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Starch metabolism in developing embryos of oilseed rape

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Abstract. The aim of this work was to characterise the metabolism of starch in developing embryos of oilseed rape (*Brassica napus* L. cv. Topaz). The accumulation of starch in embryos in siliques which were darkened or had been exposed to the light was similar, suggesting that the starch is synthesised from imported sucrose rather than via photosynthesis in the embryo. Starch content and the activities of plastidial enzymes required for synthesis of starch from glucose 6-phosphate (Glc6P) both peaked during the early-mid stage of cotyledon development (i.e. during the early part of oil accumulation) and then declined. The mature embryo contained almost no starch. The starch-degrading enzymes α-(EC 3.2.1.1) and β-amylase (EC 3.2.1.2) and phosphorylase (EC 2.4.1.1) were present throughout development. Most of the activity of these three enzymes was extraplastidial and therefore unlikely to be involved in starch degradation, but there were distinct plastidial and extraplastidial isoforms of all three enzymes. Activity gels indicated that distinct plastidial isoforms increase during the change from net synthesis to net degradation of starch. Plastids isolated from embryos at stages both before and after the maximum starch content could convert Glc6P to starch although the rate was lower at the later stage. The results are consistent with the idea that starch synthesis and degradation occur simultaneously during embryo development. The possible roles of transient starch accumulation during embryo development are discussed.

Key words: Amylase (isoforms) – *Brassica* (starch metabolism) – Embryo (developing) – Plastid – Starch phosphorylase (isoforms) – Starch (synthesis/degradation)

Abbreviations: AGPase = ADP-glucose pyrophosphorylase; DAA = days after anthesis; Glc6P = glucose 6-phosphate; NADP-GAPDH = NADP-glyceraldehyde 3-phosphate dehydrogenase; PGlcM = phosphoglucomutase

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Introduction

As a general rule, embryos that accumulate lipid as a major storage product contain very little or no starch at maturity. However, all oilseeds thus far studied accumulate starch at intermediate stages of development. Little is known about the function and importance of this starch in the carbon economy of the developing embryo. Previous studies on the synthesis of storage products in oilseed rape (*Brassica napus* L.) cotyledons have concentrated on the proteins and lipids. The limited amount of information available about starch accumulation has been gained from studies of whole seeds (Norton and Harris 1975) and thus reflects starch metabolism in the testa as well as the embryo. The aim of the work described in this paper was to characterize the accumulation of starch and the capacity for its metabolism in the developing embryo of oilseed rape.

We have established previously that plastids isolated from oilseed rape embryos at the early-mid cotyledon stage of development can take up glucose 6-phosphate (Glc6P) and convert it into starch (Kang and Rawsthorne 1994). It seems likely that this is the route by which carbon for starch synthesis enters the plastid in vivo. To characterize the capacity of the developing embryo for starch synthesis we have measured the maximum catalytic activities of enzymes required to convert Glc6P to starch [phosphoglucomutase, ADP-glucose pyrophosphorylase (AGPase) and starch synthase], and the rate at which isolated plastids convert Glc6P to starch at progressive stages in the development of the embryo.

Characterization of the capacity for starch degradation presents two problems. First, the precise pathway of degradation is not known for most plant organs (Steup 1988). We have assumed that measurement of activities of α-amylase, phosphorylase, and α-glucosidase, and of total amylolytic activity could provide a preliminary characterization of the capacity for starch degradation in oilseed rape embryos. Second, most of the enzymes of starch degradation exist as multiple isoforms, at least

some of which may be located outside the plastid. To take account of this problem we have (i) measured the fractions of these enzymes that are plastidial, and (ii) compared the isoform composition in plastids and in whole embryos at three developmental stages.

Materials and methods

Plant material. Plants of *Brassica napus* L. cv. Topas were grown as described by Kang and Rawsthorne (1994). Flowers were tagged at anthesis and embryo age measured as days after anthesis (DAA). Siliques were collected and kept in ice up to 3 h prior to the removal of testas from embryos. Embryos were either used immediately (preparation of plastids, enzyme assays and native gels), or weighed and frozen at −80 °C until use (starch and lipid measurements).

Measurement of starch and lipid content. The starch content of frozen embryos (samples of 0.3–0.5 g fresh weight) was determined enzymatically according to Smith (1988). Starch contents were also determined in the pellets remaining after centrifugation of homogenates (see *Extraction of embryos for enzyme assays*, below). Pellets were extracted six to eight times with 1.5 ml 800 ml · l^{-1} aqueous ethanol at 90 °C for 10 min prior to assay of starch. Lipid content of the embryos was determined as described by Kang et al. (1994).

Extraction of embryos for enzyme assays. Samples (0.3–0.5 g fresh weight) of embryos were extracted with ice-cold media using a mortar and pestle followed by an all-glass homogeniser. For assays of enzymes of starch synthesis the medium was 50 mM Mops (pH 7.2), 1 mM EDTA, 10 mM dithiothreitol (DTT). Samples of the homogenate were taken for assay of total starch synthase. The remainder of the homogenate was centrifuged at 10 000 · *g* for 10 min at 4 °C. The supernatant was assayed for soluble starch synthase and AGPase and the pellet was stored at −20 °C prior to measurement of starch content (see above).

For assays of enzymes of starch degradation the medium was 50 mM Mops (pH 7.0), 2 mM DTT. The homogenate was centrifuged as above. Samples of the supernatant were either assayed directly or desalted on a column of Sephadex G25 equilibrated with extraction medium (for total amylolytic activity and starch phosphorylase). The pellets were stored at −20 °C prior to measurement of starch content (see above).

ADP-glucose pyrophosphorylase (EC 2.7.7.27) was assayed as in Smith et al. (1989) except that 0.75 mM sodium pyrophosphate, 10 mM $MgCl_2$, 0.75 mM NAD and 25 µl of extract were used.

ADP-glucose-starch synthase (EC 2.4.1.21) was assayed as in Smith (1990) except that 50 mM Bicine, 0.50 M Na-citrate (pH 8.6), 4 mM ADP-[U-^{14}C]glucose at 0.74 GBq · mol^{-1}, 1.3 mg amylopectin from potato and 20 µl of extract were used.

α-Glucosidase (EC 3.2.1.20). Method A: 0.3 ml of extract was mixed with 1 ml of 1 g · l^{-1} 4-nitrophenyl-α-D-glucopyranoside in 50 mM Na-acetate (pH 5.0) and incubated at 25 °C. Samples were taken at intervals and boiled for 2 min, and optical density was determined at 400 nm (Killilea and Clancy 1978). *Method B*: 50 µl of extract was mixed with 250 µl of 50 mM maltose, 50 mM Na-acetate (pH 5.0) and incubated at 25 °C. Samples were taken at intervals, boiled for 2 min, and the amount of glucose measured enzymatically according to Lowry and Passonneau (1972).

α-Amylase (EC 3.2.1.1). The assay was based on that of Doehlert and Duke (1983). Assays containing 2 ml of 100 mM Na-acetate (pH 6.0), 3 mM $CaCl_2$, 10 g · l^{-1} starch azure (Sigma, Poole, Dorset, UK), 12.5 U · ml^{-1} of commercial β-amylase (Boehringer, Mannheim, Germany: β-amylase units as defined by Boehringer) and 50 µl extract were incubated at 25 °C. At intervals, samples were mixed with 0.3 ml of 200 g · l^{-1} trichloroacetic acid, centrifuged at 10 000 · *g* for 2 min, and the absorbance of the supernatant measured at 595 nm. The relationship between α-amylase activity and change in absorbance was determined with standards made with commercial α-amylase (Boehringer; from pig pancreas).

α–1,4-Glucan phosphorylase (EC 2.4.1.1). The assay was based on that of Bulpin and ap Rees (1978). It contained, in 1 ml, 40 mM Mops (pH 7.0), 20 mM KH_2PO_4, 0.75 mM NAD, 5 units glucose 6-P dehydrogenase (NAD-linked, from *Leuconostoc mesenteroides*), 2 units phosphoglucomutase and 100 µl desalted extract. The reaction was initiated by addition of 0.1 ml of an aqueous solution of soluble starch (50 g · l^{-1})

Total amylolytic activity. The assay (1 ml) contained 90 mM Na-acetate (pH 6.0), 0.125 mg soluble starch and 50 µl of desalted extract, and was incubated at 25 °C for 30 min. The reaction was stopped by boiling for 10 min. Reducing sugars were quantified by an adaptation of the method of Nelson (1944) using maltose as standard (Dygert et al. 1965).

Assay of other enzymes. Phosphoglucomutase (EC 5.4.2.2) was assayed according to Entwistle and ap Rees (1988), and *NADP-glyceraldehyde 3-phosphate dehydrogenase (NADP-GAPDH; EC 1.2.1.9)* and *alcohol dehydrogenase (EC 1.1.1.1)* according to Denyer and Smith (1988).

Preparation of plastids, and starch synthesis by isolated plastids. Plastids were prepared from embryos as described by Kang and Rawsthorne (1994). Incorporation of ^{14}C from [1-^{14}C]glucose 6-phosphate (Glc6P) (16–32 GBq · mol^{-1}) into starch by isolated plastids was measured as described by Kang and Rawsthorne (1996).

Subcellular distribution of enzyme activities. Activities were measured in the initial homogenate and final washed plastid fractions, as described above. Homogenate fractions were desalted prior to assay of starch phosphorylase. Alcohol dehydrogenase and AGPase were used as markers for cytosol and plastid compartments respectively.

The subcellular distribution of phosphoglucomutase (PGlcM) was measured as follows. An initial homogenate fraction was prepared as in Kang and Rawsthorne (1996) and was divided equally between six tubes which were centrifuged at 750 · *g* for 5 min to produce crude plastid pellets. The supernatant fractions were pooled and used to make a dilution series (0, 10, 30, 50, 80 and 100% supernatant fraction diluted in plastid isolation medium; Kang and Rawsthorne 1994). Each pellet was resuspended in 0.5 ml of one of these dilutions and the total activities of PGlcM and plastid and cytosolic marker enzymes (NADP-glyceraldehyde 3-phosphate dehydrogenase and alcohol dehydrogenase, respectively) were measured in each tube. The percentage of the total PGlcM activity attributable to the plastid was calculated from linear regression analysis (Denyer and Smith 1988; Kang and Rawsthorne 1994). The percentage values were used in conjunction with total PGlcM activities measured in an homogenate (in plastid isolation medium) made from the same batch of embryos from which the plastids were prepared to provide an estimate of the total plastidial PGlcM activity per embryo. The experiment was repeated on three separate batches of embryos.

Native gel electrophoresis. Extracts were made as for assay of enzymes of starch synthesis (see above) except that the extraction medium was 60 mM Mops (pH 7.0), 2 mM DTT. Phosphorylase activities were resolved by electrophoresis in non-denaturing polyacrylamide gels (0.75 mm thick, 5 cm long), followed by activity staining (Steup 1990). Amylolytic activities were resolved by electrophoresis in non-denaturing polyacrylamide gels containing 2 g · l^{-1} of soluble starch, amylopectin, starch azure or β-limit dextrins in the separation gel (Ziegler and Beck 1986). Following electrophoresis, gels were washed in two changes of 100 mM Mes (pH 6.0) for 30 min each and then stained with 14 mM KI, 10 mM I_2.

Results

Accumulation of starch and lipid. The fresh and dry weights of embryos increased until about 50 DAA (late cotyledon stage of development). Up to the early-mid cotyledon stage (until 33 DAA) the total amount of starch per embryo increased (Fig. 1). After that, it declined and by the end of development very little starch remained. This general pattern of accumulation was consistently observed on several, separately grown batches of plants. The maximum starch content on a fresh-weight basis for the batch of embryos shown in Fig. 1 was 28.5 ± 1.0 mg · g^{-1} which occurred at 26 DAA. For embryos from other batches of plants up to 36 mg starch · g^{-1} fresh weight was recorded (data not shown). Separate measurements on cotyledons and embryonic axes at four different stages through development revealed that they had very similar starch contents on a fresh-weight basis (data not shown). The embryos accumulated lipid very rapidly between 21 and 53 DAA after which there was a decrease in the total amount of lipid per embryo (Fig. 1). The maximum lipid content attained during development (at 57 DAA) was more than tenfold greater than that of starch when expressed on a fresh-weight basis.

To establish whether starch in the embryo is a product of embryo photosynthesis (Eastmond et al. 1996) or is synthesised from imported sucrose, the accumulation of starch was measured in embryos from siliques which had been covered with black bags to exclude light from 1 DAA onwards. These dark-grown embryos had the same pattern of starch accumulation (data not shown) and starch content as those in siliques exposed to the light (respectively 25.4 ± 0.3 mg · g^{-1} fresh weight at 34 DAA and 24.0 ± 0.9 mg · g^{-1} fresh weight at 33 DAA: mean ± SE of three replicate measurements on pooled embryos from at least five plants).

Activities of enzymes of starch synthesis. Assays were optimized with respect to pH and concentrations of the reagents in the assay. Rates were linear with respect to time and were proportional to the amount of extract assayed. In experiments in which young embryos (30 DAA) were co-extracted with maturing embryos (60 DAA) the activities of AGPase and starch synthase in the mixed extracts were not statistically different from those predicted from separate extracts of the two ages of embryo ($P < 0.01$). We therefore believe that our measurements are likely to reflect the maximum catalytic activities of enzymes in the embryo. In order to discover the precise relationships between the activities of enzymes and the starch content of the embryo through development, both were measured in the same extracts (Figs. 2, 4).

During development, changes in activities of AGPase and the soluble and granule-bound starch synthases showed the same pattern as changes in the total starch content of the embryo (Fig. 2). The decrease in activities of these enzymes in the later stages of development did not reflect a general decline in metabolic activity in the

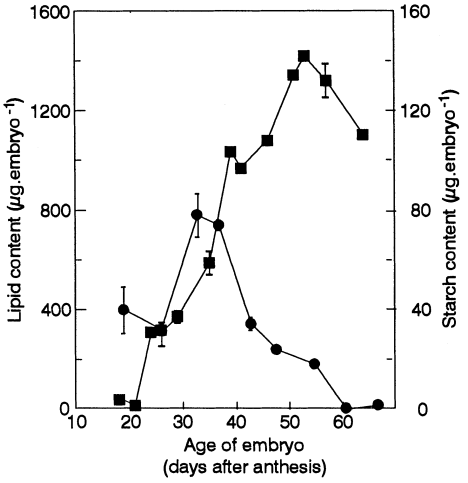

Fig. 1. Amounts of starch (●) and lipid (■) in developing embryos of oilseed rape. Starch was assayed enzymatically after extraction of embryos with ethanol and digestion of the solubilized residue with α-amylase and α-amyloglucosidase. Lipid content was measured as fatty-acid methyl esters. Values are means of measurements made on three separate samples of embryos from a single batch of plants. Standard errors of the means are shown as bars (±) where they are larger than the size of the symbol

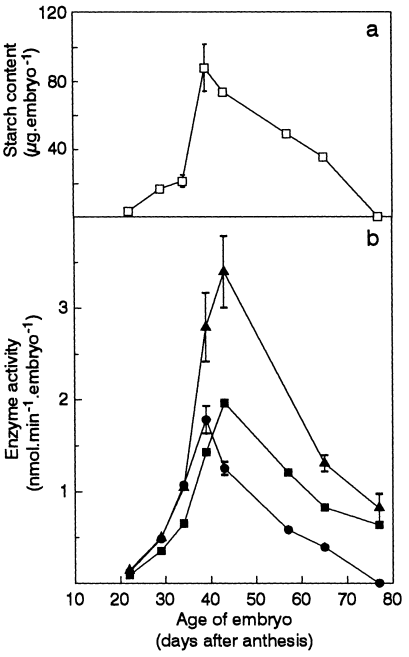

Fig. 2a, b. Activities of enzymes of starch synthesis in developing embryos of oilseed rape. Starch content of the embryos (**a**) was determined enzymatically and the activities of enzymes (**b**) were measured in the same embryo extracts (see *Materials and methods*). Total starch synthase (▲) was assayed in a crude homogenate, and soluble starch synthase (■) and AGPase (●) were assayed in the supernatant after centrifugation. Each value is the mean of measurements made on three independently extracted samples of embryos from a single batch of plants. Standard errors of the means are shown as bars (±) where they are larger than the size of the symbol

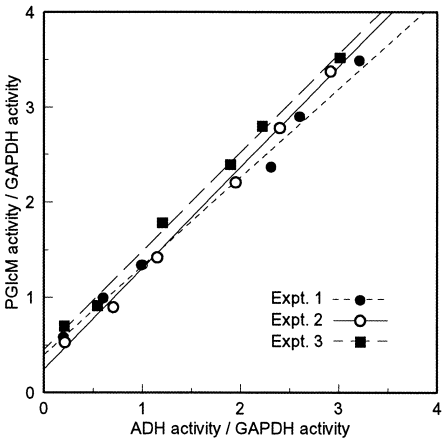

Fig. 3. Subcellular distribution of PGlcM activity in oilseed rape embryos. Homogenates and crude plastid fractions contaminated by increasing amounts of supernatant were prepared (see *Materials and methods*) and the activities of PGlcM, NADP-GAPDH (plastid marker enzyme), and alcohol dehydrogenase (cytosol marker enzyme) in each fraction were assayed. Three lines are shown, each derived from a separate experiment using early-mid stage embryos (r^2 values were all ≥ 0.99). The distribution of PGlcM activity in each experiment was determined from the following equations (see Kang and Rawsthorne 1994): Total PGlcM/PM = C_1 + C_2 (CM/PM); C_1 = **p**PGlcM/PM; C_2 = **c**PGlcM/CM. **p** and **c** denote respectively plastid and cytosolic PGlcM activities; PM, plastid marker enzyme activity; CM, cytosol marker enzyme activity

embryo: activities of NADP-GAPDH and plastidial PGlcM did not follow this pattern (see below).

The measurement of plastidial PGlcM was complicated by the presence of a very large activity of cytosolic PGlcM. In fact, the contaminating activity of cytosolic PGlcM in washed plastid preparations considerably exceeded the plastidial activity of PGlcM. Normally the plastidial activity could be calculated by subtracting the fraction of the activity known to be due to cytosolic contamination (estimated by measuring a cytosolic marker enzyme). In this case however, the fraction subtracted is very large, and the remainder very small and thus subject to large variations. We therefore chose a method in which PGlcM and cytosolic and plastidial marker enzymes were measured in samples of plastids diluted with differing amounts of a fraction enriched in cytosolic enzymes. This enabled the plastial PGlcM activity in each extract to be estimated from linear regression analysis of several data points (see *Materials and methods*). Results proved highly reproducible between extracts at each developmental stage examined (e.g. Fig. 3). Plastidial PGlcM activity per embryo did not differ significantly between the three developmental stages at which it was assayed (Table 1).

Starch synthesis by isolated plastids. The capacity of intact, washed plastids to synthesize starch was determined at the same developmental stages used for measurement of plastidial PGlcM. The recoveries of marker enzymes and latency of the plastid marker enzyme in these plastid preparations were comparable at all three stages (Table 1). When expressed on the basis of activity of NADP-GAPDH, the rate of incorporation of carbon from Glc6P into starch by these plastids was very similar at the first two stages (up to early-mid cotyledon) but then declined by 70% by the mid-late cotyledon stage (Table 1). This decline is unlikely to be due to a decrease in plastid quality since the maximum rate of fatty acid synthesis by isolated plastids increases through the three stages examined here (data not shown).

Activities of starch-degrading enzymes. Activities of α-amylase and phosphorylase followed the same general pattern as starch content during development (Fig. 4). Mature seeds still contained considerable α-amylase

Table 1. Properties of plastids in developing embryos of oilseed rape. Plastidial PGlcM activity was determined as described in Fig. 3. The activity of NADP-GAPDH (plastid marker enzyme) was determined in a total homogenate prepared from embryos using plastid isolation medium (Kang and Rawsthorne 1994). For the plastid isolation experiments, recovery of activity in the plastid preparation is expressed as a percentage of the total activity in the initial homogenate. For all enzymes the total recoveries of activity (i.e. the sum of those in the plastid fraction plus initial supernatant, expressed as a percentage of that in the initial homogenate) ranged between 92% and 111%. Starch synthesis by intact isolated plastids was measured as incorporation of carbon from Glc6P (1 mM) into methanol/KCl-insoluble material. The rate of starch synthesis is expressed per unit (μmol · min^{-1}) of NADP-GAPDH. The starch content of embryos from the same batches used for plastid preparations was determined enzymatically. Values are the mean ± SE of determinations made on at least three separate extracts/plastid preparations

Measurement	Embryo age (DAA)		
	23	32	41
Embryo characters			
Starch content (μg · $embryo^{-1}$)	39 ± 3	133 ± 11	70 ± 6
Plastidial PGlcM activity (nmol · $embryo^{-1}$ · min^{-1})	0.98 ± 0.15	1.53 ± 0.35	1.12 ± 0.40
NADP-GAPDH activity (nmol · $embryo^{-1}$ · min^{-1})	12.8 ± 2.1	21.0 ± 1.9	23.8 ± 1.3
Plastid preparations			
Recovery of NADP-GAPDH activity (%)	14.8 ± 3.8	12.5 ± 2.1	8.2 ± 1.9
Latency of NADP-GAPDH activity (%)	71.3 ± 3.9	68.4 ± 4.9	65.7 ± 6.6
Recovery of alcohol dehydrogenase (%)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Recovery of cytochrome *c* oxidase (%)	2.8 ± 0.5	2.7 ± 0.3	2.5 ± 0.5
Starch synthesis by plastids (nmol hexose · $unit^{-1}$ GAPDH · h^{-1})	456 ± 31	463 ± 40	138 ± 28

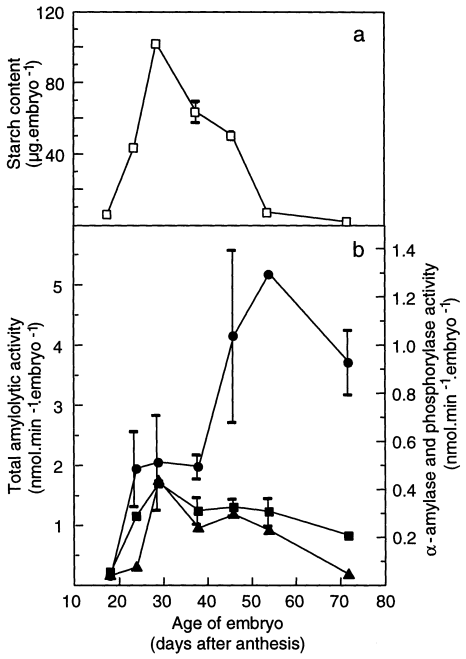

Fig. 4a,b. Activities of enzymes of starch degradation in developing embryos of oilseed rape. Starch content of the embryos (**a**) was determined enzymatically and the activities of enzymes (**b**) were measured in the same embryo extracts (see *Materials and methods*). Supernatants derived from centrifugation of crude homogenates of embryos were assayed for phosphorylase (▲), α-amylase (■) and total amylolytic activity (●). Values for total amylolytic activity and α-amylase activity are expressed on a maltose basis. Each value is the mean of measurements made on three independently extracted samples of embryos from a single batch of plants. Standard errors of the means are shown as bars (±) where they are larger than the size of the symbol

activity but no detectable phosphorylase activity. In contrast, total amylolytic activity increased through most of development and decreased only in the very late stages. Total amylolytic activity was much higher than α-amylase activity; therefore, other enzymes, most probably β-amylase, were responsible for much of the total activity. No α-glucosidase activity was detected at any stage of development. The minimum activity detectable with the methods used would have been about 0.9 nmol · min^{-1} · g FW^{-1}. Extracts of young (30 DAA) and maturing (60 DAA) embryos did not inhibit the activity of commercial α-glucosidase from yeast, indicating that inability to detect this activity is unlikely to be due to the presence of inhibitors in the extracts.

Subcellular distribution of activities of starch-degrading enzymes. Plastids were isolated from early-mid stage embryos (ca. 35 DAA), and the recoveries of activities of starch-degrading enzymes and plastidial and cytosolic marker enzymes in the washed plastid fraction were determined. Based on the recovery of plastidial and cytosolic marker enzymes in the plastid fraction, about 30% of the phosphorylase activity and less than 20% of the total amylolytic activity are attributable to the plastid (Table 2). There were no significant losses of activity of these enzymes during the preparation of plastids. To test whether activity in the plastid fraction reflected the presence of these enzymes inside plastids, the final washing step during plastid isolation was carried out in the absence of osmoticum. Total amylolytic activity in the pellet in this experiment was reduced to 0.8% of that in the unfractionated homogenate, and α-amylase and phosphorylase activities were undetectable.

Isoforms of starch-degrading enzymes. To determine whether the changes in total amylase and phosphorylase activities shown above could be ascribed to particular plastidial or extraplastidial isoforms, unfractionated homogenates and washed plastids were prepared from embryos at stages of development between early- and late-cotyledon, and subjected to native gel electrophoresis followed by activity staining (Fig. 5). Both homogenates and plastids were prepared from three stages representing the phases of net starch synthesis, maximum starch content, and net degradation (i.e. between 24 and 45 DAA from Fig. 1), and homogenates were also prepared from a later stage when starch had almost disappeared (day 60 from Fig. 1). Gels were loaded so that each lane contained the same activity of the plastidial marker enzyme NADP-GAPDH. Comparison of the intensity of bands therefore indicates which isoforms change in activity through development, while comparison of the pattern of bands derived from plastids with that derived from unfractionated homogenate reveals whether these isoforms are plastidial.

In order to detect amylase activity, electrophoresis was carried out on gels containing amylopectin

Table 2. Starch-degrading enzymes in the plastids of developing embryos of oilseed rape. Plastids were prepared from about 500 embryos at 30 DAA. Activities of the cytosolic marker enzyme alcohol dehydrogenase (ADH), the plastid marker enzyme AGPase, α-amylase, starch phosphorylase and total amylolytic activity were assayed in the homogenate and plastid fractions. Two or three replicate assays were performed on each fraction. For each experiment the compartmentation of degrading enzymes was calculated taking into account the recovery of the plastid marker and the cytosolic contamination, according to the formula: Percentage activity in plastid $= (A_{ENZ} - A_{ADH})/(A_{AGPase} - A_{ADH}) \cdot 100$ where A_X is the proportion of the activity of enzyme X recovered in the plastid fraction and ENZ is the degradative enzyme. The values presented are the mean ± SE of determinations on at least six separate plastid preparations

	AGPase	ADH	Phosphorylase	α-amylase	Total amylase
Activity in the homogenate (nmol · min^{-1})	305 ± 34	2820 ± 274	50 ± 2	340 ± 43	1249 ± 103
Activity in the plastid preparation (nmol · min^{-1})	28 ± 3	7 ± 1.7	2 ± 0.3	5 ± 0.8	41 ± 13
Proportion in the plastid (%)	100	0	30 ± 2	11 ± 2.1	18 ± 6.1

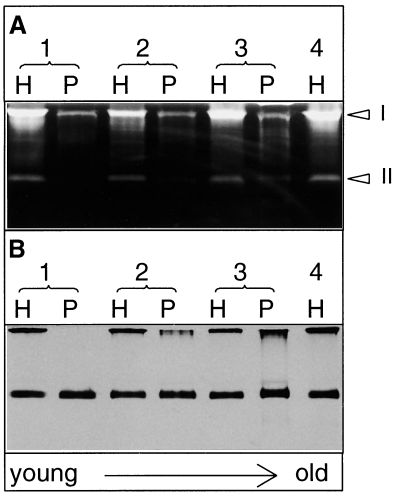

Fig. 5A,B. Isoforms of starch-degradative enzymes in embryos of oilseed rape. *Lanes H1–H4*, unfractionated homogenates of embryos from the early- (*H1*), early-mid- (*H2*), mid-late- (*H3*), and late-cotyledon (*H4*) stages. These stages correspond approximately to 24, 33, 45, and 60 DAA as shown in Fig. 1. *Lanes P1–P3*, extracts of plastids purified from the same batches of embryos as H1–H3. All lanes contained extract with the same total activity of the plastidial marker enzyme, NADP-GAPDH. **A** Amylases. Isoforms were resolved on native polyacrylamide gels containing amylopectin and visualised by staining with iodine solution after incubation at pH 6. Isoforms of classes I and II are indicated. **B** Phosphorylases. Isoforms were resolved on native polyacrylamide gels containing glycogen, and visualised by staining in iodine solution after incubation with glycogen and glucose 1-phosphate. No bands were seen when glucose 1-phosphate was omitted (not shown)

(Fig. 5A). Bands of starch-degrading activity revealed by staining with iodine were resolved into three groups: colourless bands close to the origin of the gel (Class I), light-pink bands in the lower part of the gel (Class II), and poorly resolved bands between classes I and II. Class I bands were identified as α-amylases by their ability to digest starch azure when excised and transferred to gels containing starch azure (not shown). Class II enzymes did not digest starch azure when treated similarly (not shown) and are likely to be β-amylases.

Plastids at all three developmental stages contained amylase activity (Fig. 5A). At the earliest stage this activity was primarily α-amylase (Fig. 5A, lane P1). The total amylolytic activity in the plastids and the proportion of the activity attributable to β-amylase and to poorly resolved bands between α- and β-amylase increased through development. Both α-amylase and β-amylase bands from plastids were less intense than those from homogenates, indicating that much of the total embryo activity of both enzymes was accounted for by extraplastidial isoforms.

In order to detect phosphorylase activity, electrophoresis was carried out in gels with (Fig. 5B) or without (not shown) glycogen. In the absence of glycogen, the phosphorylase activities in unfractionated homogenates appeared as a single band, or two barely resolved bands (data not shown). One of these bands was strongly retarded by the inclusion of glycogen in the gel (Fig. 5B).

Like amylase, starch phosphorylase was present in plastids at all the stages of development examined. In young embryos (Fig. 5B, lanes H1, P1), the band retarded by glycogen was absent from the plastids, consistent with the observation that extra-plastidial isoforms of phosphorylase characteristically have a higher affinity for glycogen (Steup 1990). The more mobile band was plastidial (Fig. 5B). At later stages of development a retarded band also appeared in extracts of isolated plastids. Since this band had a mobility in the gel very similar to that of the retarded extra-plastidial band, it is possible that it could represent increased contamination of the plastid fractions from older embryos by other cell fractions. This is because in loading equal amounts of plastidial marker enzyme in each lane of the gel (Fig. 5) we loaded increasing amounts of the extraplastidial part of the cell (see relative recoveries of ADH and NADP-GAPDH in plastid fractions during development, Table 1). However, we believe that this band does represent a second plastidial isoform, for two reasons. First, the increase in loading of extra-plastidial enzymes was less than two fold (Table 1), and this is therefore unlikely to explain the very large change in intensity of the band (Fig. 5B). Second, in contrast to the profiles of starch phosphorylases, the profiles of amylases (Fig. 5A) from these preparations of plastids remained different from the profiles in corresponding unfractionated homogenates throughout development.

Discussion

Our results show that net starch synthesis in oilseed rape embryos occurs in both the cotyledons and the embryonic axis prior to and during the early phase of storage lipid accumulation. The maximum starch content is less than one-tenth of the final lipid content on a fresh-weight basis. The starch accumulated in developing embryos originates not from embryo photosynthesis but from carbon imported into the embryo from vegetative parts of the plant. Subsequent net degradation, starting after one-third of the oil has accumulated, results in the almost complete disappearance of starch by maturity. This pattern of starch accumulation resembles that reported for embryos of the related species *Sinapis alba* (Fischer et al. 1988) but is different from that reported for embryos of another oilseed, soybean. Although the mature soybean seed contains little or no starch, maximum amounts during embryo development are much higher relative to amounts of oil than in oilseed rape (maximum starch content is about 50% of the final lipid content on a dry-weight basis), and net degradation does not occur until later in development (Yazdi-Samadi et al. 1977; Adams et al. 1980).

The capacity for starch synthesis of developing embryos rises very early in development, then falls from about the time that starch content starts to decline. Maximum catalytic activities of plastidial PGlcM, AGPase and starch synthase during the phase of starch accumulation are at least 20-fold greater than is required

to account for the net rate of starch synthesis (calculated for 26–33 DAA from data in Fig. 1). The rate at which starch is synthesized by plastids isolated from embryos accumulating starch is at least twice the net rate of starch synthesis in vivo when the rates per unit NADP-GAPDH at the first two stages in Table 1 are expressed on an embryo basis (taking into account the intactness of the plastids during the incubations and using the NADP-GAPDH activity measured in total homogenates of the developing embryos; Table 1) .

The capacity for starch degradation is more difficult to assess. Our data indicate that distinct isoforms of amylases and phosphorylase are present in plastids, with activities at least fivefold in excess of those required to account for the maximum net rate of starch degradation (calculated from data in Fig. 1 and Table 2). However, both measurements of activity and activity gels show that the major isoforms of these enzymes in the embryo are extraplastidial. Extraplastidial degradation of starch is known to occur in a few plant organs at certain stages of development. The amyloplast membrane disappears prior to seed germination in both cereal endosperms and pea cotyledons (Steup 1988). There is no evidence that this occurs during the development of oilseed rape embryos. Electron microscopy shows starch granules within membrane-bounded organelles at advanced stages of embryo development (D.J. Murphy, John Innes Centre, personal communication). The plastids of embryos of *Sinapis alba* also remain intact during the phase of net starch degradation (Fischer et al. 1988). We think it is likely that only plastidial isoforms of amylases and phosphorylase participate in starch degradation in oilseed rape embryos. Consistent with this, the plastidial activity of both sorts of enzymes appears to increase at the stages where net starch degradation is occurring. Activities of β-amylase and of one of two plastidial isoforms of phosphorylase increase relative to activities of α-amylase and the other plastidial phosphorylase.

Although starch-degrading capacity increases during development, plastids isolated from oilseed rape embryos have the capacity for both starch synthesis and starch degradation throughout the phases of net starch synthesis and net starch degradation. Enzymes of both synthesis and degradation are present, and isolated plastids can synthesise starch from Glc6P (Fig. 1; Table 1). This raises the possibility that starch is turned over throughout embryo development, and that the changes in content reflect the net balance between synthetic and degradative capacity rather than a synthetic phase followed by a degradative phase. It is also possible that there are developmental gradients within the embryo such that starch synthesis and starch degradation are actually happening in different cells at any given time, rather than in the same cell.

Two suggestions have been made about the role of starch accumulation in developing oilseed embryos. First, starch could serve as a reserve which is utilised later in development in the synthesis of lipids (Norton and Harris 1975). Starch synthesis in the early stages of development enables the storage of incoming carbon prior to onset of high rates of lipid synthesis. The overall photosynthetic capacity of oilseed rape plants declines when reserve accumulation starts, so an ability to store available carbon at an early developmental stage could be important to the carbon economy of the seed. Second, starch may serve as the source of carbon for the synthesis late in development of sugars such as sucrose and stachyose, which are proposed to contribute to the acquisition of desiccation tolerance during the drying-out phase (Yazdi-Samadi et al. 1977; Leprince et al. 1990). There is no direct evidence to support either of these suggestions. On the basis of this study we suggest a third possible role for the initial accumulation of starch. During early cotyledon filling, starch represents ca. 8–10% of the dry weight of oilseed rape embryos (based on data in *Results* and a fresh:dry weight ratio of 3.0–3.5: da Silva 1993). This value is comparable to that for the early stages of starch accumulation in embryos for which starch is the major product (e.g. pea, which on a dry-weight basis contains ca. 10% starch at 10 mg fresh weight: Lloyd 1995). In the oilseed rape embryo this amount of starch can provide only a very small proportion of the carbon required for the subsequent development of the embryo. Most of the carbon must come from imported sucrose. We suggest that the early development of a capacity for starch synthesis may contribute to the establishment of the embryo as a sink organ prior to the onset of lipid synthesis. However, starch accumulation per se is not prerequisite for establishment of a sink in developing oilseed embryos. A mutant of another oilseed, *Arabidopsis thaliana*, which lacks plastidial PGlcM activity and is unable to synthesize starch (Caspar et al. 1985) is able to produce viable seed.

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