Further Investigation on the Orange Cotyledons in Pea  
(*Pisum sativum* L.)

A. Haghnazari¹* and M. R. Azimi¹

ABSTRACT

Pea (*Pisum sativum* L.) is an important model plant for genetic as well as biochemical and physiological studies. A well-saturated map of pea consisting several morphological, biochemical and molecular markers has been constructed to date. Nevertheless, there are still several genes whose inheritance and map positions are not well understood. Orange cotyledon color in pea is an interesting characteristic whose precise nature of gene interactions is unknown. Genetic analysis using crosses between lines having orange cotyledon color and lines with yellow or green cotyledons revealed that the character is controlled by a single gene. It was also found that the gene *i* (producing green cotyledon color) shows an epistatic effect on the gene *Orc* (orange cotyledon color). Incomplete dominance and dominance were revealed in the loci *Orc* and *I*, respectively. Mapping analysis revealed that the gene *Orc* is located on linkage group 1 and 28.5 crossover units away from the gene *Ans* and 31.3 map units away from *Idh*. In addition, a significant linkage was detected between two genes *Pur* and *Ans* with an estimated distance of 9.9 map units. The distance between *Orc* and *Pur* was estimated as 38 map units.

Keywords: Epistasis, Incomplete dominance, Isocitrate dehydrogenase, Linkage.

INTRODUCTION

Pea (*Pisum sativum* L.) has been a model plant for genetic, biochemical and physiological studies. It is also an important crop plant whose utilization can be traced back to Neolithic times (Davies, 1993). The basic laws of inheritance were derived from studies on pea. By the early 1980s, already more than 350 genes (mostly morphological mutants) were described in pea (Weeden, 1996). The majority of these genes, along with newly identified morphological as well as biochemical and molecular markers, have been assembled into a saturated linkage map consisting of seven linkage groups (Weeden et al., 1998). Nevertheless, there are still several genes whose inheritance and map positions are not well understood.

Since Mendel’s investigations, two colors of cotyledons controlled by alleles of the gene *i* in chromosome 1 (*I* yellow, *i* green cotyledons) have been accepted in pea (Swiecicki, 1989). For the first time, a pea line (Wt 11145) with orange cotyledons was reported by Swiecicki (1982). In this line the whole seed, including seed coat, appears to be pink but the cotyledons have a deeper orange hue after the seed coat has been removed. This characteristic was called "orange cotyledon" and designated by the gene symbol *Orc*, (Blixt and Swiecicki, 1983).

Chromatographic analysis of yellow and orange cotyledon seeds by Ludwicki and Swiecicki (1983) revealed that the orange cotyledons have more than double the biologically active β-carotene as compared to the yellow cotyledons.

¹ Division of Agronomy and Plant Breeding, Faculty of Agriculture, The University of Zanjan, Zanjan, Islamic Republic of Iran.
* Corresponding author
From a cross between lines having orange and yellow cotyledons, Blixt and Swiecicki (1983) reported a monogenic dominance for orange cotyledon color. Swiecicki (1989) detected digenic segregation with a ratio of 9 brick: 3 yellow: 4 green and the epistatic effect of the gene Orc over I in a cross between lines with orange and green cotyledons.

Marx (1986) suggested that the gene Orc should be placed in linkage group 5 (Weeden et al., 1998). However, Swiecicki (1987) mapped Orc in linkage group 1 near the genes D (basal anthocyanin ring) and Idh (isocitrate dehydrogenase) isozyme locus. Because of an insufficiency of data on the inheritance and relative map position of the genes involved, the line P1546-7 with orange cotyledons was selected from the pea germplasm collection and was utilized in our study. Here we report the inheritance of orange cotyledon color and map position of the gene Orc.

**MATERIALS AND METHODS**

From a large collection of pea (*Pisum sativum* L.) germplasm maintained in the Division of Genetics of the Indian Agricultural Research Institute (New Delhi), the line P1546-7 was noted for its orange cotyledon. This line was crossed with four lines having differential expression of yellow cotyledon color. The line P1546-7 was selected as a female parent in two crosses and a male parent in the other two crosses (Table 1). Reciprocal crosses were made between lines P1546-7 and P3001 having green cotyledons (Table 2). An additional cross was made between lines P1546-7 and P1297 with green cotyledons as shown in Table 2. Four more crosses were made for linkage analysis (Table 3). In order to pair the contrasting parents for linkage analysis, the line P 1546-7-2 with orange cotyledons and red flowers was used in two of these four crosses as a female parent instead of line P 1546-7 (Table 3). All the crosses were made during the winter of 1998-99 in New Delhi. The F₁ seeds were sown during summer 1999 in the off-season nursery of the Directorate of Wheat Research, Lahaul and Spiti, Himachal Pradesh. The F₁ and F₂ seeds were screened for cotyledon color. For linkage analysis, the F₂ plants were raised during winter 1999-2000, keeping a 30 cm. spacing between and within 5m. long rows. Morphological traits were recorded at different stages of crop growth. Scoring for the gene Ans (anthocyanin coloration of seedling) was done in the early stages of plant growth. To confirm the F₂ segregation ratios for cotyledon color, a sample of seeds from each class of F₂ phenotypes in crosses number 1 and 5 (Tables 1 and 2) were raised to obtain F₃ seeds.

The purple pod coloration is determined by the simultaneous presence of two dominant genes, Pur and Pu, but only in the presence of the major gene for anthocyanin production, A. As a result, scoring of these characters is very difficult. Recently, a method has been suggested by Bogdanova et al. (1995) by which the funiculus coloration can be used for easy identification of the genes Pur and Pu. In our study, scoring for the presence/absence of the gene Pur (purple coloration of pods) was done according to the proposed method using funiculus coloration.

To visualize alleles of the isozymic locus Idh for isocitrate dehydrogenase (*Idh-idh*: Fast-slow isozymic variants) an electrophoretic separation of *Idh* on starch gel using leaf tissue was conducted following the method of Shaw and Prasad (1970). Extraction of enzyme was conducted using 0.1 M Tris-HCl, pH 8.3 and 0.6% mercaptoethanol. The χ² test was used to assess the quality of fit and linkage was estimated using the maximum likelihood method (Mather, 1957). Data were analysed by the computer program CROSS provided by Dr. S. M. Rozov (http://pisum.bionet.nsc.ru).

**RESULTS AND DISCUSSION**

In all the crosses of line P1546-7 (orange cotyledons) with four different lines having yellow cotyledons (Table 1), the F₁ seeds
had an intermediate color (light orange) which suggests incomplete dominance of the orange cotyledon color. The F2 seeds were classified into three classes of orange, light orange and yellow (Figure 1); the data were segregated into a 1: 2: 1 ratio. The $x^2$ values for each cross as well as for pooled data over all crosses were not significant (Table 1). In order to confirm the results, the F3 progenies of the F2 plants obtained from the cross P1546-7 × P1935 were tested for cotyledon color. There was no segregation for cotyledon color in F3 progenies of the F2 plants producing orange and yellow cotyledons. In the F3 progenies for the F2 plants producing light orange there were three types of segregants and 163 orange: 298 light orange: 149 yellow were obtained from six F2 plants. Data fits well into the ratio of 1: 2: 1 ($x^2$ =0.963; P> 0.5) which suggests the incomplete dominance operating at this locus. The dominance of orange over yellow cotyledons was reported by Blixt and Swiecicki (1983) and Swiecicki (1987). Similar results has also been reported in lentil, *Lens culinaris* (Wilson *et al*., 1970; Singh, 1978; Sinha, 1987; and Emami and Sharma, 1996).

The line P 1546-7 was also crossed with three lines having green cotyledons. In all crosses, the F2 seeds were again light orange (Table 2). The cotyledons of F2 seeds had a range of shades but with careful observation they were divided into four classes of: orange; light orange; yellow; and green (Figure 2). Surprisingly, even though the parental cotyledon colors were only orange and

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**Table 1. Distribution of phenotypes in F2 populations segregating for cotyledon color in pea.**

<table>
<thead>
<tr>
<th>No.</th>
<th>Cross</th>
<th>F1 Phenotype</th>
<th>F2 phenotype</th>
<th>Chi-square$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P1546-7 (Orc) × P1935 (I)</td>
<td>Light orange</td>
<td>Orange: 219</td>
<td>0.528</td>
</tr>
<tr>
<td>2</td>
<td>P1546-7 (Orc) × P1403 (I)</td>
<td>Light orange</td>
<td>Light orange: 191: 391</td>
<td>0.812</td>
</tr>
<tr>
<td>3</td>
<td>P1563 (I) × P1546-7 (Orc)</td>
<td>Light orange</td>
<td>Light orange: 168: 355</td>
<td>0.650</td>
</tr>
<tr>
<td>4</td>
<td>P1743 (I) × P1546-7 (Orc)</td>
<td>Light orange</td>
<td>Light orange: 225: 478</td>
<td>0.558</td>
</tr>
<tr>
<td></td>
<td>Pooled over four crosses</td>
<td></td>
<td>Light orange: 803: 1658</td>
<td>0.862</td>
</tr>
</tbody>
</table>

$^a$ All Chi-square values are nonsignificant.

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Figure 1. Segregation for cotyledon color in a cross between lines with orange and yellow cotyledons.
green, a new class (yellow color) also appeared. The segregation of F₂ seeds into groups of orange, light orange, yellow and green cotyledons fits well the digenic ratio of 3: 6: 3: 4 with a very high degree of probability as the $\chi^2$ values for each individual cross as well as data the pooled over the three crosses were non-significant (Table 2). From similar crosses, the ratio of 9 brick: 3 yellow: 4 green was reported by Swiecicki and Blixt (1984).

The F₃ segregation in each F₂ phenotypic classes of the cross P3001 × P1546-7 were as follows. For the class of green cotyledons, all 782 F₃ seeds obtained from the randomly selected F₂ plants had green cotyledons. For the class of yellow cotyledons out of 685 F₃ seeds, the ratio of 498 yellow: 187 green was obtained which accords well with the ratio of 3: 1 ($\chi^2 = 1.931; P > 0.1$).

Two classes of orange cotyledons were distinguished on the basis of the F₃ segregation. In one class, all 402 F₃ seeds were of orange cotyledons. In the other class out of 802 F₃ seeds, 591 seeds had orange and 211 seeds green cotyledons. The segregation ratio was in agreement with the ratio of 3:1 ($\chi^2 = 0.733; P > 0.3$). In the class of light cotyledons, two types of segregates were observed. In one class, the ratio of 196 orange: 469 light orange: 204 yellow seeds was observed which is in accordance with a ratio of 1: 2: 1 ($\chi^2 = 5.626; P > 0.05$). In the second class for the total of 744 F₃ seeds, the ratio of 126 orange: 292 light orange: 154 yellow: 172 green seeds was obtained which is in accordance with a ratio of 3: 6: 3: 4 ($\chi^2 = 4.473; P > 0.2$).

<table>
<thead>
<tr>
<th>No.</th>
<th>Cross</th>
<th>F₁ phenotype</th>
<th>F₂ phenotype</th>
<th>Chi-square $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Orange</td>
<td>Light orange</td>
<td>Yellow</td>
</tr>
<tr>
<td>5</td>
<td>P3001 (i) x P1546-7(Orc)</td>
<td>Light orange</td>
<td>83</td>
<td>168</td>
</tr>
<tr>
<td>6</td>
<td>P1546-7(Orc) x P3001 (i)</td>
<td>Light orange</td>
<td>127</td>
<td>251</td>
</tr>
<tr>
<td>7</td>
<td>P1546-7(Orc) x P1297 (i)</td>
<td>Light orange</td>
<td>92</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Pooled over three crosses</td>
<td></td>
<td>302</td>
<td>604</td>
</tr>
</tbody>
</table>

$^a$ All Chi-square values are nonsignificant.
The results can be interpreted as follows. In crosses between lines having orange (Orc Orc II) and yellow cotyledons (orc orc II), three classes of F2 plants can be expected in the following ratio. 1 orange (Orc Orc II): 2 light orange (Orc orc II): 1 yellow (orc orc II). In the crosses between lines with orange (Orc Orc II) and green cotyledons (orc orc ii), four classes of seeds are expected in the case of independent assortment in the following ratio. 3 orange (Orc Orc ii): 1 yellow (org orc ii): 4 green (orc− ii and orc orc ii). The data were large enough to conclude that this trait is under genic control and the gene Orc is hypostatic to recessive allele i. An incomplete dominance of the Orc locus and a complete dominance at I locus is also evident. The dominant allele for the gene I (II or I−) decides the degree of orange coloration in the cotyledons under the influence of the gene Orc. The presence of two dominant alleles (Orc Orc) give rise to deep orange cotyledons. However, with only one dominant allele (Orc orc) the cotyledons are light orange. The recessive homozygosity at locus I (ii) inhibits the development of orange color in the cotyledons. The loss of function at the I locus is known to prevent the development of yellow pigment in the pea seeds.

A linkage between the genes of chromosome 1 was investigated in seven crosses using multi-marker lines. In only four crosses significant linkages were observed (Table 3). The cross P1743× P1546-7 showed no linkage between the genes Orc and r (joint segregation χ²=0.852). Thus the possibility of Orc localization near the marker r on linkage group IV was eliminated.

The gene Ans (anthocyanin in seedling), a linkage group I marker, is located near the marker d (anthocyanin ring in leaf base). From the cross P1546-7-2× P1404, a highly significant linkage was obtained for the genes Ans and Orc (χ²=17.18; P<0.0001). The recombination fraction between these two genes was estimated as 28.5 ± 4.8%. This ratio was not reported earlier. The map distance is almost similar to the one reported between genes D and Orc (Świecicki, 1989).

Table 3. Joint F2 segregation and recombination of genes controlling three morphological traits in Pea.

<table>
<thead>
<tr>
<th>No.</th>
<th>Cross</th>
<th>F2 phenotype</th>
<th>Joint segregation Chi-square</th>
<th>Recombination frequency</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>P1743(r,orc) × P1546-7(R,Orc)</td>
<td>C</td>
<td>486 175 149 45</td>
<td>0.852***</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>P1404(Ans,pur) × P1935(ans, PUR)</td>
<td>R</td>
<td>207 109 94 1</td>
<td>41.75***</td>
<td>9.90</td>
</tr>
<tr>
<td>8</td>
<td>P1546-7(Orc,idh) × P1744(orc, Idh)</td>
<td>R</td>
<td>105 41 50 5</td>
<td>8.36***</td>
<td>31.34</td>
</tr>
<tr>
<td>9</td>
<td>P1546-7-2(Orc,ans) × P1404(orc, Ans)</td>
<td>R</td>
<td>182 85 74 7</td>
<td>17.18***</td>
<td>28.5</td>
</tr>
<tr>
<td>10</td>
<td>P1546-7-2(Orc, pur) × P1935(pur, Orc)</td>
<td>R</td>
<td>473 180 192 38</td>
<td>17.37</td>
<td>38.00</td>
</tr>
</tbody>
</table>

* ** P<0.01
** NS: Non-significant
**** P<0.0001

DD: Dominant alleles for both loci. DR: Dominant allele for first locus, recessive allele for second locus.
RD: Recessive allele for first locus, dominant allele for second locus. RR: Recessive alleles for both loci.

Our unpublished data show a very tight linkage between Ans and D. This is a further confirmation of a tight linkage between these genes in linkage group 1 (Weeden et al., 1998). The map distance between the isozyme locus Idh and Orc was estimated from the cross P1546-7× P1744. The linkage was significant and the distance between these two genes was estimated as 31.34 ± 6.2 crossover units.

The gene Pur is a well-known marker on linkage group I. The distance between Pur and d was shown to be 10 cM on the pea linkage map (Weeden et al., 1998). Recently, Gorel et al. (1997) reported the Pur gene to be in close vicinity of d with a recombination fraction of 8.5 ± 3.1%. A
highly significant linkage between \textit{Orc} and \textit{Pur} was obtained from the cross P 1546-7-2\times P1935 (\(x^2=17.37; P< 0.0001\)). The distance between these two genes was estimated as 38 \pm 2.8 crossover units. This linkage is also reported for the first time. Finally, the distance between \textit{Ans} and \textit{Pur} was estimated from the cross P 1404\times P1935. (\(x^2=17.37; P< 0.0001\)). The linkage between these two genes was highly significant and the recombination fraction for this gene pair was 9.9 \pm 4.8\%.

This linkage is also reported for the first time. Pooling all results and considering the order of genes on the pea linkage map (Weeden et al; 1998), the following arrangement can be proposed for the genes of linkage group I in pea.

REFERENCES

مطالعه بیشتر بر روابط لیه‌های نارنجی در خودفرنگی (Pisum sativum L.)

چکیده

خودفرنگی (Pisum sativum L.) یک گیاه مدل برای غنی‌الدیدگی‌ها، مطالعاتی از روند‌های کم‌پویش و رشد در این گیاه انجام شده است. نقشه زنتیکی اشباع شده‌ای از خودفرنگی شامل نشانگرهای متنوع مورفولوژیکی، بیوشیمیایی و مولکولی به شده است، ولی هنوز زنده‌مانده وجود دارد که غیر قوت و مکانی‌گردنی آنها شناخته شده نیست. رنگ نارنجی لیه در بذور خودفرنگی صفت جالب‌تری است که ماهیت دقيق اثرات متقابل زنی در مورد آن مشخص نیست. چگونگی زنی‌یابی با استفاده از تلاقی بین لاژن‌های دارای لیه نارنجی و لاژن‌های با لیه زرد یا سیاه نشان داد که صفت رنگ نارنجی لیه غیب یافتگذاری کند زنی غالب است. چگونگی مشخص شد که زنی (Tolidekendine رنگ سبز I) دارای اثر اپیستاتیک بر روی زنی و (Zn) می‌باشد. غلیب‌الترتیب و غلیبیت به‌تهیه‌نرم‌دار مکان‌های زنی در خودفرنگی و I (Zn) شناخته شد. چگونگی براز مکان‌یابی زنی در داد که زنی و (Zn) رای و (Zn) به (Zn) در تکرار 1 و با فاصله 28/3 و 24/6 واحد کراسینگ‌گار از (Zn) و 36/2 واحد (Zn) در فاصله (Zn) و (Zn) با فاصله 9/4 واحد دارد. بعلاوه به دلیل قرار دادن تکرار کراسینگ‌گار از (Zn) و (Zn) دارای دو زنی دارد. بعلاوه (Zn) و (Zn) با فاصله 48 واحد براز و (Zn) با دو زنی این نتیجه پرآوردن گردید.