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# The Effect of the $r_a$ and $r_b$ Loci on the Lipid Content of the Seed of *Pisum sativum*

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**Summary.** The lipid content of seed from a set of isogenic lines for the $R_a$: $r_a$ locus has been determined; the results show that this locus as well as affecting the starch, sugar and storage protein content and composition, also has a marked effect on the lipid content of the seed. Genotypes having different combinations of alleles at the $r_a$ and $r_b$ loci have also been examined; an $r_a r_a r_b r_b$ genotype had 5.57% purified total lipid in its seed – a more than 2-fold increase over that found in the round-seeded varieties ($R_a R_a R_b R_b$) usually grown for the dry sed crop.

**Key words:** Lipid content – Storage protein – Legume seeds – Genetic control – Pea – *Pisum sativum*

## Introduction

Genetic variants in which the composition of the seed has been changed have been recognised in a number of crop plants. Among the best known are the high lysine variants of maize (Mertz et al. 1964; Nelson et al. 1965) and of barley (see Munck et al. 1970; Ingverson et al. 1973), in which there is a reduction in the proportion of prolamin in the seed protein. Many mutants of maize also show differences in the proportions of starch and sugar accumulated in the seed (see Nelson 1981) and genetically induced alterations in fatty acid composition are seen in variant forms of *Brassica napus* (Downey 1963) and of *Carthamus tinctorius* (see Ladd and Knowles 1971).

The only reported major gene variants affecting the composition of a legume seed have been those involving mutations at the $r_a$ and $r_b$ loci in *Pisum sativum;* these are two quite separate loci, $r_a$ is on chromosome 7 and $r_b$ on chromosome 3 (Blixt 1972). If the dominant allele is present at both the $r_a$ and $r_b$ loci then the seed has a higher starch and lower sugar content (Kooistra 1962). Recently it has been shown that the $r_a$ locus also influences the storage protein composition of the seed; most $r_a r_a$ genotypes have lower proportions of the 11S (legumin) storage protein than genotypes in which the $R_a$ allele is present (Davies 1980). Of particular interest has been the indication that a third class of seed storage compounds may be influenced by the $r_a$ locus; Colonna et al. (1980) have shown that a wrinkled variety of peas ($r_a r_a$ genotype) had over twice as much lipid as a round variety ($R_a R_a$) (4.5 v 2.0%). In this paper we have examined whether this difference is due to the $r_a$ locus and also determined whether the $r_b$ locus has a role in the regulation of the lipid content and composition of pea seeds. Both the $r_a$ and $r_b$ loci have been shown to have an effect on the lipid content of the seeds.

## Materials and Methods

*Genotypes*

The following varieties of *Pisum sativum* were used:

| | |
|---|---|
| c. v. Birte | ($R_a R_a R_b R_b$) Round seed, simple starch grains |
| c. v. Greenshaft | ($r_a r_a R_b R_b$) Wrinkled seed, compound starch grains |
| J. I. 399 | ($R_a R_a r_b r_b$) Wrinkled seed, simple starch grains |
| J. I. 827 | ($r_a r_a r_b r_b$) Wrinkled seed, compound starch grains. |

A set of six near-isogenic lines for the $R_a$ : $r_a$ locus, derived from lines kindly provided by Dr. A. Slinkard of the University of Saskatchewan, were also used. Lines 9, 10 and 11 were derived from the cross PI 206790 × MP39; line 26 from PI 206838 × MP 39 and lines 43 and 44 from PI 206790 × Trapper. The seeds that were used were obtained from heterozygous $F_8$ plants, these having been derived in turn from heterozygous $F_7$ plants. All the near-isogenic lines were $R_b R_b$.

*Lipid Analysis*

The total lipid contents of the four lines representing the four different genotypes were determined by a slight modification

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of the method of Haydar and Hadziyev (1973). Pea samples were ground to a flour which would pass through a 75 μm sieve using a Janke and Kunkel mill. Duplicate 10 g samples of flour (5 g samples in the case of JI 399) were extracted with chloroform-methanol (2 : 1) to obtain the crude lipid extract. Non-lipid contaminants in the crude extract were removed by the method of Folch et al. (1957). Purified total lipid was then determined gravimetrically. The moisture content of the pea flours was determined by drying for 3 h at 110° followed by vacuum desiccation over $P_2O_5$ to constant weight. Lipid contents are expressed as percentages relative to the dry weight of the pea flour. Lipid class composition was determined on each purified total lipid extract by a gravimetric method employing silicic acid column chromatography for the separation of neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions (Rouser et al. 1967). Individual fatty acid composition of the total lipid and neutral lipid fractions was determined by gas chromatography of the mixtures of methyl esters obtained by transmethylation of the respective fractions.

The crude total lipid content of a set of six near-isogenic lines for the $R_a$:$r_a$ locus was determined by soxhlet extraction with dichloromethane-methanol (2 : 1) using a Tecator Soxtec apparatus. Triplicate 4 g samples of pea flour were extracted by a procedure involving 10 min extraction in the boiling solvent followed by a 30 min rinsing period. The extracted lipid was evaporated and dried to constant weight in a vacuum desiccator and then determined gravimetrically.

## Results and Discussion

Lipid contents of the paired sets of near isogenic pea lines are given in Table 1. The $R_aR_a$ lines had lipid contents ranging from 2.82 to 3.07%. For comparison the crude lipid contents of some commercial round seeded varieties (genotype $R_aR_a$) are as follows: Birte, 2.70; Filby, 3.07; Progreta, 3.15; Trapper 3.30 and Proco, 3.24%. In contrast the isogenic $r_a r_a$ lines had much higher crude lipid contents, between 4.51 and 5.15%; these values are comparable to those obtained in commercial wrinkled-seeded cultivars (genotype $r_a r_a$); Scout had 4.75 and Greenshaft 4.21. The results in Table 1 extend those of Colonna et al. (1980) who showed that a round seeded ($R_aR_a$) variety had a lower lipid content than a wrinkled form ($r_a r_a$), and demonstrate the effect of the $r_a$ locus on the total lipid content of the seeds. The observation of a consistent difference in lipid content between the members of a pair of near-isogenic lines irrespective of their background genotype makes it highly likely that it is the $r_a$ locus or a closely linked locus which is responsible for the difference in lipid content.

Lipid contents were also determined for 4 lines representative of the four genotypes of pea with the different combinations of alleles at the $r_a$ and $r_b$ loci; these are given in Table 2. In the case of these samples, the lipid contents represent purified total lipid as opposed to the crude total lipid values given in Table 1. On the basis of our experimental observations the purified total lipid contents as determined for the samples presented in Table 2 would be expected to be between 10–15% lower than the crude total lipid contents of the same samples determined by the method used for the samples presented in Table 1.

**Table 1.** Lipid content of paired sets of near isogenic pea lines

| Line (genotype) | Total crude lipid[a] % dry wt |
|---|---|
| 9 – 6w ($r_a r_a$) | 5.04 ± 0.12 |
| 9 – 6R ($R_a R_a$) | 2.82 ± 0.02 |
| 10 – 5w ($r_a r_a$) | 4.60 ± 0.04 |
| 10 – 5R ($R_a R_a$) | 2.83 ± 0.06 |
| 11 – 9w ($r_a r_a$) | 5.15 ± 0.08 |
| 11 – 9R ($R_a R_a$) | 3.03 ± 0.02 |
| 26 – 1w ($r_a r_a$) | 4.51 ± 0.03 |
| 26 – 1R ($R_a R_a$) | 2.96 ± 0.01 |
| 43 – 1w ($r_a r_a$) | 5.09 ± 0.08 |
| 43 – 1R ($R_a R_a$) | 2.96 ± 0.05 |
| 44 – 5w ($r_a r_a$) | 5.06 ± 0.08 |
| 44 – 5R ($R_a R_a$) | 3.07 ± 0.02 |

[a] Mean of three determinations

No generalisations can be made about the differences between genotypes since only one example of each of the $R_a R_a r_b r_b$ and $r_a r_a r_b r_b$ genotypes was available for study but it would certainly appear likely from Table 2 that the $r_b$ locus is also affecting lipid content in view of the results obtained with lines 399 and 827. The latter showed the highest lipid content of all the lines examined with 5.57% of the dry weight as purified total lipid.

In addition to the total lipid content, Table 2 also gives the lipid class composition for each of the four representative lines. It can be seen that as the total lipid content increases, the proportion of neutral lipid to phospholipid also increases. This finding is consistent with comparative electron microscopic observations of sections of $R_a R_a R_b R_b$ and $r_a r_a R_b R_b$ pea seeds. In the former seeds, with a low total lipid content, a greater proportion of the lipid (both neutral and polar) is assumed to be associated with cell membrane structures. In $r_a r_a R_b R_b$ pea seeds, with their much higher lipid content, electron micrographs have shown more free lipid bodies to be present, particularly in the later stages of development when many are seen surrounding the starch grains (Horowitz 1982). Of particular interest is the fact that at that stage, there is a degradation of the centre of the starch grains to give the characteristic "compound" structure which typifies the starch grains of wrinkled ($r_a r_a R_b R_b$) seeded peas. We are currently investigating further the significance of this latter observation in relation to lipid biosynthesis. The fatty acid compositions of the total lipid and

**Table 2.** Lipid content of pea lines representing different genotypes

| Line or variety | Genotype | Total lipid % dry wt | Lipid class composition in mg/g pea (% in brackets) | | |
|---|---|---|---|---|---|
| | | | NL | GL | PL |
| Birte | $R_aR_aR_bR_b$ | 2.38±0.12 | 10.9 (45.9) | 1.9 (8.1) | 10.9 (46.0) |
| Greenshaft | $r_ar_aR_bR_b$ | 4.21±0.09 | 20.6 (49.0) | 4.2 (9.9) | 17.3 (41.1) |
| JI 399 | $R_aR_ar_br_b$ | 4.75±0.19 | 26.8 (56.5) | 3.0 (6.4) | 17.6 (37.1) |
| JI 827 | $r_ar_ar_br_b$ | 5.57±0.25 | 33.1 (59.6) | 5.3 (9.5) | 17.3 (31.0) |

**Table 3.** Total lipid fatty acid composition (%)

| Line or variety | Genotype | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
|---|---|---|---|---|---|---|
| Birte | $(R_aR_aR_bR_b)$ | 12.5 | 4.6 | 24.2 | 49.7 | 9.0 |
| Greenshaft | $(r_ar_aR_bR_b)$ | 8.6 | 2.7 | 24.9 | 53.1 | 10.7 |
| JI 399 | $(R_aR_ar_br_b)$ | 12.8 | 3.6 | 27.3 | 46.3 | 10.0 |
| JI 827 | $(r_ar_ar_br_b)$ | 9.7 | 3.1 | 29.3 | 50.2 | 7.7 |

**Table 4.** Neutral lipid fatty acid composition (%)

| Line or variety | Genotype | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
|---|---|---|---|---|---|---|
| Birte | $R_aR_aR_bR_b$ | 8.6 | 3.3 | 21.8 | 51.6 | 14.6 |
| Greenshaft | $r_ar_aR_bR_b$ | 9.9 | 3.1 | 24.2 | 50.9 | 10.5 |
| JI 399 | $R_aR_ar_br_b$ | 12.5 | 3.3 | 22.7 | 48.3 | 13.2 |
| JI 827 | $r_ar_ar_br_b$ | 11.8 | 4.0 | 28.6 | 48.7 | 7.0 |

neutral lipid fraction from each of the four lines in Table 2 were also determined, and the results are presented in Tables 3 and 4. There was some variation in the fatty acid composition but in all cases it was such that $18:2 > 18:1 > 16.0 \simeq 18:3 > 18.0$ as has previously been found for pea lipid (Haydar and Hadziyev 1973).

The effect of the $r_a$ and $r_b$ loci on the biosynthesis of more than one class of storage product – starch, protein and lipid, is analogous to that observed in mutant forms of other crops. For example, some of the maize mutants (e.g. *op2*) whose primary effect is on the synthesis of the prolamin fraction of the storage protein, exhibit reductions in starch accumulation, and mutants affecting the accumulation of starch (e.g. *su1*) also affect protein synthesis (see Nelson 1981). The high lysine mutants of barley (e.g. 1508) show a reduced synthesis of both prolamin and starch; furthermore the similarity is even greater with the $r_a$ and $r_b$ mutants of the pea, in that the barley mutant 1508 has twice as much total lipid as the parent variety from which it was derived (Miflin and Shewry 1979).

It is not known whether the primary effect of the $r_a$ and $r_b$ loci is on starch, protein or lipid biosynthesis but the fact that all three storage products are affected either indicates a coordinate regulation of all three, or alternatively, that the mutation involves a change in a cellular component, such as an altered membrane structure, and which in turn affects all aspects of the seed's metabolism. It remains to be established whether the $r_a$ and $r_b$ loci affect the same pathway; the double mutant $r_a r_a r_b r_b$ has a higher lipid level (Table 2) and a lower starch content (Kooistra 1962) than either single mutant which might suggest that separate pathways are affected.

The implications of these data for the utilisation of the pea crop remain to be evaluated, and analyses of genotype-environment effects are among the many studies that need to be undertaken. However the ~230% increase in lipid content observed in the $r_a r_a r_b r_b$ mutant in comparison with Birte, one of the smooth seeded $(R_aR_aR_bR_b)$ forms currently grown as dry seed, indicates the potential scope for improvement should a breeding programme for oil content be deemed desirable. Perhaps of even greater relevance in this context is the fact that the neutral lipid content has increased by 300%.

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