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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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 (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.

Results

To determine if the wrinkled phenotype of pea resulted from a lesion in a gene encoding SBEI, we used an antiserum raised against the 114 kd protein of the isoform of starch-branching enzyme unique to RR peas, SBEI, to detect SBEI in 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

<table>
<thead>
<tr>
<th>Composition</th>
<th>RR</th>
<th>rr</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch (% dwt)</td>
<td>45-49 (4)</td>
<td>33-36 (6)</td>
<td>Kellenbarger et al. (1961)</td>
</tr>
<tr>
<td>Amylose (%) total starch</td>
<td>33-35 (3)</td>
<td>65-66 (4)</td>
<td>Greenwood and Thomson (1962)</td>
</tr>
<tr>
<td>Sucrose (% dwt)</td>
<td>36-51 (6)</td>
<td>59-68 (5)</td>
<td>Kooistra (1962)</td>
</tr>
<tr>
<td>Lipid (% dwt)</td>
<td>33-37 (6)</td>
<td>57-65 (10)</td>
<td>Schneider (1951)</td>
</tr>
<tr>
<td>Legumin (% protein)</td>
<td>24-39 (7)b</td>
<td>6-30 (7)b</td>
<td>Domoney and Casey (1965)</td>
</tr>
</tbody>
</table>

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 x 10^6 pfu of an expression library in λgt11. Hybridization of pJSE3, 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, pJSE5 (Figure 2a), was 2.7 kb long. Northern blot analysis (Figure 2b) revealed that the two EcoRI fragments of pJSE5 originated from the same transcript. The size of the transcript detected by pJSE5 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 pJSE5 was larger than in HH 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, pJSE5, showed high homology when translated (51.3% similarity as measured by BESTFIT in the WISCONSIN package) to glycogen-branching enzyme of Escherichia coli (Baeker 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 pJSE5 (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 (J115[RR] x J1194[rr] and J115[RR] x J161[rr]) was digested with EcoRI, and Southern blots were probed with the 1.3 kb EcoRI fragment of pJSE5 (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
Isolation of \( r \) Locus of Pea

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 \( \mu \)g 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) Occurrence of SBEI proteins in crude extract of embryos. (A) SDS-75% polyacrylamide gel of 20 \( \mu \)g 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.

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
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 Ipe-r (insertion P. 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
isolation of r Locus of Pea

Figure 5. Analysis of the Insertion in the SBEI Gene
(A) Restriction maps of the genomic EcoRl 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 EcoRl and probed as shown in the figure with (a) the Hincll fragment from the RR line that does not contain the insertion element or (b) the Hincll 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 sh1, sh2, bt2, ae, du, su, opaque 2, and opaque 7 mutants of maize (Misra et al., 1972, 1975) and the Riso 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 (sh2 and bt2 are mutations affecting ADP-glucose 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-
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 osmolality 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 J430 as described by Hedley et al. (1988). 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 x 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 N2. 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 links were added, and cDNA was ligated into the EcoRI site of pBluescript. About 3 x 10^5 plaques were screened for the expression of starch-branching enzyme protein using the antisera to the 114 kd protein purified from RR embryos. During screening, the antisera 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 antisera (1 mg/ml-1) at a dilution of 1:500. Antiserum binding was assayed using peroxidase-linked donkey anti-rabbit antisera 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 (Y1000) infected with individual plaques and grown at 43°C for 3 hr in 5 ml of L-broth without glucose containing ampicillin (100 μg/ml)1) and CaCl2 (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 (1986). 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 hybridized overnight with nick-translated radiolabeled DNA fragments and washed twice with 2x SSC, 0.1% (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 (C800) 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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