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The Wrinkled-Seed Character of Pea Described by Mendel Is Caused by a Transposon-like Insertion in a Gene Encoding Starch-Branching Enzyme

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Summary

We describe the cloning of the *r* (*rugosus*) locus of pea (Pisum sativum L.), which determines whether the seed is round or wrinkled. Wrinkled (*rr*) seeds lack one isoform of starch-branching enzyme (SBEI), present in round (*RR* or *Rr*) seeds. A major polymorphism in the SBEI gene between near-isogenic *RR* and *rr* lines shows 100% cosegregation with the *r* locus, establishing that the SBEI gene is at the *r* locus. An aberrant transcript for SBEI is produced in *rr* embryos. In *rr* lines the SBEI gene is interrupted by a 0.8 kb insertion that is very similar to the Ac/Ds family of transposable elements from maize. Failure to produce SBEI has complex metabolic consequences on starch, lipid, and protein biosynthesis in the seed.

Introduction

The first character described by Mendel in his study on the laws of inheritance was the "difference in the form of the ripe seeds" of pea (Mendel, 1865; Bateson, 1901). This character affected the shape of mature seeds, which were either round or wrinkled (Table 1). The production of alternative forms was governed by a single genetic locus, *rugosus* or *r* (White, 1917). The *r* locus offered two major advantages to studying the laws of inheritance: it conferred a prominent, easily recognizable phenotype, and it affected embryo development, allowing early identification of segregants in the seed of heterozygous parents. The clearly visible effect of *r* on seed phenotype results from the profound effect of this locus on the composition of the developing pea seed (Table 1). There is a marked difference in starch metabolism between round (*RR* or *Rr*) and wrinkled (*rr*) peas. In *RR* peas, starch grains are large and simple, while in *rr* peas they are small and compound (deeply fissured) (Gregory, 1903). *RR* seeds contain larger amounts of starch than *rr* seeds and have much higher ratios of amylopectin to amylose. In *rr* seeds, the levels of free sucrose are higher than in *RR* seeds, and this probably leads to the observed higher osmotic pressure and hence higher water content and greater cell size of developing *rr* seeds (Kappert, 1915; Hedley et al., 1986; Ambrose et al., 1987; Wang et al., 1987). The *rr* seeds lose a larger proportion of their volume upon seed maturation, and since the testa does not shrink with the cotyledons, it wrinkles to give the seed phenotype (Kappert, 1915). Mature *rr* seeds also contain more lipid and less of the storage protein legumin than *RR* seeds (Coxon and Davies, 1982; Davies, 1980; Domoney and Casey, 1985). Although many of these effects of the *r* locus were deduced using different round and wrinkled pea cultivars, they have been further extended and confirmed by the development of near-isogenic *RR* and *rr* lines (Hedley et al., 1986; Table 1).

An understanding of the gene at the *r* locus can provide an insight into the mechanisms controlling pea seed development and seed composition. The multiple differences observed between mature *RR* and *rr* peas could result from modification of a regulatory gene controlling all the affected processes or from a primary metabolic change, which in turn influences a number of other metabolic parameters. Considerable evidence has suggested that the primary lesion in *rr* embryos might occur in starch synthesis (Matters and Boyer, 1982; Edwards and ap Rees, 1986a, 1986b; Edwards et al., 1988; Smith, 1988). If starch biosynthesis is limited, it could result in an accumulation of free sucrose and a change in osmotic pressure of the developing embryos. The most likely cause of a reduction in starch synthesis accompanied by a failure to convert amylose to amylopectin is a loss of activity of starch-branching enzyme. Metabolic evidence and reduced starch-branching enzyme activity indicate that this enzyme is important in determining the starch content of *rr* embryos (Smith, 1988; Edwards et al., 1988). We have shown that the *r* lesion is associated with complete absense of one isoform of starch-branching enzyme (SBEI) from developing embryos (Smith, 1988). This isoform is active early in development in *RR* embryos. The absence of SBEI from *rr* embryos could be due to a lesion either in the gene encoding SBEI or in a gene regulating the production of SBEI. We describe here the cloning and characterization of the gene encoding SBEI and the establishment of its relationship to the *r* locus.

Results

Cloning and Characterization of cDNA for Starch-Branching Enzyme

To determine if the wrinkled phenotype of pea resulted from a lesion in a gene encoding SBEI, we used an antibody to this isoform to clone a cDNA and used this probe to examine the gene structure in *RR* and *rr* isolines. An antiserum raised against the 114 kd protein of the isoform of starch-branching enzyme unique to *RR* peas, SBEI, cross-reacted specifically with 114 and 108 kd proteins on Western blots of SDS–polyacrylamide gels of crude extracts of developing embryos of *RR* but not *rr* peas (Figures 1a and 1b). Incubation of crude extracts of *RR* embryos with the antiserum strongly inhibited the activity of starch-branching enzyme. Under the conditions used, preimmune serum alone inhibited activity by less than 20%, whereas antiserum alone inhibited it by 90%. The high level of specificity of this antiserum for starch-

Table 1. Composition of Mature *RR* and *rr* Pea Seeds

	RR	*rr*	Reference
Starch (% dwt)	45–49 (4)	33–36 (6)	Kellenbarger et al. (1951)
	42–43	31–32 (3)	Greenwood and Thomson (1962)
	46–54 (6)	30–36 (5)	Kooistra (1962)
Amylose (% total starch)	33–45 (4)	67–71 (6)	Kellenbarger et al. (1951)
	33	71	Colonna and Mercier (1984)
	34–35 (3)	65–66 (4)	Greenwood and Thomson (1962)
	36–51 (6)	59–68 (5)	Kooistra (1962)
	33–37 (6)	57–65 (10)	Schneider (1951)
Sucrose (% dwt)	5–7 (6)	9–12 (5)	Kooistra (1962)
Lipid (% dwt)	2.8–3.1 (6)[a]	4.5–5.2 (6)[a]	Coxon and Davies (1982)
	2.4	4.2	Coxon and Davies (1982)
Legumin (% protein)	24–39 (7)[b]	6–30 (7)[b]	Davies (1980)
	33[c]	23[c]	Domoney and Casey (1985)

Ranges refer to studies on a number of genotypes (given in parentheses). a, b, and c indicate paired studies with near isogenic lines of *RR* and *rr* plants. In study b, *RR* values for legumin were greater than *rr* values in all but one of seven near-isogenic pairs. (% dwt indicates percent dry weight).

branching enzyme proteins and the fact that cross-reaction with SBEI was very strong rendered it suitable for screening a cDNA library in λgt11.

Seven cDNA clones were identified from screening approximately 3×10^5 pfu of an expression library in λgt11. Hybridization of pJSBE3, a subclone derived from one of these seven, to other clones revealed a very high degree of homology under very stringent washing conditions. Restriction mapping of each phage clone revealed identical regions in the maps, supporting the view that the antiserum raised to the 114 kd isoform of starch-branching enzyme recognized a unique protein translated from a message represented by seven independent cDNA clones. The longest cDNA clone, pJSBE5 (Figure 2a), was 2.7 kb long. Northern blot analysis (Figure 2b) revealed that the two EcoRI fragments of pJSBE5 originated from the same transcript. The size of the transcript detected by pJSBE5 in *RR* peas was approximately 3.3 kb, which was sufficient to encode a protein of 114 kd. In *rr* embryos, the transcript complementary to pJSBE5 was larger than in *RR* embryos (4.1 kb) and about 10-fold less abundant (Figure 2b). Analysis of steady-state levels of the SBEI transcript revealed that the gene was quite highly expressed during the early stages of embryo development (50 mg fresh weight) and that transcript levels declined as the embryos matured (Figure 2c) both in *RR* and *rr* embryos. The sequence of the long cDNA clone, pJSBE5, showed high homology when translated (51.3% similarity as measured by BESTFIT in the WISCONSIN package) to glycogen-branching enzyme of Escherichia coli (Baecker et al., 1986), confirming that this clone was a cDNA for starch-branching enzyme.

Molecular Organization of the SBEI Gene

To investigate the organization of the SBEI gene in *RR* and *rr* genotypes, genomic DNA from the near-isogenic lines was digested with EcoRI, EcoRV, and HindIII and probed with pJSBE5 (Figure 3). EcoRI revealed a clear difference between the two genotypes. Using the 3′ EcoRI cDNA fragment as a probe (1.3 kb; Figure 2a), the *rr* genotype had a fragment (4.1 kb) that was 0.8 kb larger than the corresponding fragment (3.3 kb) of the *RR* genotype. No differences were observed between *RR* and *rr* plants for the 7.7 kb EcoRI fragment of genomic DNA homologous to the 5′ end (1.4 kb) of the cDNA clone. A larger band was also observed in the HindIII-digested DNA of *rr* plants compared with *RR* plants. EcoRV digestion gave four bands homologous to the cDNA clone in *rr* plants compared with three in *RR* plants, indicating an additional EcoRV site in the gene from the *rr* genotype.

Linkage Analysis of the SBEI Gene and the *r* Locus

The clear difference in the SBEI gene and the difference in size and abundance of the SBEI transcript between *RR* and *rr* near-isogenic lines suggested very strongly that this gene was at the *r* locus. This was confirmed by linkage analysis. Genomic DNA from 79 F6 plants (derived by single seed descent) from two separate crosses (JI15[*RR*] × JI1194[*rr*] and JI15[*RR*] × JI61[*rr*]) was digested with EcoRI, and Southern blots were probed with the 1.3 kb EcoRI fragment of pJSBE5 (Figure 4). Comparison of the seed phenotype of each plant with the restriction fragment length polymorphism for the SBEI gene revealed 100% linkage of the gene to the *r* locus (no recombinants out of 79 lines). This result, together with the discovery of an aberrant SBEI transcript in *rr* lines, led to the conclusion that the SBEI gene is at the *r* locus.

Molecular Analysis of the Insertion in the 3′ End of the *rr* Allele

The identification of a major difference in restriction fragments of the SBEI gene between *RR* and *rr* lines suggested that the polymorphism itself might be the cause of the absence of SBEI in *rr* peas, and that the lesion in *rr* peas might be due to an insertion of DNA toward the 3′ end of the gene. To investigate this further, the genomic

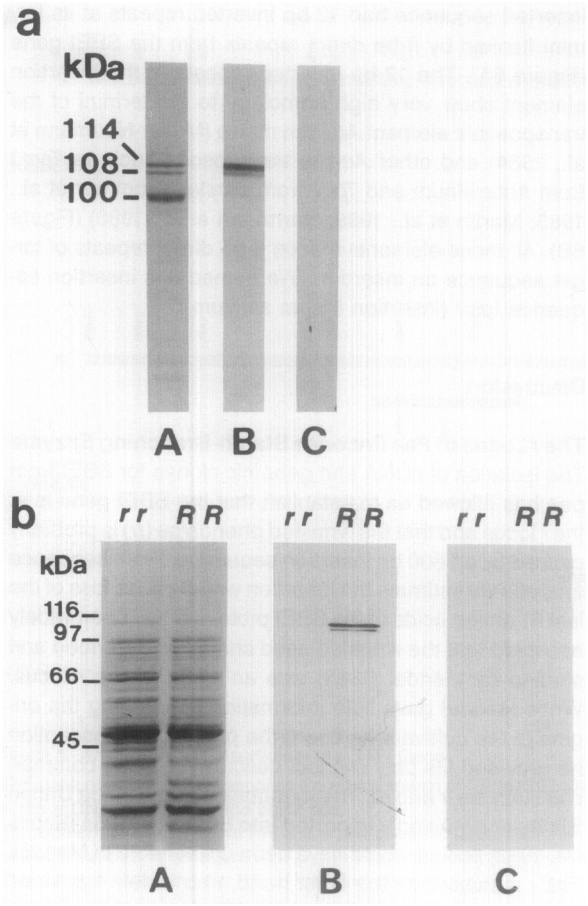


Figure 1. Cross-Reaction of Antiserum with Purified Starch-Branching Enzyme and Crude Extracts of Pea Embryos

(a) Reaction of starch-branching enzyme proteins with antibody to SBEI. (A) SDS–7.5% polyacrylamide gel of 2 μg of purified starch-branching enzyme from developing embryos of *RR* peas. (B) Western blot of gel in (A), developed with a 1/1000 dilution of 1 mg/ml^{-1} immunoglobulin fraction of antiserum to the 114 kd protein of starch-branching enzyme. (C) same as (B), but immunoglobulin fraction of preimmune serum.

(b) Occurence of SBEI proteins in crude extract of embryos. (A) SDS–7.5% polyacrylamide gel of 20 μg of protein of crude extracts of developing embryos (0.36 g fresh weight) of *RR* and *rr* peas. (B) Western blot of gel in (A) developed with a 1/100 dilution of 5 mg/ml^{-1} immunoglobulin fraction of antiserum to the 114 kd protein of starch-branching enzyme. (C) same as (B), but immunoglobulin fraction of preimmune serum. Apparent molecular sizes are indicated.

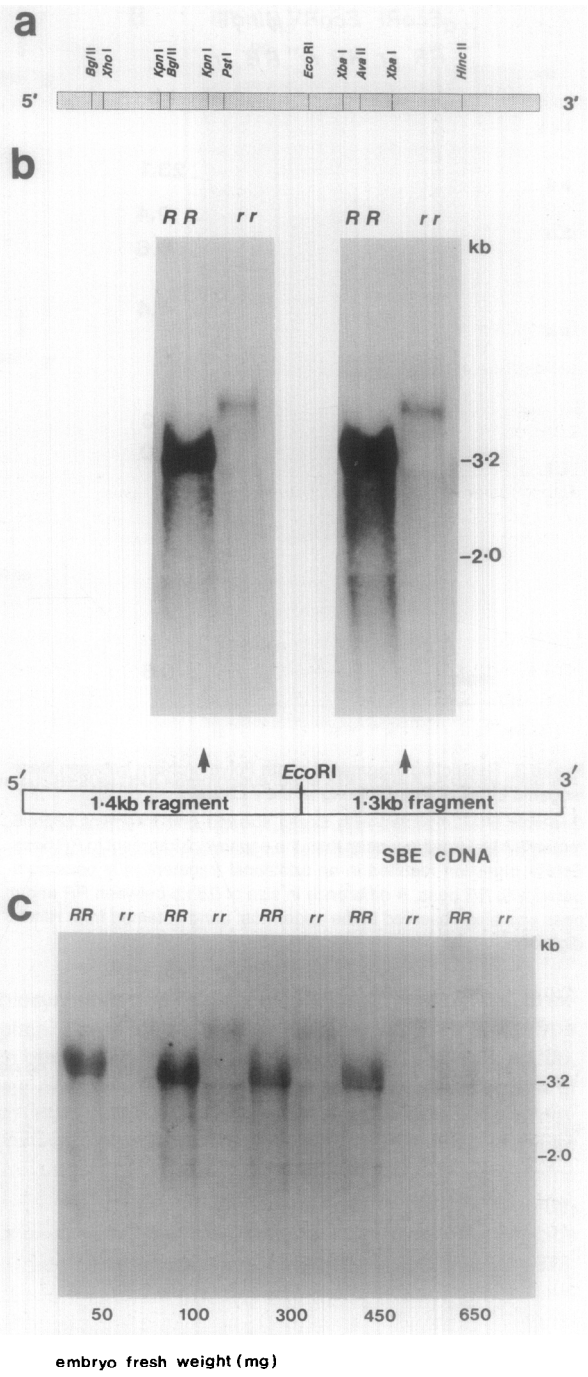


Figure 2. Analysis of the cDNA for SBEI

(a) Restriction map of the largest (2.7 kb) cDNA isolated for SBEI (pJSBE5).

(b) Northern blot of RNA from round (*RR*) and wrinkled (*rr*) embryos. Poly(A)$^+$ RNA (10 μg) from *RR* and *rr* embryos was probed with either the 1.4 kb (5′) or the 1.3 kb (3′) EcoRI fragment of pJSBE5.

(c) Northern blot of RNA from different stages of pea embryo. Total RNA (25 μg per lane) was probed with the 2.7 kb insert of pJSBE5.

region hybridizing to the 3′ end of pJSBE5 was cloned as an EcoRI fragment from both *RR* and *rr* peas; these clones were called pJSBE102 and pJSBE206, respectively. Restriction mapping (Figure 5A) revealed an extra 0.8 kb of DNA in the gene from *rr* peas, located between the XbaI and HincII sites toward the 3′ end of the gene. When Southern blots of genomic DNA were hybridized with the HincII fragments from pJSBE102 and pJSBE206 (without and with the insertion, respectively), a single band (the SBEI gene) hybridized with the probe from pJSBE102, but many fragments were revealed with the probe from pJSBE206 (Figure 5B). Thus the novel DNA in the *rr* gene must be repeated many times in the pea genome.

The sequences of the ends of the insertion in the SBEI gene, the sequences of the gene flanking the insertion (pJSBE206), and the comparable regions from pJSBE102 indicated that the insertion was located in an exon. The

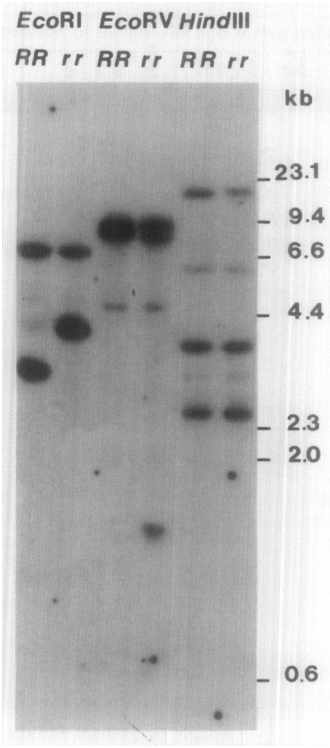


Figure 3. Restriction Fragment Length Polymorphism between Near-Isogenic Lines of Pea Differing at the *r* Locus

Genomic DNA digested with EcoRI showed one fragment approximately 0.8 kb larger in *rr* peas than the equivalent fragment in *RR* peas. EcoRV digestion resulted in an additional fragment in *rr* peas compared with *RR* peas. A difference in size of 0.8 kb between *RR* and *rr* peas was also observed in the uppermost band obtained from HindIII digestion.

inserted sequence had 12 bp inverted repeats at its termini flanked by 8 bp direct repeats from the SBEI gene (Figure 6A). The 12 bp inverted repeats of the insertion element show very high homology to the termini of the transposable element *Ac* from maize (Müller-Neumann et al., 1984) and other *Ac*-like transposons such as *Tam3* from Antirrhinum and *Tpc1* from parsley (Sommer et al., 1985; Martin et al., 1989; Herrmann et al., 1988) (Figure 6B). All these elements induce 8 bp direct repeats of target sequence on insertion. We named this insertion sequence Ips-*r* (insertion Pisum sativum-*r*).

Discussion

The *r* Locus of Pea Encodes Starch-Branching Enzyme

The isolation of cDNA and genomic clones for SBEI from pea has allowed us to establish that the SBEI gene is at the *r* locus and that the wrinkled phenotype (*rr*) is probably caused by an 800 bp insertion sequence. From sequence analysis we estimate the insertion would cause loss of the last 61 amino acids of the SBEI protein. It has been widely accepted that the wrinkled-seed character described and studied by Mendel (1865) was an allele of the *r* locus. While Mendel gave little information concerning the origins of the cultivars he used, the phenotypic description he provided fits only two loci described among commercial cultivars, *r* and *rb*. The *rb* mutation arose in the United States and was only imported into Europe in 1934 (Kooistra, 1962) and so would have been unavailable to Mendel. The *r* mutation, on the other hand, was widely dispersed in European commercial cultivars because of the sweetness of wrinkled peas, and White (1917) concluded that

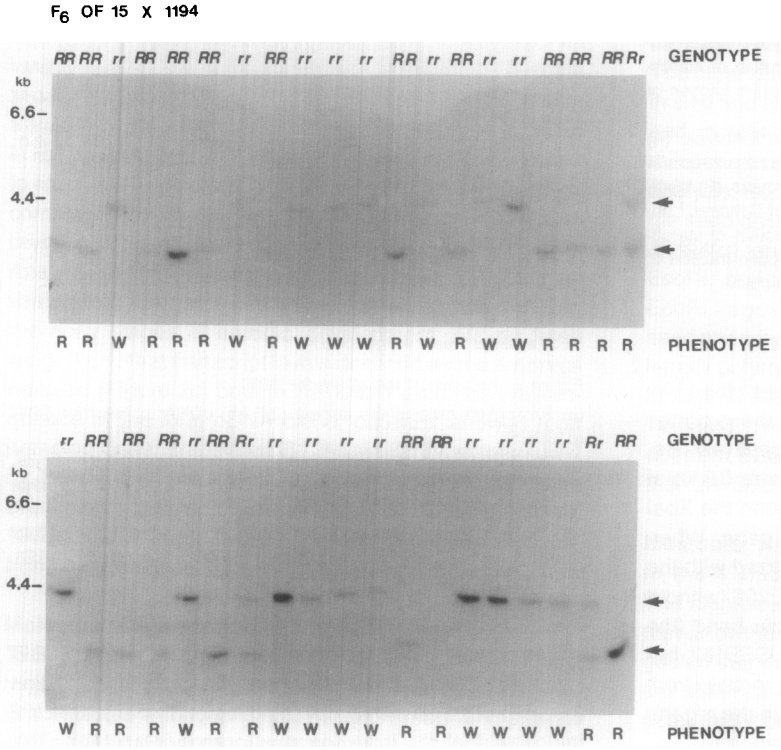


Figure 4. Example of Restriction Fragment Length Polymorphism among F6 Plants of a Cross between Lines JII15 and JII194

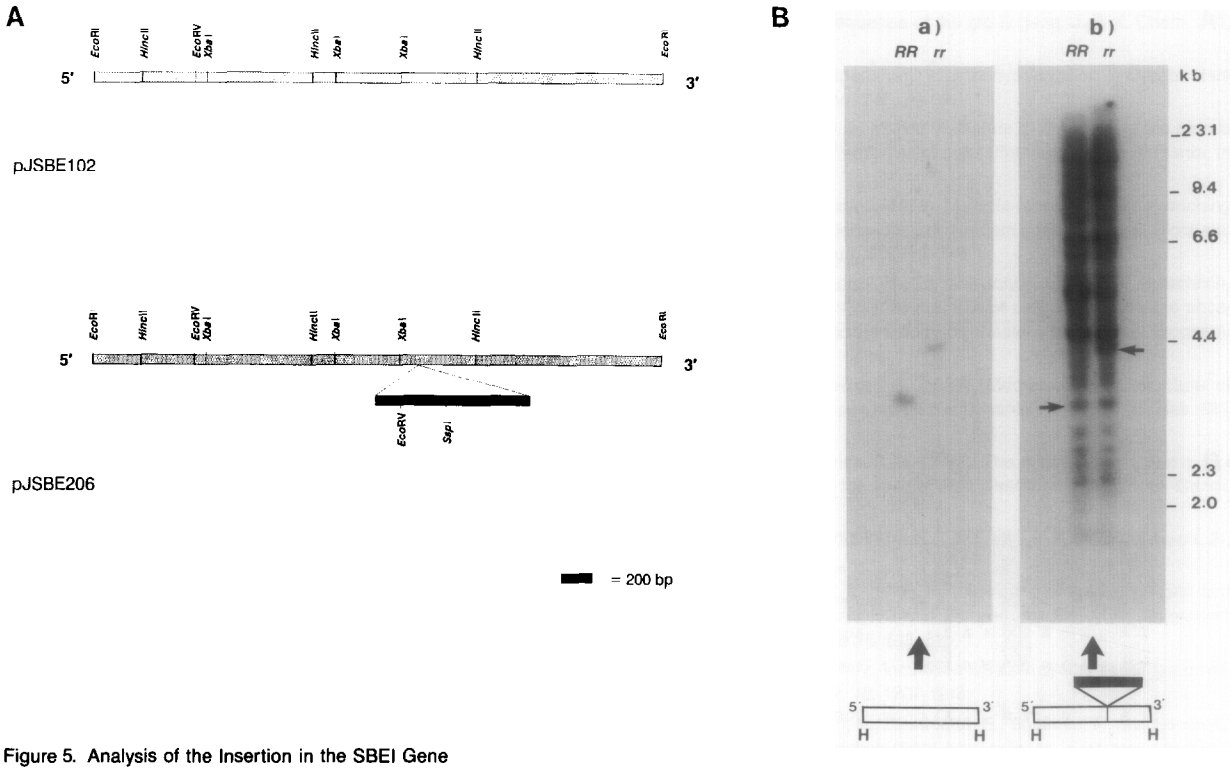


Figure 5. Analysis of the Insertion in the SBEI Gene

(A) Restriction maps of the genomic EcoRI fragments of *RR* (pJSBE102) and *rr* (pJSBE206) DNA homologous to the SBEI cDNA. A 0.8 kb insertion in the *rr* DNA is shown by the black box.

(B) Southern blot analyses of *RR* and *rr* pea genomic DNA. Genomic DNA (10 μg) obtained from *RR* and *rr* leaves was digested with EcoRI and probed as shown in the figure with (a) the HincII fragment from the *RR* line that does not contain the insertion element or (b) the HincII fragment from the line containing the 0.8 kb insertion element.

only one factor, the *r* locus, was involved in all preceding studies of wrinkled-seeded peas, including that of Mendel. Therefore, since all the data Mendel provided fit the assumption that he used the *r* mutant and since there is no evidence of another mutation available at the time, we believe that the gene we have cloned is the one studied by Mendel.

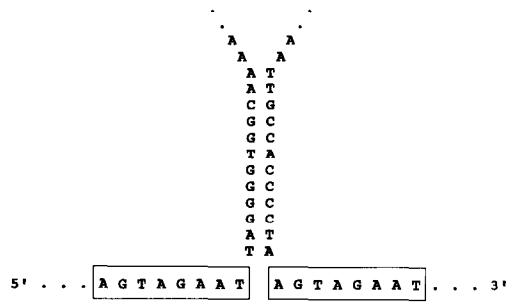
The Relationship between the *r* Locus and Seed Composition and Development

Our findings illustrate the importance of the process of starch synthesis itself in determining the overall composition of the seed. All the effects of the *r* mutation are likely to be consequences of a reduction in starch synthesis caused by a reduction in branching enzyme activity.

Loss of SBEI activity probably results in the reduced production of starch, because the substrate for starch synthase, the nonreducing ends of the glucose chains in the starch polymer, becomes limiting (Edwards et al., 1988; Smith, 1988). Reduced starch biosynthesis may, in turn, lead to the accumulation of sucrose in the developing embryos and hence to the effects on osmotic pressure, water content, cell size, and seed shape. The change in morphology of starch grains, from simple to compound, could be due to their high amylose content.

The influence of reduced activity of SBEI on legumin biosynthesis and lipid accumulation is more difficult to explain. There are mutants of a similar wrinkled phenotype in other species. These include the *sh*1, *sh*2, *bt*2, *ae*, *du*, *su*, *opaque* 2, and *opaque* 7 mutants of maize (Misra et al., 1972, 1975) and the Risø high-lysine mutants of barley (Shewry et al., 1987). All these mutations show reduced production of one class of storage proteins, the prolamins (zein or hordein), reduced starch biosynthesis, and generally elevated levels of free sucrose and amino acids (Creech, 1969; Dalby and Tsai, 1975; Di Fonzo et al., 1978; Shewry et al., 1987). Several of the mutant genes have been identified in maize, and some are lesions in starch biosynthesis (*sh*2 and *bt*2 are mutations affecting ADPG-pyrophosphorylase [Hannah and Nelson, 1976]; *wx* affects starch synthase [Nelson and Rhines, 1962]; *ae* affects starch-branching enzyme [Boyer and Preiss, 1978]), while the opaque genes appear to be regulators of zein biosynthesis. The repeated association of reduced prolamin synthesis and reduced starch production in a number of mutations of different types of gene (Di Fonzo et al., 1978) suggests a coupling of prolamin and starch synthesis through metabolic control rather than through specialized regulatory genes. The expression of the potato tuber storage protein patatin (class I) may be regulated by cellular sucrose levels or the rate of starch biosynthesis (Rocha-Sosa et al., 1989). Little is known about how le-

A



B

T A G G G G T G G C A A	... Ips*r*	Pea
T A A A G A T G G C A A	... Tam3:105 ...	*Antirrhinum*
T A G G G . T G T A A A	... Tpc*l*	Parsley
T A G G G A T G A A A	... Ac/Ds	Maize
T A A A G A T G T G A A	... Tam3	*Antirrhinum*
T A . . G . T G . . A	... CONSENSUS	

Figure 6. Sequences of the Terminal Inverted Repeats of the Insertion Element Ips-*r*

(A) Sequences of the 12 bp inverted repeats of the end of Ips-*r* and flanking 8 bp direct repeats of the SBEI target site.

(B) Homology of the inverted repeat of Ips-*r* with those of other elements. Plants from which the elements were isolated are given on the extreme right column of the figure. Common homologous sequences among the elements and the sequence of Ips-*r* are shown by dark background.

sions in storage protein biosynthesis might limit starch production.

The Role of Osmotic Pressure in Controlling Seed Composition and Development

One common factor linking starch and storage protein biosynthesis could be osmotic pressure. The wrinkled-seed phenotypes of all these mutant lines in pea, maize, and barley indicate that changes in osmotic pressure are involved. Starch and storage protein biosynthesis may both be sensitive to small changes in osmotic pressure (Oparka and Wright, 1988; Tsai et al., 1978). In pea, increases in osmotic pressure of the external medium have been found to cause a dramatic reduction in steady-state mRNA levels for legumin but not for vicilin in cultured *RR* embryos (S. Turner, personal communication), mimicking the effects of the *r* mutation on legumin mRNA. It is likely that the higher osmotic pressure of *rr* embryos leads to their greater lipid accumulation. Structural differences, including and arising from differences in cell size caused by different osmotic pressures, mean that *rr* embryos have a greater area of membrane than *RR* embryos (Ambrose et al., 1987; Bettey and Smith, 1989).

The nature of the influence of osmotic pressure on seed development and the mechanisms through which cellular processes in plants respond to osmotic change are not established. At present we can only suggest that changing osmolarity caused by sucrose accumulation is a major factor in the developmental and compositional changes brought about by the mutation at the *r* locus.

The wrinkled-seed phenotype of peas is the result of complex changes in the metabolic processes of seed development. The discovery that the *r* locus encodes SBEI helps to clarify these changes to some extent, but most importantly indicates the central role that metabolism must play in the control of plant development.

Experimental Procedures

Plant Material

Pea plants were grown in the greenhouse according to Smith (1988). Unless otherwise stated, *RR* and *rr* plants were near-isogenic lines derived from JI430 as described by Hedley et al. (1986). Other lines were obtained from the germplasm collection, John Innes Institute.

Preparation of Antibody

The 114 kd polypeptide (SBEI) of starch-branching enzyme (EC 2.4.1.18) was excised from SDS–polyacrylamide gels of the purified enzyme (Smith, 1988), electroeluted from the gel slice, and freeze-dried. Protein (75 μg) was redissolved in 0.5 ml of phosphate-buffered saline (PBS), mixed with Freund's complete adjuvant, and injected intramuscularly into the rabbit. The injection was repeated 3 weeks later. Antiserum was collected 3 weeks after the second injection. The immunoglobulin fraction was prepared by precipitation with 50% saturated ammonium sulfate and dialysis against PBS.

Western Blotting

Crude extracts of pea embryos were made by extraction of 0.5 g of embryo tissue in 2.5 ml of 100 mM MOPS (pH 7.2), 2 mM DTT and centrifugation at 10,000 × g for 10 min. The supernatant was adjusted to 2 mg/ml^{-1} protein with extraction medium, diluted 1:1 with double-strength sample buffer (Laemmli, 1970), and boiled for 1 min. Starch-branching enzyme was purified and separated by SDS–PAGE according to Smith (1988). Protein was transferred to nitrocellulose by Western blotting, and filters were incubated with the immunoglobulin fraction of rabbit serum followed by alkaline phosphatase–conjugated goat anti-rabbit antiserum (Sigma, Poole, Dorset, UK) according to Blake et al. (1984), except that the initial blocking step contained 3% (w/v) bovine serum albumin and 2% (w/v) dried milk powder.

Preparation of RNA

Embryos of comparable size were pooled and frozen in liquid N_2. Total RNA was extracted according to Prescott and Martin (1987) and Ward et al. (1989). Polyadenylated RNA was purified by two passages of RNA over a column of oligo(dT)-cellulose.

Construction and Screening of a cDNA Library

cDNA was synthesized from a mixture of poly(A)$^+$ RNA derived from *RR* embryos at two developmental stages (90–100 mg and 350–400 mg fresh weight). The first strand of the cDNA was synthesized using reverse transcriptase primed with oligo(dT) and the second strand using RNAase H and DNA polymerase I (Klenow fragment). About 1.8 μg of cDNA was obtained from a total of 2.2 μg of poly(A)$^+$ RNA. The cDNA was methylated to protect it from EcoRI digestion, EcoRI linkers were added, and cDNA was ligated into the EcoRI site of λgt11. About 3×10^5 plaques were screened for the expression of starch-branching enzyme protein using the antiserum to the 114 kd protein purified from *RR* embryos. During screening, the antiserum to the 114 kd protein was preabsorbed with E. coli lysate (10 mg/ml^{-1}) at 1:50 dilution to block nonspecific binding. The filters were then absorbed with the antiserum (1 mg/ml^{-1}) at a dilution of 1:500. Antiserum binding was assayed using peroxidase-linked donkey anti-rabbit antiserum detected with 3,3'-diamino-benzidine tetrahydrochloride.

The cDNA clones obtained through these screening procedures were subcloned into the EcoRI site of plasmid vector pUC1813 (Kay

and McPherson, 1987), and the resulting plasmids were called pJSBE3, pJSBE5, pJSBE12, pJSBE14, pJSBE20, and pJSBE21.

Isolation of DNA and DNA Hybridization

Minipreparations of DNA from recombinant λgt11 clones were made from small-scale cultures of E. coli (Y1090) infected with individual plaques and grown at 43°C for 3 hr in 5 ml of L-broth without glucose containing ampicillin (100 $\mu g/ml^{-1}$) and $CaCl_2$ (5 mM). Large-scale preparations of DNA from recombinant λgt11 clones were prepared according to Maniatis et al. (1982). Minipreparations and large-scale preparations of plasmid DNA from recombinant pUC1813 clones were carried out according to Holmes and Quigley (1981). Genomic DNA was prepared from pea leaves either according to Ellis et al. (1984), or as large-scale preparations from 5–10 g leaves, where the DNA was purified over CsCl (Martin et al., 1985).

Southern Blotting and Hybridization

DNA digested with various restriction enzymes was separated on 0.6%–0.8% agarose gels and blotted onto nitrocellulose filters according to Southern (1975) and Wahl et al. (1979). Radioactive probes were prepared by nick translation (Maniatis et al., 1982). Blots were routinely washed with 0.1× SSC, 0.5% (w/v) SDS at 65°C.

Northern Blot Analysis

Total or poly(A)$^+$ RNA from developing embryos was separated in agarose gels containing formaldehyde and transferred to nitrocellulose (Lehrach et al., 1977). Blots were hybridized overnight with nick-translated radioactive DNA fragments and washed twice with 2× SSC, 0.5% (w/v) SDS at 65°C.

Cloning and Sequencing of Genomic DNA

Genomic DNA obtained from *RR* and *rr* leaves was completely digested with EcoRI. Fragments homologous to the cDNA clone for starch-branching enzyme (pJSBE5) were size fractionated on agarose gels and cloned into the EcoRI site of λNM1149 (Murray, 1983). Recombinant plaques were grown in E. coli (C600) and screened with the 1.3 kb EcoRI fragment obtained from pJSBE5. A 3.3 kb EcoRI fragment from *RR* peas and a 4.1 kb EcoRI fragment from *rr* peas were subcloned into pUC1813, and resulting plasmids were called pJSBE102 and pJSBE206, respectively.

The major differences between the plasmid clones derived from *rr* plants and those derived from *RR* plants were identified by restriction mapping. These regions of pJSBE21 and also pJSBE102 were subcloned into M13 and sequenced following the method of Sanger et al. (1977).

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