activity in the developing embryo of transgenic tobacco plants, the Bn10 desaturase promoter was highly expressed in other oleogenic tissues such as the endosperm, the tapetum in developing flowers, and in pollen grains taken from anthers prior to dehiscence. Lipid bodies accumulate in the tapetal cells during development of the flower in oilseed rape. After the tapetum disintegrates, the lipid relocates to the microspores to become a component of the pollen coat. Accumulation by synthesis in situ also occurs inside the pollen grain, and this process peaks slightly later than accumulation within the tapetum (Evans et al., 1992).

Similar patterns of expression in the tapetum and pollen grains were observed with the *A1* ACP gene from *Arabidopsis* expressed in tobacco (Baerson and Lamppa, 1993). Sequence similarities were found in the desaturase promoter to three DNA motifs (LAT 52/56 box, LAT 56/59 box, and PB core motif) from late pollen-expressing genes of tomato (Fig. 7). These motifs were shown to regulate expression in tobacco pollen grains on the basis of deletion and substitution studies using transient expression assays (Twell et al., 1991). Single matches were observed to the LAT 52/56 box (83% identity) and the LAT 56/59 box (70% identity). Five matches were found to the PB core motif, all with 71% identity. Expression of this lipid synthesis gene in oleogenic tissues may be determined by separate tissue-specific determinants or by a single factor common to oleogenic tissues. This latter possibility could be of significance in the use of lipid synthesis gene promoters for producing novel lipids in the seeds of transgenic crop plants. For example, the expression of such lipids in the pollen of crop plants may represent an environmental problem.

In addition to induction of this desaturase promoter in oleogenic tissues of tobacco plants, high levels of activity were also observed in parts of the plant undergoing rapid growth. The promoter was active in all parts of germinated seedlings, and in developing flowers it was expressed strongly in the tips of petals, anthers, carpel, ovary placenta, and ovules. This is consistent with the promoter meeting a requirement for elevated levels of membrane lipid synthesis. Similar patterns of expression in floral parts of tobacco were also observed with the *Arabidopsis A1* ACP gene promoter (Baerson and Lamppa, 1993). Expression in the stigma was linked by these authors to the observation that solanaceous plants accumulate oil bodies in the epidermal and subepider-

LAT 52/56 BOX		TTTAGTTATATA	
1	-1480	Tg TgGTTATATA	-1469
PB CORE MOTIF		TGTGGTT	
2	-942	TGTGGac	-948
3	-852	cGTGGTg	-858
4	-686	cGTGGTa	-680
5	-654	cGTGGgT	-648
6	- 12	TGTGGac	- 18
		A	
LAT 56/59 BOX		GAATTTGTGA	
7	-191	Gt gATa GTGA	-182

Figure 7. Comparison of sequences from the stearoyl-ACP desaturase promoter (Fig. 1B) with the LAT 52/56 box, LAT 56/59 box, and PB core motif of pollen-specific genes from tomato (Twell et al., 1991).

mal cells of this organ, which eventually contribute to the stigmatal exudate.

Stearoyl-ACP desaturase transcript and protein levels in the seed of *B. napus* at maximal levels are 100-fold higher than in the leaf (Slocombe et al., 1992). However, the specific GUS activity in tobacco generated by the 2-kb promoter fragment from Bn10 was only 2.5-fold greater in the seed than in young leaves. It is possible that the levels of specific GUS activity if described in terms of total protein may be underestimated in the seed relative to the leaf if a portion of the seed protein is solubilized in the extraction buffer. The discrepancy could also be due to the use of a heterologous system for the analysis of the rape promoter (Benfey and Chua, 1990). Nevertheless, these results suggest that this particular promoter may make a relatively minor contribution to the accumulation of desaturase message seen in the rape embryo. Variation in seed specificity between the four stearoyl-ACP desaturase *B. napus* described here is possible given that different regulation has been observed between seed-expressed ACP genes from *B. napus* (ACP05) and *Arabidopsis* (*A1*) (DeSilva et al., 1992; Baerson and Lamppa, 1993). In tobacco, the former expresses at 100-fold greater levels in the seed relative to the leaf, whereas the expression pattern for the latter was closer to that of the desaturase gene reported here.

In conclusion, a stearoyl-ACP desaturase gene (Bn10) promoter from *B. napus* was shown to drive GUS expression in tobacco seeds in a manner subject to temporal regulation. However, the levels of activity in the developing seeds were not greatly in excess of those in the leaf, suggesting that this promoter may not be a major contributor to seed transcript accumulation. The promoter also directed high levels of expression in a number of other specific tissues associated with TAG accumulation or with a likely requirement for high rates of membrane lipid synthesis. Potential regulatory sequences were identified within the promoter relevant to ABA induction and pollen grain expression. It would appear highly likely that the desaturase promoter is a multifunctional promoter where combinatorial mechanisms operate to generate a complex pattern of tissue-specific expression (Benfey and Chua, 1990).

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LITERATURE CITED

Abián J, Gelpi E, Pagès M (1991) Effect of abscisic acid on the linoleic acid metabolism in developing maize embryos. Plant Physiol **95:** 1277–1283

Baerson SR, Lamppa GK (1993) Developmental regulation of an acyl carrier protein gene promoter in vegetative and reproductive tissues. Plant Mol Biol **22:** 255–267

Benfey PN, Chua N-H (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science **250:** 959–966

Bensi G, Raugei G, Klefenz H, Cortese R (1985) Structure and expression of the human haptoglobin locus. EMBO J **4:** 119–126

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram amounts of protein using the principle of protein-dye binding. Anal Biochem **72:** 248–254

Covey SN, Hull R (1981) Transcription of cauliflower mosaic virus DNA. Detection of transcripts, properties and location of the gene encoding the virus inclusion body protein. Virology **111:** 463–474

Cummins I, Hills MJ, Ross JHE, Hobbs DH, Watson MD, Murphy DJ (1993) Differential, temporal and spatial expression of genes involved in storage oil and oleosin accumulation in developing rapeseed embryos: implications for the role of oleosins and the mechanisms of oil-body formation. Plant Mol Biol **23:** 1015–1027

DeLisle AJ, Crouch ML (1989) Seed storage protein transcription and mRNA levels in *Brassica napus* during development and in response to exogenous abscisic acid. Plant Physiol **91:** 617–623

DeSilva J, Robinson SJ, Safford R (1992) The isolation and functional characterisation of a *B. napus* acyl carrier protein 5′ flanking region involved in the regulation of seed storage lipid synthesis. Plant Mol Biol **18:** 1163–1172

Domoney C, Casey R (1985) Measurement of gene number for seed storage proteins in *Pisum*. Nucleic Acids Res **13:** 687–699

Evans DE, Taylor PE, Singh MB, Knox RB (1992) The interrelationship between the accumulation of lipids, protein and the level of acyl carrier protein during the development of *Brassica napus* L. pollen. Planta **186:** 343–354

Holbrook LA, Magus JR, Taylor DC (1992) Abscisic acid induction of elongase activity, biosynthesis and accumulation of very long chain monounsaturated fatty acids and oil body proteins in microspore-derived embryos of *Brassica napus* L. cv Reston. Plant Sci **84:** 99–115

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple method for transferring genes into plants. Science **227:** 1229–1231

Jefferson RA (1987) The GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Joshi CP (1987) An inspection of the domain between putative TATA box and translational start site in 79 plant genes. Nucleic Acids Res **15:** 6643–6653

Kater MM, Koningstein GM, Nijkamp HJH, Stuitje AR (1991) cDNA cloning and expression of the *Brassica napus* enoyl-acyl carrier protein reductase in *Escherichia coli*. Plant Mol Biol **17:** 895–909

Kavanagh T, Murphy G (1988) Speeding up the sequencing of double-stranded DNA. Nucleic Acid Res **16:** 5198

Knutzon DS, Thompson GA, Radke SE, Johnson WB, Knauf VC, Kridl JC (1992) Modification of a *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. Proc Natl Acad Sci USA **89:** 2624–2628

Lütcke HA, Chow KC, Mickel FS, Moss KA, Kern HF, Scheele GA (1987) Selection of the AUG initiation codons differs in plants and animals. EMBO J **6:** 43–48

MacKintosh RW, Hardie DG, Slabas AR (1989) A new assay procedure to study the induction of β-ketoacyl-ACP synthase I and II, and the complete purification of β-ketoacyl-ACP synthase I from developing seeds of oilseed rape (*Brassica napus*). Biochim Biophys Acta **1002:** 114–124

McKeon TA, Stumpf PK (1982) Purification and characterisation of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J Biol Chem **257:** 12141–12147

Murphy DJ, Cummins I (1989) Biosynthesis of seed storage products during embryogenesis in rapeseed, *Brassica napus*. J Plant Physiol **135:** 63–69

Nishida I, Beppu T, Matsuo T, Murata N (1992) Nucleotide sequence of a cDNA clone encoding a precursor to stearoyl-(acyl-carrier-protein) desaturase from spinach, *Spinacia oleracea*. Plant Mol Biol **19:** 711–713

Ohlrogge JB, Browse J, Somerville C (1991) Review: the genetics of plant lipids. Biochim Biophys Acta **1082:** 1–26

Pettersson M, Schaffner W (1987) A purine-rich DNA sequence motif present in SV40 and lymphotropic papovavirus binds a lymphoid-specific factor and contributes to enhancer activity in lymphoid cells. Genes Dev **1:** 962–972

Prescott A, Martin C (1987) A rapid method for the quantitative assessment of levels of specific mRNAs in plants. Plant Mol Biol Rep **4:** 219–224

Roesler WJ, Vandenbark GR, Hanson RW (1988) Cyclic AMP and the induction of eukaryotic gene transcription. J Biol Chem **263:** 9063–9066

Ryan AJ, Royal CL, Hutchinson J, Shaw CHS (1989) Genomic sequence of a 12S seed storage protein from oilseed rape (*Brassica napus* cv. Jet Neuf). Nucleic Acids Res **17:** 3584

Safford R, Windhust JHC, Lucas C, DeSilva J, James CM, Hellyer A, Smith CG, Slabas AR, Hughes SG (1988) Plastid-localised seed acyl-carrier protein of *Brassica napus* is encoded by a distinct, nuclear multigene family. Eur J Biochem **174:** 287–295

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sato A, Becker CK, Knauf VC (1992) Nucleotide sequence of a complementary DNA clone encoding stearoyl-acyl carrier protein desaturase from *Simmondsia chinensis*. Plant Physiol **99:** 362–363

Schussler JR, Brenner ML, Brun WA (1984) Abscisic acid and its relationship to seed filling in soybeans. Plant Physiol **76:** 301–306

Shanklin J, Somerville CR (1991) Stearoyl-acyl carrier protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc Natl Acad Sci USA **88:** 2510–2514

Slabas AR, Sidebottom CM, Hellyer A, Kessell RMJ, Tombs MP (1986) Induction, purification and characterisation of NADH-specific enoyl acyl carrier protein reductase from developing seeds of oil seed rape (*Brassica napus*). Biochim Biophys Acta **877:** 271–280

Slocombe SP, Cummins I, Jarvis RP, Murphy DJ (1992) Nucleotide sequence and temporal regulation of a seed-specific *Brassica napus* cDNA encoding a stearoyl-acyl carrier protein (ACP) desaturase. Plant Mol Biol **20:** 151–155

Slocombe SP, Fairbairn D, Bowra S, Taylor R, Murphy DJ (1993) Investigation of temporal and tissue-specific regulation of the stearoyl-acyl carrier protein (ACP) desaturase in oilseed rape. *In* A Cherif, GB Miled-Daoud, B Marzouk, A Smaoui, M Zarrouk, eds, Metabolism, Structure and Utilization of Plant Lipids. Centre Nationale Pédagogique, Tunis, Tunisia

Stanton BG, Schilperoort RA, Verma DPS (1988) Plant Molecular Biology Manual. Kluwer Academic Publishers, Amsterdam

Taylor MA, Smith SB, Davies HV, Burch LR (1992) The primary structure of a cDNA clone of the stearoyl-acyl carrier protein desaturase gene from potato (*Solanum tuberosum* L.) Plant Physiol **100:** 533–534

Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearoyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. Proc Natl Acad Sci USA **88:** 2578–2582

Twell D, Yamaguchi J, Wing RA, Ushiba J, McCormick S (1991) Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. Genes Dev **5:** 496–507

Van der Leede-Plegt LM, van de Ven BCE, Bino RJ, van der Salm TPM, van Tunen AJ (1992) Introduction and differential use of various promoters in pollen grains of *Nicotiana tabacum* and *Lilium longiflorum*. Plant Cell Rep **11:** 20–24

Weselake RJ, Pomeroy MK, Furukawa TL, Golden JL, Little DB, Laroche A (1993) Developmental profile of diacylglycerol in maturing seeds of oilseed rape and safflower and microspore-derived cultures of oilseed rape. Plant Physiol **102:** 565–571

Williams ME, Foster R, Chua N-H (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein building. Plant Cell **4:** 485–496

Yamaguchi-Shinozaki K, Mino M, Mundy J, Chua N-H (1990) Analysis of an ABA-responsive gene promoter in transgenic tobacco. Plant Mol Biol **15:** 905–912