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Fatty acid synthesis in developing leaves of *Brassica napus* in relation to leaf growth and changes in activity of 3-oxoacyl-ACP reductase

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Abstract In young expanding leaves of *Brassica napus*, the demand for fatty acids is met by de novo biosynthesis of fatty acid synthase components, as demonstrated by 3-oxoacyl-ACP reductase. Using a novel radio-chemical assay for 3-oxoacyl-ACP reductase and specific antibodies, we have demonstrated a direct relationship between the increase in activity and synthesis of polypeptide. The maximum rate of fatty acid synthesis was between 4 and 7 days post-emergence, but slowed after this point even though 3-oxoacyl-ACP reductase activity was high. Leaf area continued to expand in a linear fashion after reductions in both enzyme activity and the rate of fatty acid synthesis.

Key words: 3-Oxoacyl-ACP reductase; Fatty acid synthase; [^{3}H]NADPH; *Brassica napus*

1. Introduction

Fatty acid biosynthesis is an essential process for both animals and plants, providing membrane components in all cells and energy reserves in specialised cells. Synthesis is catalysed by a complex enzyme system involving acetyl-CoA carboxylase and fatty acid synthase (FAS). In plants, and most prokaryotes, FAS enzymatic activities reside on separate, freely dissociable, polypeptides (type II) which is in contrast to the arrangement in animals and yeast where the activities are located on one or two multifunctional polypeptides (type I).

The temporal behaviour of FAS components is well characterised in plant seeds during embryogenesis. For example, the demand for enoyl-ACP reductase activity increases throughout the period of storage triglyceride formation; activity is mirrored by an increase in the levels of enoyl-ACP reductase protein [1] and follows an increase in the levels of mRNA [2]. The profile of expression of 3-oxoacyl-ACP reductase in seed tissue is the same as that for enoyl-ACP reductase. [3] These temporal patterns are consistent with the increased demand for fatty acid biosynthesis being met by increased transcription and translation of FAS components. However, there are no reports of the activity of any FAS component during early leaf development when increased amounts of membrane lipids are required for leaf expansion. Equally, no correlation between leaf size, the activity of FAS components and the synthesis of fatty acids has been made to date.

We have generated a number of *Brassica napus* lines that contain an antisense construct against 2-oxoacyl-ACP reductase in order to investigate the effect of reduced activity on fatty acid supply to storage triglycerides in seeds and to membrane lipids in leaves. Two requirements of this programme are the accurate determination of 3-oxoacyl-ACP reductase activity in extracts from single leaves and an understanding of temporal and spatial activity in leaves of wild-type plants. Whilst activity can be measured spectrophotometrically [4–6] sensitivity is limited by interfering compounds in plant extracts that absorb at 340 nm. This study reports the use of a novel radio-chemical assay for 3-oxoacyl-ACP reductase in the investigation of the relationship between activity, fatty acid levels and growth in developing *B. napus* leaves.

2. Materials and methods

2.1. Chemicals

D-[1-^{3}H]Glucose (15.3 Ci/mmol) was from NEN. Nicotinamide adenine dinucleotide phosphate ($NADP^+$); nicotinamide adenine dinucleotide phosphate reduced form (NADPH); Glucose dehydrogenase (EC 1.1.1.47 from *Bacillus megaterium*) were from Sigma. Diketene was from Aldrich and high purity isopropanol from Fischer.

2.2. Purification of ACP and synthesis of acetoacetyl-ACP (AcAc-ACP)

Acyl carrier protein was prepared from *Escherichia coli* essentially by the method of Prescott and Vagelos [7]. Electrophoretically homogeneous ACP was reduced by the addition of dithiothreitol to a concentration of 5 mM, dialysed against several changes of Milli Q water and freeze-dried in aliquots before storage at −80°C.

AcAc-ACP was synthesised chemically from the reaction of diketene with reduced *E. coli* ACP [8], using isopropanol as the solvent for diketene. Following the reaction AcAc-ACP was separated from unreacted diketene by gel filtration using a Pharmacia PD-10 column equilibrated and chromatographed with Milli Q water; fractions containing the product were lyophilised in aliquots and stored at −80°C.

2.3. Synthesis and purification of [proS-^{3}H]NADPH

Tritium was introduced, specifically, at the *proS* (or β-) face of NADPH by the enzymatic transfer of ^{3}H from D-[1-^{3}H]glucose to the C4 position of the nicotinamide ring of $NADP^+$ using glucose dehydrogenase from *B. megaterium* according to Valera et al., [9]. Purification of the reaction products was simplified considerably by substituting a single Sephadex G25 gel filtration step using a PD-10 column (Pharmacia). The column was equilibrated and eluted in Milli Q water that had been adjusted to pH 7.5 by the addition of ammonium hydrogen carbonate. GDH eluted at the void volume, labelled [4S-^{3}H]NADPH shortly after the void volume (at 3.5 ml) and the unused glucose at 6.5 ml Fractions containing labelled [4S-^{3}H]-NADPH were combined, adjusted to 36 Ci/mol by the addition of

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unlabelled NADPH, and stored in aliquots at −80°C after lyophilisation.

2.4. Growth of plant material

B. napus Westar plants were grown in M3 Levington compost in individual 4 cm pots with a 16 h light period (20°C) and an 8 h dark period (15°C) at 60% humidity. Illumination, measured with a Macam Quantum Radiometer/photometer, was at a photon flux density of 250 μE m^{-2} s^{-1}. Leaf samples were removed from plants, measured and immediately frozen in liquid nitrogen before storing at −80°C until required. Leaf areas were determined from scanned images using the NIH *Image* software.

2.5. Preparation of protein extracts

Leaves, from −80°C storage, were allowed to equilibrate to 4°C before extraction. Each leaf was ground with a micropestle (Treff) in 200 μl of extraction buffer (50 mM potassium phosphate, pH 7.5; 10% (w/v) glycerol; 1 mM EDTA). The extract was centrifuged for 3 min at 13 000 × *g*, and the supernatant transferred to clean tube and spun at 35 000 × *g* for 30 min. The volume of the supernatant was recorded and aliquots flash frozen before storage at −80°C. One aliquot was used for each experiment and never refrozen.

2.6. Fatty acid analysis

Fatty acid methyl esters (FAMES) were prepared from individual leaves [10] with addition of 25 μg of heptadecanoic acid as an internal standard. Hexane extracted esters (1.0 μl) were analysed on a Shimadzu GC14A gas chromatograph using a fused silica capillary column (30 m × 0.25 mm i.d., film thickness 0.2 mm) coated with a stabilised polycyanopropyl substituted phase (Supelco SP-2380). Fatty acids were identified by reference to standards and were quantified by electronic integration.

2.7. Radio-chemical assay

Activity of 3-oxoacyl-ACP reductase was measured in a reaction mixture containing 0.1 M potassium phosphate buffer, (pH 6.0); AcAc-ACP. (50 μM); [4S-^{3}H]NADPH, (160 μM; 18 Ci/mol) and BSA, (50 μg) in a final volume of 50 μl. The BSA was included to stabilise enzyme activity and as a carrier for radiolabelled acyl-ACP at the end of the assay. The reactions were pre-incubated for 5 min at 30°C and initiated by the addition of [4S-^{3}H]NADPH. The reaction was terminated and proteins precipitated by the addition of an equal volume of ice-cold 20% TCA. After 5 min. incubation on ice, the precipitate was collected by centrifugation (13 000 × *g* for 5 min), washed in 10% cold TCA and finally suspended in 100 μl of 0.1 M NaOH. The solution was neutralised by the addition of 100 μl 0.1 M HCl prior to scintillation counting.

2.8. Spectrophotometric assay

Activity of 3-oxoacyl-ACP reductase was measured spectrophotometrically by monitoring the rate of AcAc-ACP-stimulated oxidation of NADPH at 340 nm [4], in a 100 μl reaction volume.

2.9. Western analysis

3-Oxoacyl-ACP reductase polypeptide was identified using an antigen-specific polyclonal antibody. Crude leaf proteins were separated by SDS–PAGE before transfer to nitrocellulose membrane (Hybond C, Amersham). Antigen–antibody interactions were visualised using a chemiluminescent substrate (Super Signal West Pico, Pierce).

3. Results and discussion

3.1. Evaluation of the spectrophotometric assay

Using the spectrophotometric assay, the best estimate of 3-oxoacyl-ACP reductase activity was achieved with an extract from the fourth-true leaf at 4 days post-emergence. This method gave an activity of 6.7 nmol min^{-1} $leaf^{-1}$, which is comparable with the radio-chemical assay. However, it was not possible to measure the rate with half or twice the volume of leaf extract, due to lack of sensitivity with lower volumes and high absorbance of the sample at 340 nm when larger volumes were used. Therefore, it would not be possible to determine lower enzymatic rates using the spectrophotometric assay. The ability to accurately measure low 3-oxoacyl-ACP reductase levels is a necessary requirement of our present and future aims of understanding the temporal levels of activity in leaves and the effect of antisense 3-oxoacyl-ACP reductase on activity. It is clear that an assay with increased sensitivity is required to measure activity in individual rape leaves.

3.2. Rationale of radio-chemical assay

3-Oxoacyl-ACP reductase catalyses the transfer of the *proS* proton of [4S-^{3}H]NADPH to convert a C=O bond to a C–OH bond. The labelled acyl product, 3-hydroxyacyl-ACP, can be collected by precipitation at low pH leaving unused [4S-^{3}H]NADPH in solution.

3.3. Optimisation of radio-chemical KR assay in crude leaf homogenates

3.3.1. Stabilisation of activity. Purified 3-oxoacyl-ACP reductase from *B. napus* seeds, avocado and *E. coli* is inactivated by dilution, [6,11,12] but is partly protected by the presence of NADPH. In our experiments, 3-oxoacyl-ACP reductase activity measurements were performed with 35 000 × *g* supernatants of whole leaf homogenates which showed much greater enzymatic stability. Catalytic activity was stable to flash-freezing in liquid nitrogen and storage at −80°C for several months. Dilution of the crude extract in the presence of 5 mg ml^{-1} BSA resulted in the retention of greater than 92% of the activity after 1 h on ice. Assays routinely utilised freshly thawed and diluted aliquots of leaf homogenate to ensure measurement of optimal activity.

3.3.2. Control assays. To investigate the influence of small molecules on activity, an aliquot of leaf homogenate was subjected to gel filtration and elution in extraction buffer, using a Micro-Biospin 6 (exclusion limit 6000 Da) column to remove small molecules, including NADPH, from the sample. The 3-oxoacyl-ACP reductase activity in the void volume was the same as that of the crude extract, indicating that there were no interfering compounds in the homogenate and the level of NADPH was insufficient to interfere with measurements.

We routinely carried out control assays in the absence of AcAc-ACP and these gave very little precipitation of radioactivity; the level of AcAc-ACP in the crude leaf homogenate did not interfere with the assay.

3.3.3. Determination of kinetic constants for 3-oxoacyl-ACP reductase in crude leaf homogenates. It is important to know the Michaelis constants for 3-oxoacyl-ACP reductase in crude extracts so that appropriate substrate concentrations can be used for rate measurements. Previously published kinetic data for this enzyme were determined on purified proteins, either from different tissues (*B. napus* seed) or from different species (spinach leaf). We determined the kinetic properties of 3-oxoacyl-ACP reductase in crude leaf homogenates under steady-state conditions. Initial rates of incorporation of ^{3}H into 3-hydroxyacyl-ACP were measured at various concentrations of AcAc-ACP (5–60 μM) using 290 μM [4S-^{3}H]NADPH (Fig. 1A). The initial rates were also determined with a fixed concentration of AcAc-ACP (50 μM) and various [4S-^{3}H]-NADPH concentrations (20–200 μM; Fig. 1B). The enzyme followed normal Michaelis–Menten kinetics giving rectangular hyperbolic plots on *v* against [S] graphs. Hanes plots were used to determine kinetic constants (Fig. 1A,B, insets); the apparent K_m for AcAc-ACP was found to be 18.5 ± 2.3 μM,

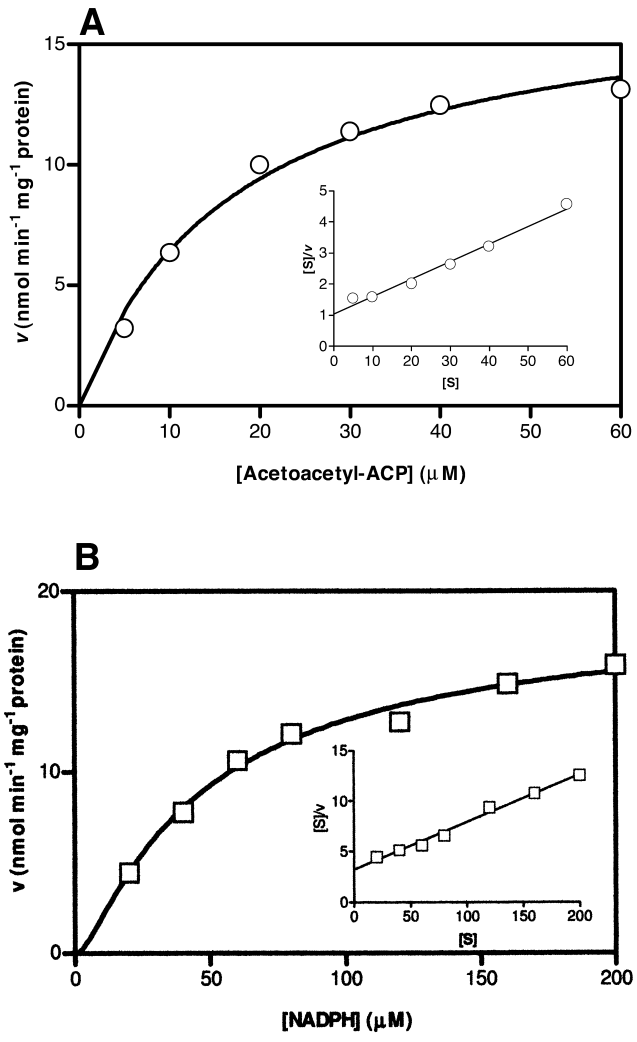

Fig. 1. Determination of Michaelis constants for 3-oxoacyl-ACP in crude extracts of *B. napus* leaves. Apparent K_m for (A) AcAc-ACP (○) was 18.5 ± 2.3 μM, and that for (B) NADPH (□) 68.6 ± 7.6 μM (determined from the inset Hanes plots; r^2 values > 0.98).

and that for NADPH 68.6 ± 7.6 μM under these conditions. These figures are higher than the published apparent K_m values for the *B. napus* enzyme purified from seeds of 3 μM for AcAc-ACP and 25 μM for NADPH [6]. The apparent reduced affinity of 3-oxoacyl-ACP reductase, for each of its two substrates, when measured in crude leaf extracts compared to purified seed enzyme may be due to the different source of enzyme. Alternatively, it may be that non-specific binding of substrates to other proteins in the crude extract causes reduced the availability of the substrates. However, the quality of data presented here gives us confidence in the kinetic determinations in crude extracts and most importantly indicates the concentrations of each substrate required to carry out activity measurements. Standard assays were conducted at substrate concentrations approximately 2.5-fold greater than their apparent K_ms: NADPH was used at 160 μM and AcAc-ACP at 50 μM.

3.3.4. Linearity of assay under optimised conditions. 3-Oxoacyl-ACP reductase activity in a leaf extract was determined under standard assay conditions for 1, 3, 5, 10 and 20 min in duplicate. The assay remained linear for 20 min under these conditions with a rate of 8 nmol min^{-1}; the data were analysed by linear regression and the r^2 value was greater than 0.99. Further assays were carried out with three time points, usually 1, 3 and 5 min; three individual leaf extracts were each assayed in duplicate at each time point and the mean data ± S.E.M. plotted on each graph.

3.4. Temporal characterisation of 3-oxoacyl-ACP reductase in leaves of B. napus

Young, developing leaves of *B. napus* increased in area with a relationship that can be described by a second order polynomial (Fig. 2A) Initial expansion was slow, but increased dramatically after 4 days. The growth from days 4–14 can be fitted in a linear fashion to yield a growth rate of 1.95 cm^2 per day (r^2 0.99). The start of the rapid rate of growth was concomitant with an increase in activity of 3-oxoacyl-ACP reductase (Fig. 2A). A dramatic increase in activity, of approximately 100% occurred between days 3 and 4. The high level of activity continued to day 10, but was reduced by approximately 30% in the day 14 sample; although the activity declined in this period, leaf expansion continued at the same rate.

The amount of 3-oxoacyl-ACP reductase polypeptide is closely correlated to the enzyme activity. Western blot analysis of crude leaf proteins (equivalent to 5% of the protein from a single leaf) demonstrated an increase in 3-oxoacyl-ACP reductase throughout the first 10 days following emergence (Fig.

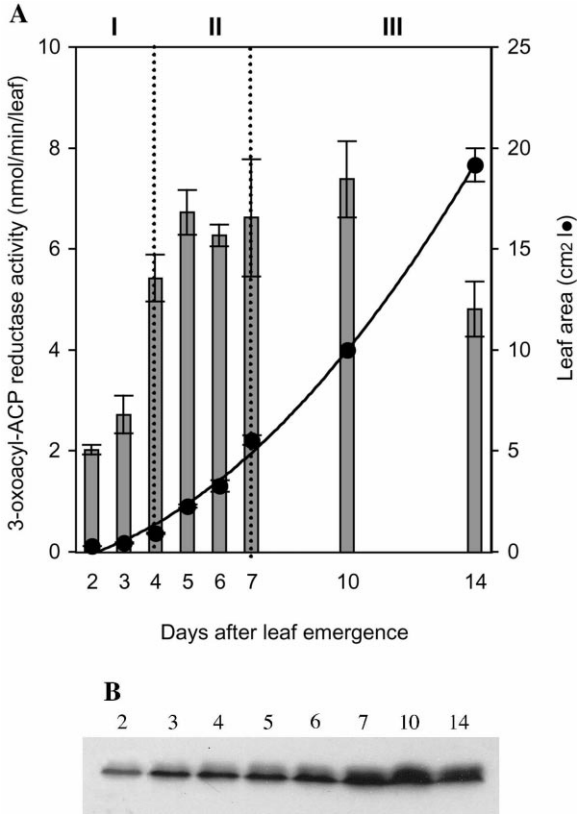

Fig. 2. Relationships between leaf area, 3-oxoacyl-ACP reductase polypeptide and activity. A: Activity of 3-oxoacyl-ACP reductase per leaf and leaf area were determined in three independent samples. Data are represented as means (±S.E.M.). B: Western analysis of 3-oxoacyl-ACP reductase polypeptide in crude leaf protein extracts equivalent to 5% of each leaf. Two independent analyses were performed and gave essentially identical results.

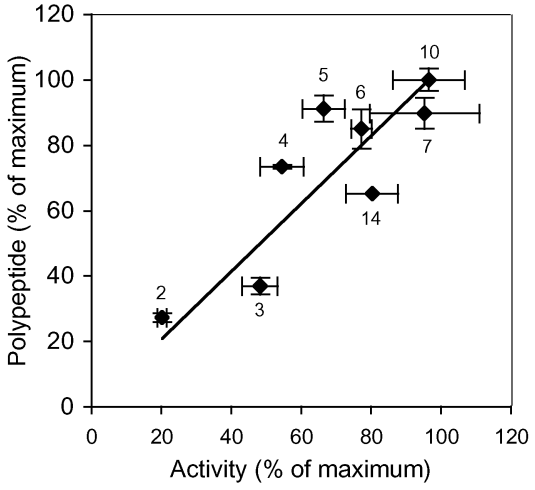

Fig. 3. Correlation between relative levels of 3-oxoacyl-ACP polypeptide and activity. The mean of three independent activity measurements (±S.E.M.), as a percentage of maximum activity, are plotted against the mean (and range) of two measurements of 3-oxoacyl-ACP polypeptide, as a percentage of maximum. Linear regression of the data gave an r^2 value of 0.71.

2B). In the 14 day sample, a decrease in both protein and activity was measured. When mean 3-oxoacyl-ACP reductase activity was plotted (as a percentage of maximum activity) against mean 3-oxoacyl-ACP reductase polypeptide (as a percentage of maximum protein), the correlation coefficient was 0.71 (Fig. 3). These data support the view that increased demand for fatty acid biosynthesis in expanding leaves is met by de novo synthesis of 3-oxoacyl-ACP reductase; this mirrors the situation previously reported in seed tissue [1].

The increase in 3-oxoacyl-ACP reductase activity precedes an increase in the rate of synthesis of fatty acids by 1 day. The rate of synthesis of fatty acids in young leaf tissue was non-linear, but can be described by three linear phases. Up to day 4 post-emergence the rate of synthesis of fatty acids was 2.6 μg per leaf per day (phase I, $r^2 = 0.72$). Between day 4 and 7 the rate increased approximately 5.5-fold, to 14.5 μg per leaf per day (phase II, $r^2 = 0.97$), and then decreased by 2.5-fold to 5.7 μg per leaf per day (phase III, $r^2 = 0.99$) over the next week (Fig. 4). The rate of fatty acid synthesis in these three phases does not correlate directly with leaf area measurements or 3-oxoacyl-ACP reductase activity. The activity of 3-oxoacyl-ACP reductase in phase II may contribute to the control of pathway flux through FAS. In contrast, the observation that enzyme activity in phase III remained high as the rate of fatty acid synthesis dropped would indicate that 3-oxoacyl-ACP reductase activity is not responsible for the reduction in biosynthetic rate. Therefore the majority of control of pathway flux must reside elsewhere during this time.

3.5. Activity of 3-oxoacyl-ACP in different leaves

Initial estimations of 3-oxoacyl-ACP reductase activity were made on the fourth true leaf to ensure that there was no embryo influence on the measurements. We determined the activity in several leaves as an indicator of the synthetic capability of each leaf. The 3-oxoacyl-ACP reductase activity of the first, second, fourth and sixth true leaves at 4 days post-emergence were similar, although there was more variability in the activity of leaf one (Fig. 5). The leaves emerged at considerably different times after germination, but had similar levels of this enzyme, suggesting that they were metabolically equivalent in terms of fatty acid biosynthesis.

Using a novel, sensitive, radio-chemical assay for 3-oxoacyl-ACP reductase, we have determined the relationship between activity of a FAS component enzyme, the rate of fatty acid

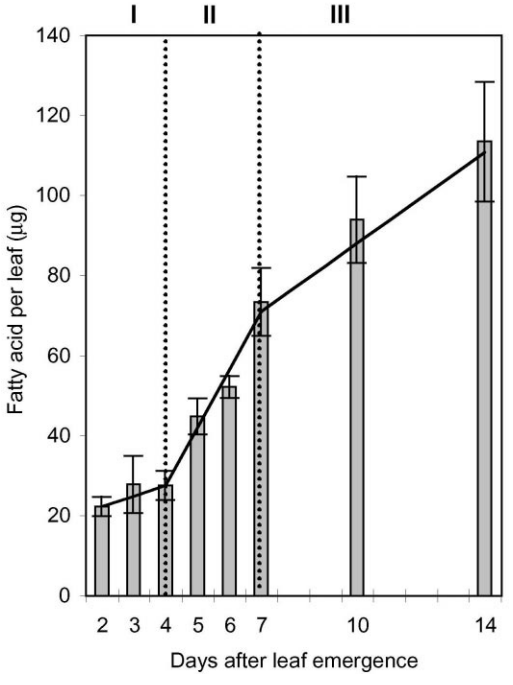

Fig. 4. Fatty acid content and rate of fatty acid synthesis in expanding *B. napus* leaves. The lines represent the rate of fatty acid synthesis in each phase: phase I, 2.6 μg per leaf per day ($r^2 = 0.72$); phase II, 14.5 μg per leaf per day ($r^2 = 0.97$) and phase III, 5.7 μg per leaf per day ($r^2 = 0.99$). Data are presented as means of three replicates (±S.E.M.).

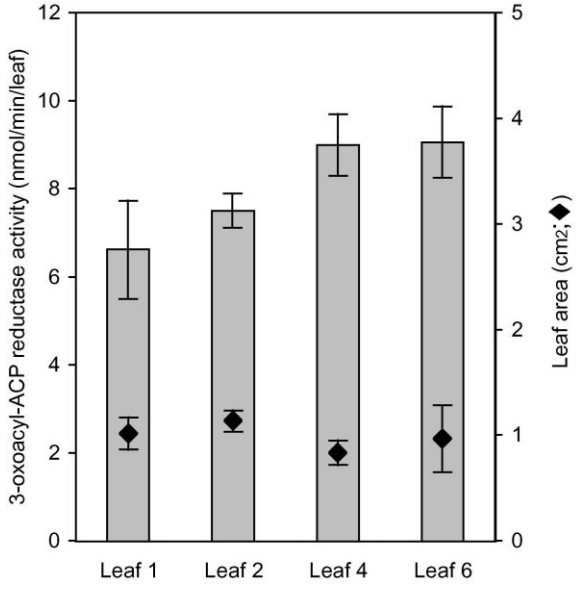

Fig. 5. Activity of 2-oxoacyl-ACP reductase in the first, second, fourth and sixth true leaves at four days post-emergence. The leaf area is also shown (◆). Data represents means of three replicates (±S.E.M.).

synthesis and expansion of young *B. napus* leaves. The increase in demand for fatty acid synthesis was satisfied by de novo biosynthesis of more active enzyme components, in a similar way to that seen in seed tissue. This information will allow us to investigate the levels of activity in antisense 3-oxoacyl-ACP reductase lines and correlate activity to the reduced fatty acid content and aberrant phenotypes seen in those plants.

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