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Biotin carboxyl carrier protein and carboxyltransferase subunits of the multi-subunit form of acetyl-CoA carboxylase from *Brassica napus*: cloning and analysis of expression during oilseed rape embryogenesis

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In the oilseed rape *Brassica napus* there are two forms of acetyl-CoA carboxylase (ACCase). As in other dicotyledonous plants there is a type I ACCase, the single polypeptide 220 kDa form, and a type II multi-subunit complex analogous to that of *Escherichia coli* and *Anabaena*. This paper describes the cloning and characterization of a plant biotin carboxyl carrier protein (BCCP) from the type II ACCase complex that shows 61 % identity/79 % similarity with *Anabaena* BCCP at the amino acid level. Six classes of nuclear encoded oilseed rape BCCP cDNA were cloned, two of which contained the entire coding region. The BCCP sequences allowed the assignment of function to two previously unassigned *Arabidopsis* expressed sequence tag (EST) sequences. We also report the cloning of a second type II

ACCase component from oilseed rape, the *β*-carboxyltransferase subunit (*β*CT), which is chloroplast-encoded. Northern analysis showed that although the relative levels of BCCP and *β*CT mRNA differed between different oilseed rape tissues, their temporal patterns of expression were identical during embryo development. At the protein level, expression of BCCP during embryo development was studied by Western blotting, using affinity-purified anti-biotin polyclonal sera. With this technique a 35 kDa protein thought to be BCCP was shown to reside within the chloroplast. This analysis also permitted us to view the differential expression of several unidentified biotinylated proteins during embryogenesis.

INTRODUCTION

Plant acetyl-CoA carboxylase (ACCase; EC 6.4.1.3) is one of the pivotal enzymes of fatty acid biosynthesis in both leaf and seed tissue [1]. It catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA and is thought to be an important regulatory step in *de novo* fatty acid synthesis in chloroplasts [2]. Its central role reflects its importance as a target for the commercial aryloxyphenoxypropionate-type herbicides used presently [3]. The herbicides' action differentiates between the two forms of ACCase found in dicotyledonous plants [4].

All the ACCases so far studied have a biotin moiety, covalently bound to a lysine residue, that is thought to act as a molecular arm to pass the carboxy group between the subunit active sites [5]. However, the molecular organization of the enzyme differs according to the source of the enzyme. The mammalian form (type I) is a large multifunctional polypeptide [6–8] of at least 200 kDa made up of three fused domains [biotin carboxylase (BC), biotin carboxylase carrier protein (BCCP), and the carboxyltransferase (CT)]. The protein represents one of the four biotinylated enzymes in mammalian cells and one of three in yeast [9,10]. In contrast, the *Escherichia coli* form (ACCase type II) consists of a polypeptide complex [11–13] consisting of BC dimer, BCCP dimer and CT tetramer ($\alpha_2\beta_2$) subunits. These are encoded by four separate genes, which in *E. coli* are named *acc*C,

*acc*B, *acc*A and *acc*D respectively. The BCCP subunit is biotinylated *in vivo* but, in contrast with BCCP from mammals and yeast, represents the only biotinylated protein in *E. coli* [10]. In higher plants at least six biotinylated proteins are present in crude extracts [14].

Initially, plant ACCase was thought to be of the type I form, as indicated by the isolation of both plant/photosynthetic algal ACCase protein and cDNA/genomic clones indicative of the 220 kDa form [15–25]. The early understanding of ACCase in plants as being solely of the mammalian-like type I form was in retrospect over-simplistic. As far back as 1972 Kannagara and Stumpf reported a smaller plant ACCase indicative of type II [26]. In addition, functional ACCases similar to the multi-subunit complexes in *E. coli* were observed in spinach chloroplasts, barley chloroplasts and avocado plastids [26–29]. However, until recently these observations were largely discounted because ACCase I was shown to be highly susceptible to protease degradation [30], and most reports studied a form of at least 220 kDa.

In 1992 the sequencing of the *acc*D gene, encoding the *β*-subunit of the carboxyltransferase (*β*CT) subunit of the *E. coli* ACCase [13], enabled a putative function to be assigned to an open reading frame (ORF) reported in the chloroplast genome of several plant species. The ORFs included pea [31,32], tobacco [33], the parasitic plants *Cuscuta reflexa* (G. Haberhausen;

EMBL accession number X69803) and *Epifagus virginiana* [34], *Pinus thunbergii* [35], *Marchantia polymorpha* [36], *Physcomitrella patens* [37] and *Angiopteris lygodiifolia* [38]. Despite the knowledge of these homologous ORFs, much less was known about their expression and the association between their predicted products and ACCase activity. Indeed, the pea chloroplast DNA ORF was first designated as a putative DNA binding protein containing a zinc-finger motif [39]. The transcriptional analysis of the *accD* homologues in plants has so far been confined to pea, where it has been shown to be complex as a result of multiple transcription start sites and co-transcription with genes both 5′ and 3′ [31,39].

The realization that there were two forms of ACCase in dicotyledonous plants was brought about by Li and Cronan [40] initially, and more exhaustive studies followed soon afterwards [41,42]. Antibodies generated to an oligopeptide derived from the pea βCT sequence were used to immunoprecipitate an 87 kDa *accD*-like protein from pea leaf chloroplast extract [42]. This led to the loss of ACCase activity from the extract. The protein antigen co-precipitated with two other proteins of 91 and 35 kDa, suggestive of a complex *in vivo* [42]. These polypeptide subunits were shown to be organized into a large complex similar to that of *E. coli* ACCase. The 35 kDa protein was biotinylated *in vivo* and was located in the chloroplast, highly indicative of its being BCCP, whereas the 91 kDa associated protein was thought to be the BC subunit. A recent study by Shorrosh et al. [25] described the cloning and characterization of the tobacco BC subunit from type II ACCase.

With two forms of ACCase present in dicotyledonous plants the possibility arose that only one or both types might play a part in *de novo* lipid synthesis. In pea leaves it has been shown that both types are present, but in different cellular and subcellular compartments [41]. The larger type I is found in the cytosol of epidermal cells whereas type II is in the chloroplasts of mesophyll cells. It has been suggested that because plant ACCase I is induced by UV irradiation and fungal elicitors, its role is mainly biosynthesis of flavonoids and waxes [43,44] through a fatty acid chain extension activity. The chloroplast is thought to be the site for *de novo* fatty acid synthesis in mesophyll cells [45] and BCCP resides within the chloroplast. It is therefore reasonable to suppose that a type II *Brassica napus* ACCase would be mainly associated with *de novo* lipid synthesis.

As part of the *Arabidopsis* genome project, random cDNA clones are continually being partly sequenced and compared with the available databases [46]. If no significant similarity is shown the clone remains unassigned. Although *Arabidopsis* provides an ideal model for plant genetic manipulation it is not suitable as an agricultural crop. Oilseed rape is the major oil-bearing crop in Europe and is amenable to genetic engineering. Several groups are working on the manipulation of its fatty acid content [47] and on the production of alternative products such as polyhydroxybutyrate [48].

Because both BC [25] and βCT [31–38,42] have been cloned from different plants, a full understanding of the type II ACCase requires a full-sized ORF BCCP subunit cDNA clone. We report here the successful use of an EST sequence to clone *B. napus* BCCP cDNAs. The BCCP sequence data obtained allowed us to assign an identity to two previously unassigned ESTs i.e. 5′ BCCP gene. Using degenerate primers we also cloned a second member of the ACCase II complex, the chloroplast-encoded βCT. Both the BCCP and βCT oilseed rape clones were used as a tool for the first comparative study of the RNA expression of two type II ACCase subunits during oilseed rape embryo development. Western analysis of biotinylated protein levels during oilseed rape embryogenesis allowed us to view the temporal expression of BCCP and other unidentified biotinylated proteins.

MATERIALS AND METHODS

cDNA library construction

The cDNA library used was generated by using poly(A)$^+$ mRNA isolated by the method of Logemann et al. [49] from mid-cotyledon-stage developing embryos of *B. napus* cv. Jet neuf. First-strand synthesis was performed with poly(dT) oligonucleotide primers in accordance with the manufacturers' instructions (Amersham International). The resulting cDNAs generated were cloned into the *Eco*RI/*Xho*I sites of λ-ZAP II as recommended by the manufacturers (Stratagene). The host bacterium used was XL-1 Blue (Stratagene).

Strategy for βCT cloning and sequencing

Degenerate oligonucleotide primers were designed against the conserved peptide motifs MDEDMV and FGMLGD (residues 293–298 and 432–437 respectively in the tobacco protein) found in the predicted amino acid sequences of all the published *accD* homologues. These oligonucleotides were used with PCR to amplify a single product of approx. 440 bp from (i) *B. napus* chloroplast DNA, (ii) plasmid psbLa containing a 4.9 kb *Hin*dIII insert of *Arabidopsis thaliana* chloroplast DNA shown previously to encode an *accD* homologue (as judged by partial sequence analysis; W. Schuster, unpublished work), and (iii) total DNA extracted from *B. napus* (results not shown). The PCR product from total *B. napus* DNA was cloned into the *Xba*I/*Bam*HI sites of pBluescript (pβCT1) and sequenced. Sequence analysis showed that pβCT1 encoded an *accD* homologue. On the basis of amplification of a PCR product of identical size from both chloroplast DNA and total DNA, the insert from pβCT1 was used as a probe to screen a total genomic library of *B. napus* cv. Jet neuf. Several clones were isolated. A *Hin*dIII fragment of approx. 5 kb was present in several overlapping genomic clones and was recognized by pβCT1. The *Hin*dIII fragment from one clone was subcloned into pBS (pβCT2) and sequenced 5′ and 3′ from within the coding region identified in pβCT1 by using overlapping primers.

Probe preparation and labelling

The cDNA probes used for screening the rape libraries were generated by appropriate restriction endonuclease digestion of cDNA containing plasmids. The DNA fragment required was separated from vector DNA by TAE agarose electrophoresis and isolated by using the GeneClean II kit (Bio 101) or by freezing and ultrafiltration.

The probe (200–300 ng) was either radiolabelled with [α-^{32}P]dCTP with the Megaprime kit as recommended by the manufacturers (Amersham International) to a level of 5×10^9 c.p.m./μg or 3′ end labelled with Klenow polymerase. Unincorporated label was removed by using Biospin chromatography columns (Bio-Rad).

Library screening

For the rape poly(dT)-primed cDNA libraries 150000 plaque-forming units were screened as previously described [20]. The filters were hybridized at 65 °C and washed four times with $2 \times$ SSC, 0.1 % SDS within 30 min at 65 °C. Plasmid rescue of cDNA clones was carried out as described in the Stratagene protocol for *in vivo* excision of pSK from λ-ZAP II.

Sequencing of DNA clones

Sequencing was performed with an Applied Biosystems Inc. 373A DNA sequencer (Ms. J. Bartley, Durham University Sequencing Service). Both forward and reverse primers (−21m13 and M13RP1) were used initially for all clones. Further sequencing was performed with a combination of forward and reverse primers and synthesized oligonucleotide primers. Primers were made by using a 381A DNA synthesizer (Applied Biosystems Inc.). Computer analysis of DNA sequence was performed with the SEQNET GCG package from the SERC facility at Daresbury, and DNA Strider shareware [50].

Northern blot analysis

Total RNA (for βCT blot) and poly(A)$^+$ mRNA (for BCCP blot) was prepared from 5 g of young leaf, 5 g of root and 5 g of embryos harvested at 15, 22, 29, 36, 42 and 49 d after anthesis, with the recommended procedure (Pharmacia mRNA purification kit). Poly(A)$^+$ mRNA (1–5 μg) and total RNA (10 μg) were loaded onto a 1 % (w/v) formamide/formaldehyde agarose gel for electrophoresis. The Northern blot procedure was performed as described previously [20].

B. napus protein extracts

Embryos were removed from seeds of *B. napus* at defined days after flowering and stored in liquid nitrogen until required. Ten embryos from different days after flowering were used for protein extraction. Embryos were ground in liquid nitrogen with a pestle and mortar, weighed and kept frozen in 15 ml polypropylene tubes. Ten volumes of boiling extraction buffer [50 mM Tris, 1 mM EDTA, 0.5 mM DTT, 10 % (v/v) glycerol, 1 mM PMSF, 0.5 % Triton X-100 and 2 % (w/v) SDS] were added, vortexed and placed in a vigorously boiling water bath for 20 min. The extracts were cooled and spun at 10 000 g. The supernatant from between the fat layer and the pellet was removed and used for SDS/PAGE analysis. Root and leaf extracts were also prepared by this method.

Chloroplasts were isolated from 10-day-old rape seedlings by pelleting through a 40 % Percoll layer (a gift from Dr. I. M. Evans).

Western blot analysis

SDS/PAGE was performed with 12 % gels. Equal loading of all protein extracts were made. Biotinylated SDS molecular mass markers (Sigma) were also loaded. The protein was transferred to nitrocellulose (Hybond C extra, Amersham) by wet-blotting in 25 mM Tris, 192 mM glycine, 0.038 % SDS, 20 % (v/v) methanol at 40 mA for 16 h. The nitrocellulose was then exposed to 2 % (w/v) SDS at 100 °C and allowed to cool. After extensive washing with Milli-Q water and PBS-T [137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.75 mM KH$_2$PO$_4$, 0.1 % (v/v) Tween-20, pH 7.4] the nitrocellulose was blocked in 3 % (w/v) BSA in PBS-T containing 0.02 % azide for 2 h. This and all subsequent steps were performed at 4 °C. The nitrocellulose was incubated with a 1:250 dilution of affinity-purified sheep anti-biotin antibodies for 2 h in 1 % BSA in PBS-T containing 0.02 % thimerosal. After being washed three times for 10 min with PBS-T, the membrane was incubated for a further 2 h with donkey anti-sheep IgG conjugated with alkaline phosphatase (Serotec) in 1 % BSA in PBS-T containing 0.02 % thimerosal. The membrane was washed a further three times for 10 min with PBS-T. The blot was developed with BCIP/NBT for approx. 5 min at room temperature until the background began to develop, at which

time the membrane was transferred to distilled water for extensive washing.

RESULTS

BCCP cDNA cloning

ACCase catalyses a pivotal step in fatty acid synthesis. Isolation of the ACCase II genes from oilseed rape is therefore desirable for the manipulation of fatty acid synthesis in the crop *B. napus*. To clone the BCCP subunit from the multi-subunit form of ACCase we required a probe. Previous data from this laboratory had shown that *Arabidopsis* DNA sequences are highly homologous with those of rape (results not shown). As a first step we analysed the *Arabidopsis* EST database for any *E. coli* BCCP-like sequences. A 410 bp sequence entry was identified that showed similarity to the *accB* gene (BCCP subunit) from *E. coli*. It contained a homologous biotin-binding domain sequence but its ORF was shorter than that of *E. coli* because a large part of the clone was 3′ untranslated region (UTR). The EST cDNA clone (EST-ID:ATTS1191, GenBank-ID:Z25714) was obtained from the Ohio State Genetic Resource Centre.

Having identified a heterologous probe we used it to screen 150 000 plaque-forming units from both leaf- and embryo-derived poly(dT)-primed mRNA libraries from *B. napus* cv. Jet neuf. The primary round of leaf library screening yielded no hybridizing plaques. However, with the embryo library 24 positive plaques were identified, which were taken through three rounds of screening to plaque purity. Complete sequencing of several clones revealed six different classes (Figure 1) typified by pBP1 (842 bp), pBP2 (421 bp), pBP3 (925 bp), pBP4 (1008 bp), pBP6 (1133 bp) and pBP7 (720 bp), which all had significant similarity to other bacterial and algal BCCP sequences. The six classes fell into two groups based on their 3′ UTR and ORF similarity (group 1, pBP1 and pBP2; group 2, pBP3, pBP4, pBP6 and pBP7). The percentage derived amino acid sequence similarity/identity between pBP1 and pBP2 (subgroup 1) was 89.4 %/83.3 %, pBP4 and pBP6 (representatives of subgroup 2) 85.7 %/80.1 %, and pBP1 and pBP4 (representatives of subgroups 1 and 2) 65.9 %/50.8 %. Other inter- and intra-group comparisons showed very similar figures, further demonstrating the existence of the two subgroups. The *Arabidopsis* EST cDNA (EST-ID:ATTS1191) used to isolate the clones showed similarity to all the clones in the ORF region but only to the group 1 clones in the 3′ UTR region. The pBP2 insert was 79 % identical at the DNA level, throughout the whole length, with the EST cDNA sequence. Analysis of the two longest clones pBP4/pBP6 identified a putative translational start methionine codon, which showed 100 % homology with the consensus sequence for plants AACAAUGGC [51]. In addition both clones had classical poly(A) tails at their 3′ ends. Both features together suggest that pBP4 and pBP6 are full-length clones with respect to the coding region. Computer analysis, based on the method of von Heijne [52], of the sequence identified a putative transit peptide sequence cleavage site that is conserved between both full-length ORF clones, pBP4 and pBP6.

Comparison of the derived amino acid sequence from the longest clone (pBP6) with that of *Anabaena* and *E. coli* BCCPs shows that the similarity between the proteins is primarily at their N- and C-termini (Figure 2). A comparison of *Anabaena* and *E. coli* BCCPs is also presented pictorially to demonstrate the known divergences (Figure 2). The direct amino acid GAP comparison (BBSRC Computing Facility, Daresbury) with the *E. coli* BCCP shows similarity throughout the length of the *E. coli* protein with the longest continuous stretch occurring around the biotinylation site (Figure 2). It has been shown previously
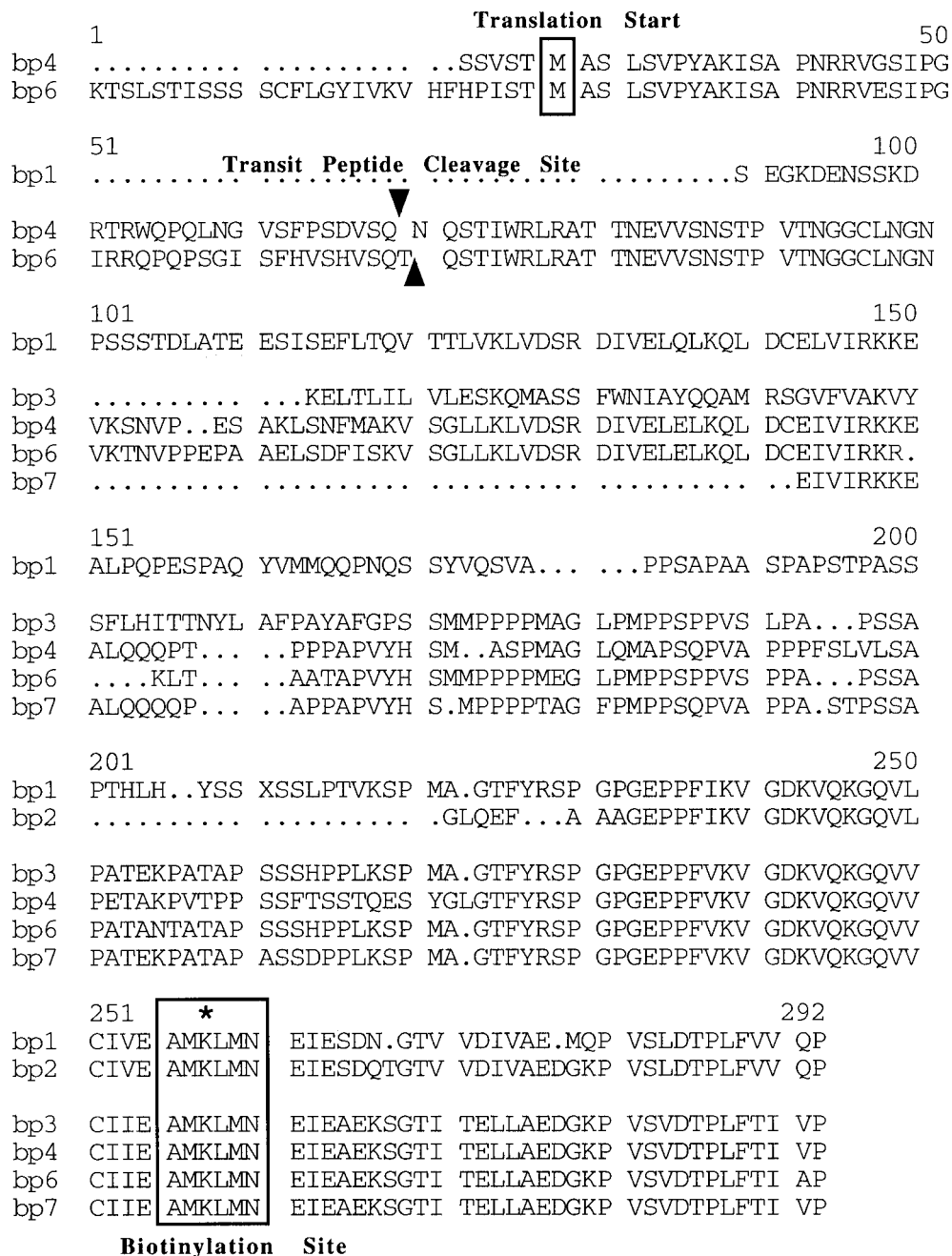
```
                                      Translation   Start
           1                                                                50
    bp4    .......... ...........  ..SSVST M AS  LSVPYAKISA  PNRRVGSIPG
    bp6    KTSLSTISSS SCFLGYIVKV  HFHPIST M AS  LSVPYAKISA  PNRRVESIPG

           51                                                               100
    bp1    .......... ...Transit Peptide Cleavage Site ..........S EGKDENSSKD

                                         ▼
    bp4    RTRWQPQLNG VSFPSDVSQ N QSTIWRLRAT TNEVVSNSTP VTNGGCLNGN
    bp6    IRRQPQPSGI SFHVSHVSQT▲ QSTIWRLRAT TNEVVSNSTP VTNGGCLNGN

           101                                                              150
    bp1    PSSSTDLATE ESISEFLTQV TTLVKLVDSR DIVELQLKQL DCELVIRKKE

    bp3    .......... ...KELTLIL VLESKQMASS FWNIAYQQAM RSGVFVAKVY
    bp4    VKSNVP..ES AKLSNFMAKV SGLLKLVDSR DIVELELKQL DCEIVIRKKE
    bp6    VKTNVPPEPA AELSDFISKV SGLLKLVDSR DIVELELKQL DCEIVIRKR.
    bp7    .......... .......... .......... .......... ..EIVIRKKE

           151                                                              200
    bp1    ALPQPESPAQ YVMMQQPNQS SYVQSVA... ...PPSAPAA SPAPSTPASS

    bp3    SFLHITTNYL AFPAYAFGPS SMMPPPPMAG LPMPPSPPVS LPA...PSSA
    bp4    ALQQQPT... ..PPPAPVYH SM..ASPMAG LQMAPSQPVA PPPFSLVLSA
    bp6    ....KLT... ..AATAPVYH SMMPPPPMEG LPMPPSPPVS PPA...PSSA
    bp7    ALQQQQP... ..APPAPVYH S.MPPPPTAG FPMPPSQPVA PPA.STPSSA

           201                                                              250
    bp1    PTHLH..YSS XSSLPTVKSP MA.GTFYRSP GPGEPPFIKV GDKVQKGQVL
    bp2    .......... .......... .GLQEF...A AAGEPPFIKV GDKVQKGQVL

    bp3    PATEKPATAP SSSHPPLKSP MA.GTFYRSP GPGEPPFVKV GDKVQKGQVV
    bp4    PETAKPVTPP SSFTSSTQES YGLGTFYRSP GPGEPPFVKV GDKVQKGQVV
    bp6    PATANTATAP SSSHPPLKSP MA.GTFYRSP GPGEPPFVKV GDKVQKGQVV
    bp7    PATEKPATAP ASSDPPLKSP MA.GTFYRSP GPGEPPFVKV GDKVQKGQVV

           251        *                                                     292
    bp1    CIVE AMKLMN  EIESDN.GTV VDIVAE.MQP VSLDTPLFVV QP
    bp2    CIVE AMKLMN  EIESDQTGTV VDIVAEDGKP VSLDTPLFVV QP

    bp3    CIIE AMKLMN  EIEAEKSGTI TELLAEDGKP VSVDTPLFTI VP
    bp4    CIIE AMKLMN  EIEAEKSGTI TELLAEDGKP VSVDTPLFTI VP
    bp6    CIIE AMKLMN  EIEAEKSGTI TELLAEDGKP VSVDTPLFTI AP
    bp7    CIIE AMKLMN  EIEAEKSGTI TELLAEDGKP VSVDTPLFTI VP
                Biotinylation   Site
```

Figure 1   Derived amino acid sequences of BCCP

ORF-derived amino acid sequences of the *B. napus* BCCP cDNA clones pBP (shown as bp in the figure) 1/2/3/4/6/7 (EMBL accession numbers X90727–X90732 respectively). Sequences were aligned by using the CLUSTAL programme from Daresbury. The boxed regions show putative translational start and transit peptide cleavage sites as well as the conserved biotinylation site.

that approximately 30 amino acid residues on each side of the biotinylated site are important for biotinylation *in vivo* [53–55]. Although the rape BCCP biotinylation site identified in the derived amino acid sequences of all the clones differs from that of *E. coli* BCCP it is identical with that of *Anabaena* (Ala-Met-Lys-Leu-Met). A TBLASTN search [56] of all available databases with the pBP6-derived amino acid sequence showed *E. coli*, *Pseudomonas* and *Anabaena* BCCPs to have the highest scores of 237 (44 % identical/59 % similar over the last 104 residues), 255 (48 % identical/64 % similar over the last 102 residues) and 248

(61 % identical/79 % similar over the last 72 residues) respectively. Scores of over 100 are generally thought to be significant. The only other non-BCCP biotinylated protein that featured in the top 50 derived protein sequences was human propionyl-CoA carboxylase, with a score of 97. Most of the other proteins that showed similarity with the translated pBP6, with the TBLASTN method, had a high proline amino acid content, a key feature of the central region of the translated BCCP gene (Figure 1). The derived BCCP sequences showed an extraordinary number of proline/alanine residues, consisting of 35 % of the amino acids
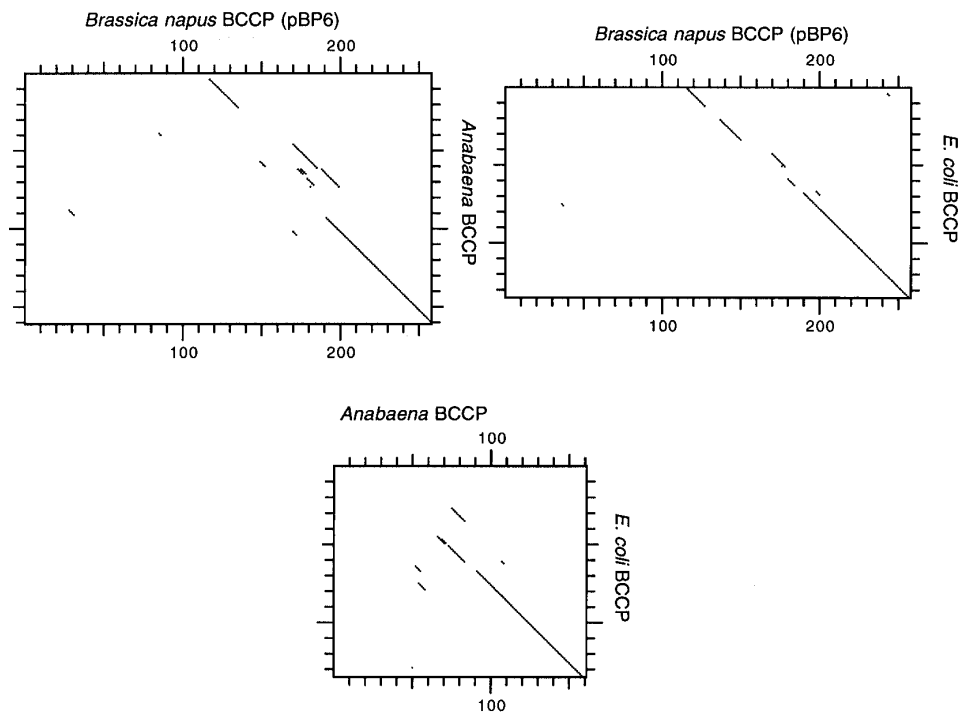
Figure 2   ACCase type II BCCP subunit sequence analysis

Comparison of *B. napus* BCCP with *E. coli* and *Anabaena* BCCP sequences at the derived amino acid level. Dot-matrix analyses (DNA strider Stringency 7, Window 23) of *B. napus* BCCP against *Anabaena* and *E. coli* BCCP in addition to that of *Anabaena* against *E. coli* are shown for comparison.

between residues 150 and 250 (Figure 1), also a characteristic of *E. coli* BCCP [13].

Assignment of function to ESTs

The overlapping, as yet unidentified, ESTs 97C18T7 and 115I6T7 (accession numbers T21716 and T43109 respectively) were shown to be homologous with the 5′ end of pBP4 and pBP6 with identity in 70 % of the derived amino acid sequence (results not shown). The clones can therefore with some confidence be assigned as *Arabidopsis* BCCP.

*β*CT cloning and sequencing

Using all the available *acc*D homologue sequence data, degenerate oligonucleotides were used to amplify a single product of approx. 440 bp from *B. napus* total DNA. A PCR product of identical size was amplified from both rape chloroplast DNA and an *Arabidopsis* subclone containing the *acc*D homologue. The 440 bp PCR product was subcloned and fully sequenced (p*β*CT1), which showed it to be an *acc*D homologue. The 440 bp was used to screen a total genomic library of *B. napus* cv. Jet neuf and several clones were isolated. A *Hin*dIII fragment of approx. 5 kb was present in several overlapping genomic clones that hybridized to the p*β*CT1 insert. The *Hin*dIII fragment from one clone was subcloned (p*β*CT2) and sequenced (results not shown, accession number Z50868).

The ORF within p*β*CT2 encodes a protein of 489 amino acids that has 66 % identity and 78 % similarity over its full length with the *acc*D homologue encoded by the tobacco chloroplast DNA [33]. Three methionine residues are found very close to the first stop codon at the N-terminus. The residue giving the longest

ORF is assumed to be the N-terminus on the basis of similarity with the N-termini of the tobacco, *E. virginiana* and *C. reflexa* ORFs (Figure 3). Broad comparison of the *B. napus acc*D amino acid sequence with other published plant sequences is not straightforward as they fall into distinct subclasses. In the region homologous with that encoded by the full length of *E. coli acc*D, the proteins from mosses, liverworts, ferns and *Pinus* have slightly lower similarities/identities with the *B. napus* protein than those from tobacco, *Epifagus*, *Cuscuta* and pea (see Table 1). These relatively small differences are a reflection of much greater divergence between these proteins in their N-termini. Although all of them contain a region homologous with that encoded by the full length of *E. coli acc*D (Table 1) [13] they diverge in their N-termini.

The mosses, liverworts and ferns [36–38] and *Pinus* [35] form a group in which the *acc*D proteins all possess a 25-residue extension, with a high degree of identity, beyond the N-terminus of the *E. coli* protein. Alignment of the five other plant protein sequences, which are much longer, revealed that the *B. napus*, tobacco and *E. virginiana* ORFs and amino acid sequences were most similar (Figure 3). The pea ORF contains two large inserts, one in each of the N- and C-terminal halves that result from repeated DNA sequences identified previously [31,32]. The alignment also showed the deletion of a region from the pea protein (from between Ile-72 and Thr-73) that is conserved in *B. napus*, tobacco, *E. virginiana* and *C. reflexa* (Figure 3). The ORF from *C. reflexa* also contains an insert (from Ser-69 to Asn-112) by comparison with the other amino acid sequences but is otherwise very similar. The functional significance of the N-terminal halves of the higher-plant polypeptides can, at present, only be speculated on. The presence of substantial regions of identity within this domain suggests conservation of structure

Figure 3   ACCase type II β-carboxyltransferase subunit sequence analysis

Amino acid alignment of the N-terminal halves of the predicted higher-plant polypeptides with similarity to the βCT subunit of ACCase [40]. Sequences were aligned by using the PILEUP and LINEUP programs and were taken from pea [31], tobacco [42], *Epifagus virginiana* [4] and *Cuscuta reflexa* (G. Haberhausen; EMBL accession number X69803). Identical residues are boxed.

and/or function. A search of the translation products from the EMBL Database with the conserved polypeptide motifs found within this region failed to identify any significant relationship with any other polypeptides. *B. napus* βCT is exceptional within the C-terminal region of the *acc*D homologues. The zinc-finger motif previously identified [31,32] is found as $CXXCX_{15}CXXC$ (where X is any amino acid) in all sequences, including that of *E. coli*, except for *B. napus* where the central domain contained only 12 residues (Figure 3). Apart from this difference and the insert present in pea, the C-terminal halves of all the *acc*D homologues are remarkably similar (Table 1).

Northern blot analysis of BCCP and βCT expression

Storage lipid synthesis occurs in *B. napus* seed with a maximal rate of production during the middle stage of embryogenesis. Turnham and Northcote [56] showed that ACCase enzymatic activity in rape embryos followed the rate of lipid deposition; more recently Kang et al. [57], using different extraction techniques, showed that ACCase activity increased before lipid

deposition. In both cases the activity decreased before the maximal accumulation of lipid occurred. To study the mRNA expression of the BCCP genes, which are thought to be involved in *de novo* storage lipid synthesis, we screened a developmental Northern blot. Each lane on the blot had an equal amount of root, leaf or embryo poly(A)$^+$ mRNA from different stages of *B. napus* embryogenesis. The blot shown in Figure 4A was generated by screening with the pBP7 insert and was identical when all six cDNA types were used on separate occasions (results not shown). During embryogenesis, BCCP mRNA expression (1.2 kb full length) rose steeply, peaking during the middle portion of embryogenesis. Maximal mRNA expression occurred at 54 d, after which the level of BCCP mRNA dropped markedly. This profile mimicked that of the other *B. napus* fatty acid synthesis components enoyl reductase, β-keto reductase and ACCase I [20]. Although there was a notable amount of BCCP mRNA present in root there was a comparatively low level in leaf. The integrated phosphoimager data showed that the level of BCCP mRNA expression in 54-day embryos is 26 times that of leaves, which is itself only twice that of background.

Table 1   Comparison of the predicted amino acid sequence from *B. napus* with those of all other published sequences with similarity to the βCT subunit of ACCase [40]

The comparison was made by using the BESTFIT program and is restricted to residues 220–489 of the *B. napus* sequence. To enable sensible comparison the insert in the pea sequence (residues 234–394) has been deleted. Sequences are taken from the references described in Figure 3 and also: *Angiopteris lygodiifolia* [38], *Marchantia polymorpha* [36], *Physcomitrella patens* [37] and Black pine [35].

| | *B. napus* | |
Species	βCT identity (%)	βCT similarity (%)
Tobacco	79	86
E. virginiana	77	87
C. reflexa	72	83
Pea	68	81
M. polymorpha	69	79
Black pine	67	77
P. patens	66	76
A. lygodiifolia	66	76
E. coli	41	62

The *B. napus* chloroplastic genome-encoded βCT subunit was used in a Northern blot against total RNA from the same batch of whole seeds used for the BCCP Northern data (see Figure 4A). The cDNA insert hybridized to several bands of 2.4, 1.6, 1.3 and 1.1 kb (Figure 4B), which were expressed proportionally through-out embryogenesis, the strongest association being with a band of approx. 2.4 kb. Because all the bands are proportional we made the assumption that they all contain the βCT gene. Northern analysis of pea βCT was complicated in a similar way

as a result of multiple transcription start sites and co-transcription with genes both 5′ and 3′ [31,39]. Nagano et al. [31] showed that three transcripts with sizes of 5.0, 2.8 and 2.4 kb contained the entire ORF of *acc*D and ORFs of both *trn*Q and *psa*I genes. The blot also shows (Figure 4B) that in leaf material there is one extra hybridizing RNA species not present in embryo tissue (2.7 kb) and a band visible in embryos (2.6 kb) not present in leaves.

It can be seen from a comparison of Figures 4A and 4B that the rape βCT mRNA expression was very similar to that of BCCP during embryogenesis. Because both proteins are thought to associate *in vivo* to form part of the type II ACCase complex, this was perhaps to be expected. The peak of expression occurred at 54 d after anthesis, followed by the characteristic decrease in expression. However, in contrast with BCCP the relative amount of βCT expression in leaf was higher with an embryo:leaf ratio of 7:2.

Anti-biotin Western blot of rape embryos

To gain a real insight into BCCP expression it was important to study protein levels as well as mRNA. In the absence of specific BCCP antibodies, and because BCCP is biotinylated *in vivo*, biotin-specific antiserum was used for this purpose. To generate biotin-specific antibodies, keyhole limpet haemocyanin was coated with biotin and used as an antigen in rabbits. To minimize false background signals in a Western blot the antibodies generated were affinity-purified. This was achieved by column chromatography on a biotin-agarose matrix. The generated antibodies were more specific and had a higher titre than those available commercially.

Previous work describing the prokaryotic BCCP studies showed that the proline/alanine-rich region of *E. coli* BCCP confers an SDS gel anomaly [13]. The protein runs at 35 % less
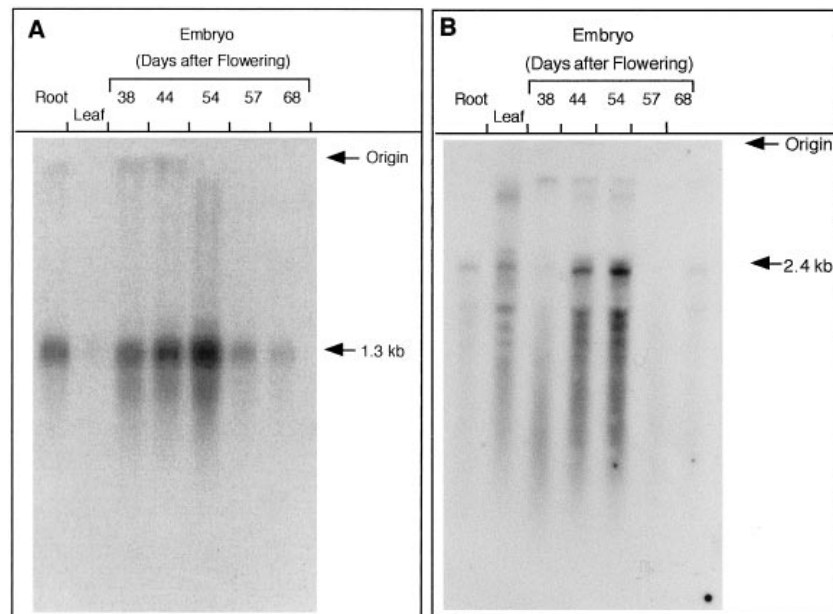

Figure 4   Northern blot analysis of ACCase type II BCCP and βCT subunit expression during rapeseed embryogenesis

(**A**) Northern analysis with the BCCP cDNA (pBP7 insert) as a probe: 1 μg of poly(A)⁺ RNA from embryos 38, 44, 54, 57 and 68 d after anthesis and from root and young leaf was used for the blot. Hybridization and washing conditions were as described in the Materials and methods section. Phosphorimaging screens were exposed for 2 h. Molecular mass markers were revealed by ethidium bromide/UV. (**B**) Northern analysis with the rape β-carboxyltransferase domain DNA: 10 μg of total RNA from the same batch of embryos as in (**A**) was used for the blot. Hybridization and washing conditions were the same as in (**A**). The hybridizing bands were revealed by autoradiography with an exposure time of 7 d.
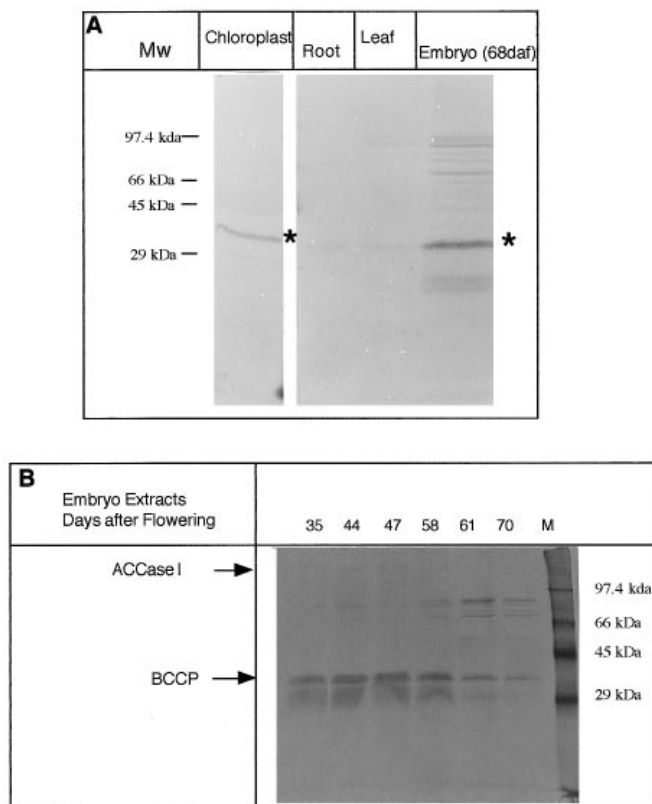
Figure 5   Western blot analysis of biotinylated proteins present in *B. napus* and identification of BCCP protein

(**A**) Extracts were derived from leaf chloroplasts, root, leaf and late embryogenesis embryos (as determined by morphology at 68 d after anthesis). SDS/PAGE was performed on 12% gels with an equal loading of root, leaf or embryo protein extract (5 μg total protein per lane). Intact chloroplasts (1 μg of total protein) and biotinylated SDS molecular mass markers (M) were also loaded in the respective lanes. Western analysis was performed as described in the Materials and methods section. The primary antibody was sheep anti-biotin and the secondary an alkaline phosphatase-conjugated antiserum. The bands marked with an asterisk represent BCCP protein at 35 kDa. All the lanes shown were run on the same gel. (**B**) Western analysis of biotinylated proteins. Western analysis of biotinylated proteins present in *B. napus* was performed on embryo extracts representative of the whole of embryogenesis (judged by morphology and days after anthesis). Volume-equalized loadings of extracts (adjusted for the respective mass of embryos used, i.e. 30–50 ng of embryos) were separated by SDS/PAGE on 12% gels. Biotinylated SDS molecular mass markers (M) were also loaded. Western analysis was performed as described in the Materials and methods section. The primary antibody was rabbit anti-biotin and the secondary an alkaline phosphatase-conjugated antiserum. The bands marked with the ACCase label indicate the position of the type I 220 kDa ACCase.

mobility than that predicted from the sequence. Because the rape BCCP sequence also contains a similar proline/adenine-rich region (Figure 1) the exact molecular mass could not be predicted from SDS gel mobility. The theoretical sizes were predicted as 22.7 and 21.9 kDa from the two longest clones pBP4 and pBP6 respectively (without the 4.7 kDa putative transit peptide). Earlier work in Durham showed that rape embryos contained several biotinylated proteins (results not shown). To analyse BCCP protein expression we needed to know with confidence which biotinylated protein band on SDS/PAGE was BCCP. The only other plant precedents [58,59] showed that the one bio-tinylated protein in pea chloroplasts was the 35 kDa BCCP subunit of ACCase. Similarly a Western blot of *B. napus* chloroplast protein showed that only one biotinylated protein resides within the chloroplast (Figure 5A). It is very probable

that this represents the BCCP protein allowing identification of the 35 kDa biotinylated protein in root, leaf and embryo as BCCP (indicated by the asterisks in Figure 5A). Purification of plastids from rapeseed embryos also revealed that the 35 kDa biotinylated polypeptide is localized within the plastid [60]. Although the same levels of protein were loaded in each lane, BCCP levels were low in both root and leaf in comparison with that in embryo. In the rape embryo BCCP is the major biotinylated protein present (approx. 40%). There is also in-dependent evidence that BCCP migrates more slowly on SDS/PAGE than expected from its predicted size. The pBP4 insert was cloned into an overexpression vector and overexpressed by inducement by isopropyl β-D-thiogalactoside (results not shown). The expressed protein (22.7 kDa theoretical size without the putative targeting sequence) was shown to be biotinylated *in vivo* and ran at 33 kDa on SDS gels (results not shown).

As in all organisms the presence of polypeptides, and their amounts, vary throughout embryogenesis. Previously the ques-tion of how many biotinylated proteins are in plants had been addressed, and it was shown to be about six. So far nobody had shown (i) how many were present in rapeseed and (ii) the differential expression in the embryo during embryogenesis. Figure 5B shows the Western blot of the embryos at different stages. During the first three stages there were three biotinylated proteins present at 220, 82 and 35 kDa, which most probably represented ACCase I, methylcrotonyl-CoA carboxylase (MCCase) and BCCP from the ACCase II complex. The smeary bands below the BCCP were most probably partial degradation products of BCCP because they were much more pronounced, and the 35 kDa BCCP levels lower, when samples were prepared by the relatively slow method of maceration/ammonium sulphate precipitation/dialysis (results not shown). At 58 d after anthesis three more biotinylated proteins appeared at 50, 70 and 88 kDa. Their levels rose to a peak at 61 d and dropped slightly at 70 d, just before the desiccation stage. Another protein band of 75 kDa appeared at 61 d and stayed present at the same level until 70 d. In contrast, the putative MCCase levels remained fairly consistent throughout embryogenesis. This profile was supported by a Northern blot (results not shown) with *Arabidopsis* MCCase sequenced at Durham (a gift from Dr. Reddy, Colorado State University). This contrasts with the 220 kDa ACCase I and 35 kDa BCCP protein levels, which decreased significantly from 47 to 70 d. The relative amounts of ACCase I and BCCP protein mimicked each other throughout embryogenesis. Although the overall level of ACCase I detected was much lower, a quantitative comparison cannot be made because the Western blot was optimized for BCCP analysis. Because ACCase I is much larger the efficiency of transfer from gel to membrane was much lower.

DISCUSSION

In recent years it has become apparent that dicotyledonous plant species contain two distinctive forms of the enzyme ACCase: type I, a single polypeptide of more than 220 kDa; and type II, a complex of smaller proteins BC, BCCP and CT. To achieve the full potential for the genetic manipulation of lipid products from crops a detailed understanding of fatty acid synthesis is required. This is dependent on a full characterization of the proteins involved, and more specifically on the pivotal enzyme ACCase. We have previously described the cloning of partial-length cDNAs from the *B. napus* type I ACCase [20].

In this paper we have studied two constituents of the multi-peptide type II ACCase from *B. napus*. We have outlined the analysis of BCCP from a plant source at the mRNA, DNA and

protein level and have described the cloning of several cDNAs. In addition we have described the first cloning and characterization of the chloroplast-encoded *B. napus* βCT cDNA. We isolated six distinct BCCP clones, of which two contained full-length ORFs encoding theoretical proteins of 22.7 and 21.9 kDa. The *B. napus* BCCP is therefore derived from a multi-gene family of at least six members. The size of the coding region was significantly greater than that of *E. coli* or *Anabaena* but correlated with the Northern data showing the full length to be 1.2 kb. On analysis of the pBP4 and pBP6 sequence the cDNAs revealed that they contained a putative 4.7 kDa 40 amino acid transit peptide at the 5′ end. The UTR and ORF sequence similarity showed that the cDNAs fall loosely into two different groups, group 1 (pBP1/2) and group 2 (pBP3/4/6/7). All six showed a strong degree of similarity to each other (at the 3′ ends of the coding regions) around the biotinylation site. This similarity also extended to the known prokaryotic BCCP sequences but showed no similarity to type I ACCase sequences. The biotinylation site, although divergent from that of *E. coli* BCCP, is identical with that for the *Anabaena* BCCP. The divergent 3′ UTRs of BCCP will permit the isolation of the corresponding six different promoter sequences. This study has shown that temporal expression is highly regulated and almost identical with other fatty acid synthesis genes that we have isolated. Using a comparison of all the promoter sequences from BCCP, and other fatty acid synthesis genes available in our laboratory, we might be able to identify specific sequences representing binding sites for the key time-specific transcription factors that control their expression.

By using the cDNAs obtained for two components of type II ACCase we were able to compare their expression directly at the RNA level. The βCT and BCCP mRNA expression levels during embryogenesis were very similar. Previous work has shown that rape type I ACCase and several other lipid synthesis genes are expressed in a very similar manner [20]. However, the Northern analysis of the βCT was complicated by the presence of several hybridizing bands. This was also observed by Sasaki et al. in their analysis of pea leaf βCT [42]. Given that the βCT primary transcript size is 2.4 kb in *B. napus* and yet the ORF is less than 1.5 kb long, there may be another ORF on the transcript. In the chloroplast DNAs of several other species such as pea the *acc*D gene is followed closely (by less than 180 bp) by the *psa*I gene, which has a very small ORF (120 bp in pea [39]). As in pea there may be several transcription start signals at the 5′ end of the ORFs that may all be used, giving rise to several bands seen on the Northern analysis.

The Western blot data that we present here show that rape leaf chloroplasts contain the biotinylated 35 kDa BCCP protein, which in turn implies that the putative transit peptide sequence is chloroplastic. In addition because leaf-to-embryo BCCP protein ratios are higher than that for mRNA we can only assume that either protein turnover is much greater in embryos or the translational control is much less prevalent in the leaf. The protein was larger on Western analysis than the theoretical size expected but the cDNA sequence showed that, like *E. coli* BCCP, the central region was rich in alanine and proline. This particular sequence characteristic has been shown previously to reduce the mobility of proteins on SDS/PAGE, i.e. the *E. coli* BCCP runs as a 22 kDa band in contrast with its 17 kDa theoretical size. The rape BCCP similarity to *E. coli* BCCP also extends to the *E. coli* BCCP N-terminus. It has been shown before that the N-terminal region represents the site for protein–protein interaction [61], whereas the proline–alanine central region is thought to act as a flexible arm to allow the carboxy group to move between subunit active sites. The BCCP genes will allow the construction of

antisense constructs to study the function of type II ACCases *in vivo*. We have already generated antisense type I ACCase transgenic rape plants, which in combination with BCCP antisense plants will allow us to differentiate between the roles of both types of ACCase within dicotyledonous species.

Using the anti-biotin antibodies generated for this study we analysed all the biotinylated proteins during rapeseed development. The major biotinylated protein in the embryo was the BCCP, which showed expression correlating to its mRNA expression. This is in marked contrast with that in pea where the BCCP has not been detected in the embryo [62]. This may reflect the different major storage products used by the two plants. Peas have only 2% of their mass as oil whereas rape has approx. 45%. With embryo morphology as a marker, the peak of BCCP expression occurred simultaneously to that of maximal lipid synthesis, after which expression was markedly reduced. We obtained enough affinity-purified antibody to generate an anti-biotin immunochromatography column. This could be used to eventually purify the BCCP from seed extracts for protein analysis.

Several other biotinylated proteins were identified, some of which can be tentatively attributed to type I ACCase and MCCase. Without functional analysis the identification of the other proteins was impossible but previous work detected pyruvate carboxylase activity in embryo extracts, a biotinylated enzyme (results not shown). Some of the bands might also represent the rape equivalent of the biotin-binding protein in pea embryos [62] and geranoyl-CoA carboxylase recently partly purified from maize [63].

Recent research has isolated the biotin carboxylase [49] and previous research the β-carboxyltransferase subunit clones [31–38,42] from different plant sources. In this report we have described the first isolation of plant full-sized BCCP and in addition a βCT clone from the same species. With the isolation of the α-carboxyltransferase subunit and the purification of expressed gene products the stage will be set for the full characterization of type II ACCase from plants at the protein level. Because the subunits are thought to form a complex *in vivo* by protein–protein interactions it may be important to study the subunits from the same plant source. In isolating two of the three or four subunit types we have gone some way to achieving this in the agriculturally relevant crop *B. napus*.

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