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Evidence that the *rb* Locus Alters the Starch Content of Developing Pea Embryos through an Effect on ADP Glucose Pyrophosphorylase[1]

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ABSTRACT

The aim of this work was to discover whether the *rb* locus of peas (*Pisum sativum* L.) affects seed starch content through action on an enzyme of starch synthesis in the developing embryo. The phenotypic effects of this locus are like those of the better characterised, unlinked *r* locus, which affects seed starch content through action on starch-branching enzyme. Embryos recessive at one or both of these loci (*RRrbrb*, *rrRbRb*, *rrrbrb*) have lower starch contents from an early stage of development than embryos dominant at these loci (*RRRbRb*). Maximum catalytic activities of enzymes of the pathway from sucrose to starch (sucrose synthase EC 2.4.1.13, UDP glucose pyrophosphorylase EC 2.7.7.9, ADP glucose pyrophosphorylase EC 2.7.7.27, ADP glucose-starch synthase EC 2.4.1.21, starch-branching enzyme EC 2.4.1.18) were compared in developing embryos of three lines of *rbrb* peas and four lines of *RbRb* peas. The only consistent difference between the two sorts of embryo was in the activity of ADP glucose pyrophosphorylase, which was at least tenfold lower in *rbrb* than in *RbRb* embryos. The activity in *rbrb* embryos was in most cases less than the estimated rate of starch synthesis of *RRRbRb* embryos. We conclude that the effect of the *rb* locus on the starch content of pea seeds is mediated through an alteration in the activity of ADP glucose pyrophosphorylase in the developing embryo.

The unlinked *r* (*rugosus*) and *rb* loci of the pea (*Pisum sativum*) have similar effects on seed morphology and composition. Mature seeds containing embryos that are homozygous dominant at both these loci (referred to as *RRRbRb* embryos) are round and have high levels of starch (about 50% of final dry weight) and low levels of lipid and sucrose (about 2 and 5%, respectively, of final dry weight). Mature seeds containing embryos that are homozygous recessive at one or both of these loci (referred to as *rrRbRb*, *RRrbrb* and *rrrbrb* embryos) are wrinkled, and have much lower levels of starch (about 30% of final dry weight) and higher levels of lipid and sucrose (about 4 and 9%, respectively, of final dry weight) than seeds containing *RRRbRb* embryos (1, 13). We have shown previously (17) that the *r* locus affects the activity of one isoform of starch-branching enzyme in the developing embryo. The absence of this isoform from *rr* embryos results in a considerably reduced activity of the enzyme and hence a lower rate of starch synthesis (17). Many of the other effects of this locus may be consequences of its effect on starch synthesis (17). The aim of the work described in this paper was to discover whether the *rb* locus also has a primary effect on an enzyme or enzymes of starch synthesis.

In order to locate any lesion in the pathway of starch synthesis during development that might account for the low starch content of the mature *rbrb* embryo, we compared the starch contents and the maximum catalytic activities of enzymes of the pathway from sucrose to amylopectin in eight different genotypes of pea. Four of these are known to be *RbRb*, and three are known to be *rbrb*. The eighth line (JI 2108, cultivar Ace) has wrinkled seeds, and has been tentatively described as *RRrbrb* on the basis of starch grain morphology, but has not been genetically characterised (2). In order to discover any interactions of the *rb* and *r* loci, two of the lines of peas which we chose were *RRRbRb*, two were *RRrbrb*, two were *rrRbRb* and one was *rrrbrb*. Any lesion in the pathway of starch synthesis in *rbrb* embryos would be identified by two criteria. First, the maximum catalytic activity of the enzyme(s) concerned would be substantially lower in embryos of all of the *rbrb* lines than in embryos of all of the *RbRb* lines, at a stage of embryo development when starch accumulation was substantially lower in embryos of *RRrbrb* lines than in those of *RRRbRb* lines. Second, at this stage of embryo development the maximum catalytic activity of the enzyme(s) concerned would be insufficient in embryos of all of the *rbrb* lines to account for the rate of starch synthesis of embryos of the *RRRbRb* lines.

For the purposes of this study, we have assumed that the pathway of synthesis of amylopectin from sucrose proceeds via sucrose synthase and UDP glucose pyrophosphorylase to glucose 1-P in the cytosol. Glucose 1-P then enters the plastid and is converted to amylopectin via ADP glucose pyrophosphorylase, starch synthase, and starch-branching enzyme. There is evidence that in pea embryos the cytosolic part of this putative pathway is the major route of sucrose catabolism (4, 5), and the pathway of conversion of glucose 1-P to amylopectin in plastids is well established (16). The nature of the metabolite entering the plastid as the substrate for starch synthesis is not known for pea embryos and most other plant organs. However, there is recent evidence for wheat endosperm that this metabolite is glucose 1-P (12, 19).

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MATERIALS AND METHODS

Pea (*Pisum sativum* L.) plants were grown in a greenhouse at a minimum temperature of 12°C and fed twice weekly with a low-nitrogen fertilizer. Lines of peas used are shown in Table I. For measurements of enzyme activities, pods were removed from the plants onto ice and seeds were used within 30 min. For measurements of starch, seeds with their testas removed were frozen at −70°C for up to 2 months before use. All experiments were carried out on embryos (cotyledons + axis) from the seeds.

Radiochemicals were obtained from Amersham International plc, Amersham, Bucks, UK.

Enzyme Assays

For measurement of all enzymes except ADP glucose pyrophosphorylase, embryos (0.2–1.0 g fresh weight) were extracted in 0.8 to 4.0 mL ice-cold 100 mM Hepes (pH 7.8), 5 mM DTT with a pestle and mortar followed by an all-glass homogeniser. Homogenates were centrifuged at 10,000*g* for 10 min at 4°C. Supernatants were desalted on a column (10 × 0.6 cm i.d.) of Sephadex G25 equilibrated with extraction medium, and were kept on ice prior to assay.

For ADP glucose pyrophosphorylase the procedure was the same except that the extraction medium was 100 mM Mops (pH 7.2), 5 mM DTT, 5 mM $MgCl_2$, 100 mg mL^{-1} bovine serum albumin. The Sephadex column was equilibrated with this medium without bovine serum albumin. Addition of ethanediol (20% [v/v]), chymostatin (10 μg mL^{-1}), and phosphate (30 mM) to the extract medium did not affect the activity in the extract.

Assay procedures are given in full for extracts of embryos of BC1/7RR. Differences for other lines of peas are given in parentheses. All assays were done at 25°C.

Sucrose Synthase (EC 2.4.1.13)

The assay contained, in 100 μL, 50 mM 3-[di-methyl (hydroxymethyl)methylamino]-2-hydroxypropanesulphonic acid (Ampso, pH 9.4), 5 mM UDP glucose, 20 mM sucrose, 7.5 mM fructose, and 10 μL extract (BC1/7rr, pH 9.2; JI 1068, 5 mM sucrose, and 15 mM fructose). The reaction was initiated with extract and stopped after 10 min by addition of 100 μL 1 M perchloric acid, 1 mM diaminoethanetetraacetic acid. After 10 min at 2°C the mixture was neutralized with 5 M K_2CO_3, centrifuged to remove K perchlorate, and assayed enzymatically for UDP by the method of Lowry and Passonneau (14) for ADP. Blanks did not contain fructose.

Table I. *JI Accession Number and Genotypes of Lines of Peas Studied*

Names of cultivars most similar to the lines used are also given.

JIIGC[a] Accession No.	Genotype	Cultivar Name
JI 430		
(BC1/7rr)[b]	*rrRbRb*	
(BC1/7RR)	*RRRbRb*	
JI 1068	*RRRbRb*	Birte
	rrRbRb	Kelvedon Wonder[c]
JI 1156	*RRrbrb*	Minnesota Early Sweet
JI 399	*RRrbrb*	Cennia
JI 827	*rrrbrb*	
JI 2108[d]		Ace

[a] John Innes Germplasm Collection. [b] BC1 lines are essentially isogenic except at the *r* locus, and were derived from JI 430 by Hedley *et al.* (11). [c] From a commercial source (Sharpe and Co., Sleaford, Lincs, UK), not the JI Germplasm Collection. [d] Not genetically characterized: wrinkled seed, genotype deduced to be *RRrbrb* from starch grain morphology (2).

UDP Glucose Pyrophosphorylase (EC 2.7.7.9)

The assay contained, in 1 mL, 80 mM Bicine (pH 8.6), 1.5 mM Na PPi, 1 mM $MgCl_2$, 0.4 mM NAD, 0.8 mM UDP glucose, 10 units glucose 6-P dehydrogenase (NAD-linked, from *Leuconostoc mesenteroides*), 4 units phosphoglucomutase, and 10 to 50 μL of a 10-fold dilution of extract in extraction medium. (BC1/77rr, 0.15 mM Na PPi; JI 1068, 2 mM $MgCl_2$; Kelvedon Wonder, 0.3 mM Na PPi and 2 mM $MgCl_2$; JI 1156, 0.3 mM Na PPi; JI 399, 0.15 mM Na PPi; JI 827, 0.3 mM Na PPi and 3 mM $MgCl_2$; Ace, 0.3 mM Na PPi and 1.5 mM UDP glucose). The reaction was initiated with UDP glucose, and was monitored spectrophotometrically at 340 nm.

ADP Glucose Pyrophosphorylase (EC 2.7.7.27)

The assay contained, in 1 mL, 75 mM Hepes (pH 7.9), 1.5 mM Na PPi, 5 mM $MgCl_2$, 0.4 mM NAD, 1 mM ADP glucose, 5 units glucose 6-P dehydrogenase (NAD-linked, from *Leuconostoc mesenteroides*), 2 units phosphoglucomutase and 20 to 50 μL extract (BC1/7rr, 2 mM $MgCl_2$ and 2 mM ADP glucose; Kelvedon Wonder, 10 mM $MgCl_2$ and 2 mM ADP glucose, JI 339, pH 7.5; JI 827, pH 7.5 and 2.5 mM $MgCl_2$; JI 2108, pH 7.0, 3 mM Na PPi, 10 mM $MgCl_2$ and 2 mM ADP glucose). The reaction was initiated with Na PPi, and was monitored spectrophotometrically at 340 nm.

ADP Glucose-Starch Synthase (EC 2.4.1.21)

The assay contained, in 240 μL, 40 mM Bicine, 10 mM K acetate, 200 mM Na citrate (pH 8.6), 2 mM ADP [U-^{14}C] glucose at 7.35 GBq mol^{-1}, 8 mg amylopectin, and 50 μL extract (BC1/7rr, 4 mM ADP glucose; JI 1068, 4 mM ADP glucose; Kelvedon Wonder, 4 mM ADP glucose; JI 2108, 4 mM ADP glucose). The reaction was initiated with extract, and stopped after 30 min by incubation at 100°C for 2 min. Glucose polymer was precipitated by addition of 3 mL 75% (v/v) aqueous methanol containing 1% (w/v) KCl, and washed according to Hawker *et al.* (10: method for starch branching enzyme). Blanks contained extract that had been incubated at 100°C for 2 min. For measurement of total starch synthase, assays were carried out as above except that the reaction was initiated with crude, uncentrifuged homogenate. Radioactivity was determined by liquid scintillation spectroscopy.

Starch-Branching Enzyme (EC 2.4.1.18)

Radiometric phosphorylase-stimulation assays were carried out according to Smith (17).

Determination of Starch

Starch was extracted and assayed according to Smith (17).

RESULTS

Starch Content

The amount of starch in embryos of all the lines of peas rose on a fresh weight basis between 100 and 500 mg embryo weight (Table II). The amount in *RRRbRb* embryos was considerably greater than in *RRrbrb*, *rrRbRb* or *rrrbrb* embryos throughout this period of development.

Assay of Enzymes

For each enzyme, the activity reported was dependent upon the presence in the assay of all of the appropriate substrates and cofactors. The concentrations of all components of the assay and its pH were optimised to give the maximum rate. Activity in the assay was in all cases proportional to the amount of extract added, over the range of amounts used in making measurements. The rate of the reaction was linear with respect to time for at least 4 min in spectrophotometric assays, and for at least 10 and 30 min in assays of sucrose synthase and starch synthase, respectively. For starch-branching enzyme, activity was calculated from the rate of reaction during the phase of the assay in which it was linear with respect to time.

Criteria for making reliable measurements of starch-branching enzyme in extracts of developing pea cotyledons have been established previously (17). In this study we have experienced particular difficulty in making reliable measurements of ADP glucose pyrophosphorylase and ADP glucose-starch synthase. The problems and our solutions to them are as follows.

ADP Glucose Pyrophosphorylase

In the widely used radiometric assay, [^{14}C]glucose 1-P is converted to ADP [^{14}C]glucose, which is separated from unreacted glucose 1-P by phosphatase treatment and anion exchange chromatography (8). In assays of desalted extracts of pea embryos, glucose 1-P was rapidly converted into other hexose monophosphates. In some assays, 75% of glucose 1-P was converted into other hexose monophosphates within 5 min. The decrease in the size of the hexose monophosphate pool during the assay was greater than could be accounted for by the synthesis of ADP glucose. In view of the complexity of reactions occurring in this assay, we concluded that it was unsuitable for extracts of pea embryos. In the spectrophotometric assay, ADP glucose is converted to glucose 1-P which is converted via phosphoglucomutase and glucose 6-P dehydrogenase to 6-phosphogluconate with reduction of NAD(P) (18). In assays of desalted extracts of pea embryos, in the absence of glucose 6-P dehydrogenase, ATP and hexose monophosphate were produced at the same rate. This rate was about 80% of the rate of NAD reduction in assays in which glucose 6-P dehydrogenase was included. This was true for embryos of all genotypes. We concluded that this assay gives a reasonably reliable estimate of ADP glucose pyrophosphorylase activity.

ADP Glucose-Starch Synthase

In several starch-storing organs including peas there are known to be both soluble and starch-granule-bound forms of ADP glucose-starch synthase (7, 15, 20). We estimated the maximum catalytic activity of soluble starch synthase in pea embryos by optimizing the assay conditions on extracts from which starch had been removed by centrifugation. To estimate total starch synthase, assays were done under the same conditions on crude, uncentrifuged homogenates. Total starch synthase activity was always greater than soluble starch synthase activity (Table III), and the difference was substantially accounted for by starch-granule-bound activity. When washed starch grains were prepared (15) from an homogenate of pea embryos for which total and soluble starch synthase activity had been measured, the activity associated with washed grains accounted for 75% of the difference between total and soluble

Table II. *Starch Contents of Developing Embryos*

Tissue (0.5–3.0 g fresh weight, 3–8 embryos) was extracted in boiling 80% (v/v) aqueous ethanol. The residue was autoclaved and starch in it was digested with α-amylase and amyloglucosidase. Glucose released was determined enzymatically. Values are means ± SE of measurements made on four separate batches of embryos, except where individual measurements are given or the number of batches used is given in parentheses.

Line of Pea	Genotype	Starch Content (embryo fresh weight, mg)			
		100–199	200–299	300–399	400–500
		mg g^{-1} fresh wt			
BC1/7RR[a]	*RRRbRb*	47 ± 4	90 ± 6	128 ± 6 (6)	237 ± 14 (7)
BC1/7rr[a]	*rrRbRb*	5 ± 0.4	10 ± 1 (3)	16 ± 2	28 ± 8
JI 1068	*RRRbRb*	ND[b]	156 ± 10	280 ± 19	296 ± 37
Kelvedon Wonder	*rrRbRb*	10, 11	13, 14	ND	68 ± 9
JI 1156	*RRrbrb*	23 ± 6 (3)	65 ± 6	72 ± 8	89 ± 4
JI 399	*RRrbrb*	37 ± 6	ND	80 ± 3	106 ± 2
JI 827	*rrrbrb*	10 ± 1	12 ± 2	18 ± 3	17 ± 2
JI 2108[c]		14 ± 2	33 ± 1	77 ± 10	91 ± 4

[a] Data from Smith (17). [b] Not determined. [c] Genotype not known (see Table I).

Table III. *Activities of Enzymes of Starch Synthesis in Developing Pea Embryos*

Except for total ADP glucose starch synthase, activities of enzymes were assayed in starch-free, desalted extracts of embryos of the lines and genotypes shown below. Total ADP glucose starch synthase was assayed on crude homogenates of embryos. Values are means ± SE of measurements made on four separate extracts, except for those marked * which are from three separate extracts, and those for which individual values are given. Detection limit for activity in the starch-branching enzyme assay was 0.1 μmol min^{-1} g^{-1} fresh weight; failure to detect activity is shown as <0.1.

Enzyme	Fresh Weight of Embryo	Enzyme Activity							
		BC1/7RR (*RRRbRb*)	BC1/7rr (*rrRbRb*)	JI 1068 (*RRRbRb*)	Kelvedon Wonder (*rrRbRb*)	JI 1156 (*RRrbrb*)	JI 399 (*RRrbrb*)	JI 827 (*rrrbrb*)	JI 2108[a]
	mg	*μmol min⁻¹ g⁻¹ fresh wt*							
Sucrose synthase	100–199	2.45 ± 0.17*	2.02 ± 0.15	1.73 ± 0.03	1.78 ± 0.04	3.31 ± 0.41	3.09 ± 0.24	2.87 ± 0.25	1.61 ± 0.15
	200–299	2.45 ± 0.08	2.29 ± 0.27	1.51 ± 0.06	2.16 ± 0.07	3.35 ± 0.13	2.89 ± 0.19	3.27 ± 0.39	1.69 ± 0.07
	300–399	1.62 ± 0.21	2.12 ± 0.11	ND[b]	2.12 ± 0.03	2.88 ± 0.18	2.90 ± 0.17	2.94 ± 0.18	1.68 ± 0.05
	400–500	1.52 ± 0.25*	2.57 ± 0.10*	0.60 ± 0.04	2.02 ± 0.05	2.71 ± 0.14	2.09 ± 0.14	2.73 ± 0.20	1.53 ± 0.05
UDP glucose pyrophosphorylase	100–199	12.0 ± 0.5*	11.1 ± 1.2	42.6 ± 1.6	30.4 ± 4.4	12.2 ± 0.6	16.2 ± 2.4	11.1 ± 1.6	14.6 ± 0.9
	200–299	11.6 ± 0.7	15.6 ± 2.3	55.4 ± 4.5	34.9 ± 4.5	26.6 ± 2.8	23.0 ± 2.7	30.3 ± 4.7	37.1 ± 3.2
	300–399	27.0 ± 3.3	40.2 ± 6.9	ND	52.5 ± 3.3	44.8 ± 4.2	35.1 ± 4.1	44.1 ± 1.5	44.1 ± 4.2
	400–500	29.3 ± 2.3*	37.2 ± 2.0	35.1 ± 1.6	54.2 ± 3.3	53.7 ± 2.5	48.0 ± 7.2	61.5 ± 4.4	49.9 ± 2.1
ADP glucose pyrophosphorylase	100–199	1.13 ± 0.02	1.36 ± 0.22	0.67 ± 0.02	ND	0.04 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.80 ± 0.09
	200–299	1.18 ± 0.02	1.67 ± 0.04	ND	1.13	0.05 ± 0.01	0.09 ± 0.01	0.03 ± 0.01	1.30 ± 0.07
	300–399	2.74	2.93 ± 0.11	2.76, 3.10	1.28 ± 0.25*	0.09 ± 0.01	0.13 ± 0.01	0.05 ± 0.01	2.02 ± 0.32
	400–500	2.29, 2.23	3.21 ± 0.31*	2.29, 1.74	2.79 ± 0.10*	0.11 ± 0.01	0.16 ± 0.03	0.11 ± 0.02	1.74 ± 0.18
ADP glucose[c]-starch synthase soluble	100–199	0.12 ± 0.01*	0.09 ± 0.01	0.08 ± 0.01	0.10 ± 0.01*	0.11 ± 0.03	0.13 ± 0.02	0.12 ± 0.01	0.09 ± 0.01*
	200–299	0.10 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01*	0.11 ± 0.01	0.08 ± 0.01	0.11 ± 0.01	ND
	300–399	0.06 ± 0.01	0.05 ± 0.01	ND	0.07 ± 0.01*	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.05 ± 0.01*
	400–500	0.05 ± 0.01*	0.05 ± 0.01*	0.03 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	ND
ADP glucose[c] starch synthase:total	300–399	0.32 ± 0.03*	0.21 ± 0.02*	0.33 ± 0.02	0.16 ± 0.01*	0.38 ± 0.01	0.33 ± 0.01*	0.16 ± 0.01*	0.25 ± 0.03*
Starch-branching[d] enzyme	100–199	10.3 ± 0.4[e]	<0.1[e]	20.2 ± 0.7	<0.1	ND	17.4 ± 2.7	<0.1 (3), 0.64	0.16 ± 0.03
	200–299	13.0 ± 0.7	<0.1 (1), 0.75	25.2 ± 0.8	<0.1 (3), 0.67	25.5 ± 3.7	15.8 ± 1.9	1.83 ± 0.43	0.85 ± 0.30*
	300–399	15.6 ± 1.4	1.72 ± 0.39	ND	0.77 ± 0.17	38.5 ± 5.3	29.1 ± 3.7	4.62 ± 0.62	1.47 ± 0.36
	400–500	20.4 ± 1.3	2.93 ± 0.64	37.3 ± 2.2	1.34 ± 0.17	34.6 ± 4.5	28.0 ± 4.8	8.84 ± 0.74	4.54 ± 0.40

[a] Genotype not known (see Table I). [b] Not determined. [c] Activity is μmol glucose from ADP glucose incorporated into methanol-insoluble polymer min^{-1} g^{-1} fresh weight. [d] Activity is stimulation by extract of incorporation by phosphorylase a of glucose from glucose 1-phosphate into methanol insoluble-polymer, as μmol glucose min^{-1} g^{-1} fresh weight. [e] Values for activity of starch-branching enzyme in BC1/7 lines are from Smith (17).

activity. Measurement of all of the starch-granule-bound activity in many plant organs including peas requires that starch grains are mechanically and enzymically disrupted (7, 15). Our measurement of total starch synthase is therefore likely to underestimate the maximum catalytic activity of this enzyme.

Activities of Enzymes in Developing Embryos

To check whether loss of enzyme activity through inhibition or degradation was occurring during extraction of a particular genotype of embryo, recovery experiments were carried out. Two, replicate samples of embryos BC1/7RR and two of another line of peas, were prepared. One sample of each sort was extracted separately, and the remaining two were mixed and extracted together. Activity in the mixed extracts was compared with that predicted from the activities in the separate extracts of the two sorts of embryo. These experiments were carried out for embryos of 300 to 400 mg fresh weight of all of the lines of peas studied, and in addition for embryos of 100 to 200 mg of JI 399 and JI 827. For all of the enzymes studied, the activities in mixed extracts were within 17% of those predicted. It is therefore unlikely that there are major losses of activity of any of the enzymes during extraction of embryos of any of the lines of peas studied.

Activities of sucrose synthase, UDP glucose pyrophosphorylase, and starch synthase differed little between different genotypes of embryos (Table III). Total starch synthase activity, measured at 300 to 400 mg embryo weight, was at least twofold higher than soluble starch synthase activity in all the embryos studied (Table III). There were major differences in activity of starch-branching enzyme between embryos of different lines of peas (Table III). In *RRRbRb* and *RRrbrb*

embryos, activity on a fresh weight basis was high, whereas in *rrRbRb* and *rrrbrb* embryos and embryos of JI 2108, it was extremely low or undetectable at 100 to 200 mg embryo weight and rose considerably as embryo weight increased. Activity in *rrRbRb*, *rrrbrb*, and JI 2108 embryos at all stages of development was less than in *RRRbRb* and *RRrbrb* embryos.

There were major differences in activity of ADP glucose pyrophosphorylase between embryos of different lines of peas. Activity in *RRrbrb* and *rrrbrb* embryos at each stage of development was less than one-tenth of that in *RRRbRb*, *rrRbRb*, and JI 2108 embryos at the same stage of development. Activity in all genotypes rose two- to fourfold on a fresh weight basis between 100 and 500 mg embryo fresh weight.

DISCUSSION

Both the *r* and *rb* loci affect starch synthesis in embryos at an early stage of development. Amounts of starch in embryos of 100 to 500 mg were lower in all wrinkled-seeded lines of peas (*RRrbrb*, *rrRbRb*, *rrrbrb*) than in the round-seeded lines (*RRRbRb*). Any lesion in the pathway of starch synthesis caused by the *rb* locus should be manifest during this period of development.

Comparison of activities of enzymes of the pathway of starch synthesis in *RbRb* and *rbrb* embryos reveals a single, major, consistent difference. The activity of ADP glucose pyrophosphorylase is one-tenth or less in *rbrb* embryos of that in *RbRb* embryos. Recovery experiments show that this is likely to be a real difference in maximum catalytic activity of the enzyme in the two sorts of embryo, rather than a result of inhibition or degradation of the enzyme during extraction of *rbrb* embryos. ADP glucose pyrophosphorylase is thus the only candidate for a site of effect on the *rb* locus on the pathway of starch synthesis.

To discover whether the low activity of ADP glucose pyrophosphorylase in *rbrb* embryos could account for their low starch contents, we calculated the rate of starch synthesis in embryos of the high starch, *RRRbRb* line, BC1/7RR. This was done using the relationship between starch content and fresh weight (17) and the relationship between fresh weight and age of embryo (11) of this line of peas. For embryos of 300 to 400 mg, a stage of development with rapid starch accumulation which is linear with respect to increase in embryo weight, the rate of starch synthesis is 0.08 μmol glucose units incorporated min^{-1} g^{-1} fresh weight. This is a minimum estimate since it does not allow for any turnover of starch.

At all of the stages of embryo development of all of the lines of peas we studied, activities of sucrose synthase and UDP glucose pyrophosphorylase were much greater than required to account for the rate of starch synthesis of *RRRbRb* embryos. Although soluble starch synthase activity in some embryos at some stages of development was lower than required to account for the rate of starch synthesis, total starch synthase activity in all embryos of 300 to 400 mg was at least twice as great as required. The maximum catalytic activity of starch synthase is likely to be even higher than our estimates of total starch synthase suggest (see "Results"). The activity of starch-branching enzyme cannot be compared meaningfully with the rate of starch synthesis. However, all *RR* embryos have high activities of this enzyme whereas all *rr* embryos have low activities. There is good evidence that the effect of the *r* locus on starch synthesis in pea embryos is mediated through alteration of the activity of starch-branching enzyme (6, 17). It is likely that the low starch contents of *rrRbRb* embryos are due to their low activities of starch-branching enzyme.

Activity of ADP glucose pyrophosphorylase in *RbRb* embryos throughout development is 9- to 48-fold greater than is required to account for the rate of starch synthesis of *RRRbRb* embryos. In contrast, throughout development of most *rbrb* embryos the maximum catalytic activity of this enzyme is considerably less than or only equal to the rate of starch synthesis of *RRRbRb* embryos. Thus, the low starch contents of *RRrbrb* embryos are probably attributable to their low activities of ADP glucose pyrophosphorylase. We suggest that the *rb* locus manifests its effects on the starch contents of embryos through a large and specific alteration in the maximum catalytic activity of ADP glucose pyrophosphorylase. In this sense it is analogous to the *brittle-2* and *shrunken-2* loci of maize, which are believed to manifest their effects on endosperm starch content through action on ADP glucose pyrophosphorylase (3, 9).

Embryos of the *rrrbrb* genotype (JI 827) have very low activities of both ADP glucose pyrophosphorylase and starch-branching enzyme. ADP glucose pyrophosphorylase activity is the same as in *RRrbrb* embryos, and starch-branching enzyme activity is the same as in *rrRbRb* embryos. Activities of the other enzymes of starch synthesis are similar to those of embryos of other genotypes. This indicates that the effects of the *r* and *rb* mutations on enzymes of the pathway of starch synthesis are independent of each other.

Our results show that the tentative classification of the line JI 2108 (cultivar Ace) as *RRrbrb* (Table I) is probably incorrect. JI 2108 embryos have high activities of ADP glucose pyrophosphorylase, like those of *RbRb* embryos. They have very low activities of starch-branching enzyme, like those of *rr* embryos. It seems likely that this cultivar has the genotype *rrRbRb*. Preliminary hybridization experiments support this hypothesis. Hybridization of JI 2108 with JI 399 (*RRrbrb*) gives round seeds, showing that JI 2108 is *RbRb*. Hybridization with BC1/7rr gives wrinkled seeds, showing that JI 2108 is *rr* (data not shown).

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LITERATURE CITED

1. **Coxon DT, Davies DR** (1982) The effect of r_a and r_b loci on the lipid content of the seed of *Pisum sativum*. Theor Appl Genet **64:** 47–50
2. **Cousin R** (1974) Le Pois, Vol 2. INRA, Paris, p 11
3. **Dickinson B, Preiss J** (1969) Presence of ADP-glucose pyro-

phosphorylase in *shrunken-2* and *brittle-2* mutants of maize endosperm. Plant Physiol **44:** 1058–1062

4. **Edwards J, ap Rees T** (1986) Sucrose partitioning in developing embryos of round and wrinkled varieties of *Pisum sativum.* Phytochemistry **25:** 2027–2032
5. **Edwards J, ap Rees T** (1986) Metabolism of UDP-glucose by developing embryos of round and wrinkled varieties of *Pisum sativum.* Phytochemistry **25:** 2033–2039
6. **Edwards J, Green JH, ap Rees T** (1988) Activity of branching enzyme as a cardinal feature of the R_a locus in *Pisum sativum.* Phytochemistry **27:** 1615–1620
7. **Frydman RB, Cardini CE** (1967) Studies on the biosynthesis of starch. II. Some properties of the ADP glucose starch glucosyltransferase bound to the starch granule. J Biol Chem **242:** 312–317
8. **Ghosh HP, Preiss J** (1966) Adenosine diphosphate glucose pyrophosphorylase- a regulatory enzyme in the biosynthesis of starch in spinach leaf chloroplasts. J Biol Chem **241:** 4491–4504
9. **Hannah CL, Nelson OE** (1975) Characterization of adenosine diphosphate glucose pyrophosphorylases from developing maize seeds. Plant Physiol **55:** 297–302
10. **Hawker JS, Ozbun JL, Ozaki H, Greenberg E, Preiss J** (1974) Interaction of spinach leaf adenosine diphosphate glucose α-1,4-glucan α-4-glucosyl transferase and α-1,4-glucan, α-1,4-glucan-6-glycosyl transferase in synthesis of branched α-glucan. Arch Biochem Biophys **160:** 530–551
11. **Hedley CL, Smith CM, Ambrose MJ, Cook S, Wang TL** (1986) An analysis of seed development in *Pisum sativum* II. The effect of the *r*-locus on the growth and development of the seed. Ann Bot **58:** 371–379
12. **Keeling PL, Wood JR, Tyson RH, Bridges IG** (1988) Starch biosynthesis in developing wheat grain. Evidence against the direct involvement of triose phosphates in the metabolic pathway. Plant Physiol **87:** 311–319
13. **Kooistra E** (1962) On the differences between smooth and three types of wrinkled peas. Euphytica **11:** 357–373
14. **Lowry OH, Passonneau JV** (1972) A flexible system of enzymatic analysis. Academic Press, New York
15. **MacDonald FD, Preiss J** (1983) Solubilization of the starch-granule-bound starch synthase of normal maize kernels. Plant Physiol **73:** 175–178
16. **Preiss J** (1982) Biosynthesis of starch and its regulation. In Plant carbohydrates I. Intracellular carbohydrates. *In* FA Loewus, W Tanner, eds, Encyclopedia of Plant Physiology (New Series) Vol 13A. Springer-Verlag, Berlin pp 397–417
17. **Smith AM** (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.). Planta **175:** 270–279
18. **Turner JF** (1969) Physiology of pea fruits. IV Changes in uridine diphosphate glucose pyrophosphorylase and adenosine diphosphate glucose pyrophosphorylase in the developing seed. Aust J Biol Sci **22:** 1145–1151
19. **Tyson RH, ap Rees T** (1988) Starch synthesis by isolated amyloplasts from wheat endosperm. Planta **175:** 33–38
20. **Vos-Scheperkeuter GH, de Boer W, Visser RGF, Feenstra WJ, Witholt B** (1986) Identification of a granule-bound starch synthase in potato tubers. Plant Physiol **82:** 411–416