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

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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 drived 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.

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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 F_1 seeds were sown during summer 1999 in the off-season nursery of the Directorate of Wheat Research, Lahaul and Spiti, Himachal Pradesh. The F_1 and F_2 seeds were screened for cotyledon color. For linkage analysis, the F_2 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 F_2 segregation ratios for cotyledon color, a sample of seeds from each class of F_2 phenotypes in crosses number 1 and 5 (Tables 1 and 2) were raised to obtain F_3 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% mercapthoethanol. The x^2 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 DISSCUSION

In all the crosses of line P1546-7 (orange cotyledons) with four different lines having yellow cotyledons (Table 1), the F_1 seeds

Table 1. Distribution of phenotypes in F_2 populations segregating for cotyledon color in pea.

No.	Cross	F_1 Phenotype	F_2 phenotype			Chi-square[a] (1:2:1)
			Orange	Light orangw	Yellow	
1	P1546-7 (*Orc*)× P1935 (*I*)	Light orange	219	434	230	0.528
2	P1546-7 (*Orc*)× P1403 (*I*)	Light orange	191	391	208	0.812
3	P1563 (*I*)× P1546-7 (*Orc*)	Light orange	168	355	166	0.650
4	P1743 (*I*) × P1546-7 (*Orc*)	Light orange	225	478	235	0.558
	Pooled over four crosses		803	1658	839	0.862

[a] All Chi-square values are nonsignificant.

had an intermediate color (light orange) which suggests incomplete dominance of the orange cotyledon color. The F_2 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 F_3 progenies of the F_2 plants obtained from the cross P 1546-7 × P 1935 were tested for cotyledon color. There was no segregation for cotyledon color in F_3 progenies of the F_2 plants producing orange and yellow cotyledons. In the F_3 progenies for the F_2 plants producing light orange there were three types of segregants and 163 orange: 298 light orange: 149 yellow were obtained from six F_2 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 F_2 seeds were again light orange (Table 2). The cotyledons of F_2 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

Figure 1. Segregation for cotyledon color in a cross between lines with orange and yellow cotyledons.

Table 2. Distribution of phenotypes in F_2 populations, segregating for cotyledon color in pea.

No.	Cross	F_1 phenotype	F_2 phenotype				Chi-square[a] (3:6:3:4)
			Orange	Light orangw	Yellow	Green	
5	P3001 (*i*) x P1546-7(*Orc*)	Light orange	83	168	92	120	0.815
6	P1546-7(*Orc*) x P3001 (*i*)	Light orange	127	251	141	182	1.63
7	P1546-7(*Orc*) x P1297(*i*)	Light orange	92	185	97	130	0.343
	Pooled over three crosses		302	604	330	432	2.59

[a] All Chi-square values are nonsignificant.

green, a new class (yellow color) also appeared. The segregation of F_2 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 x^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 F_3 segregation in each F_2 phenotypic classes of the cross P3001× P1546-7 were as follows. For the class of green cotyledons, all 782 F_3 seeds obtained from the randomly selected F_2 plants had green cotyledons. For the class of yellow cotyledons out of 685 F_3 seeds, the ratio of 498 yellow: 187 green was obtained which accords well with the ratio of 3: 1 (x^2 = 1.931; P > 0.1).

Two classes of orange cotyledons were distinguished on the basis of the F_3 segregation. In one class, all 402 F_3 seeds were of orange cotyledons. In the other class out of 802 F_3 seeds, 591 seeds had orange and 211 seeds green cotyledons. The segregation ratio was in agreement with the ratio of 3:1 (x^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 (x^2 = 5.626; P > 0.05). In the second class for the total of 744 F_3 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 (x^2 = 4.473; P > 0.2).

Figure 2. Segregation for cotyledon color in a cross between lines with orange and green cotyledons.

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 F_2 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 (*OrcOrc 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*): 6 light orange (*Orc orc I–*): 3 yellow (*orc orc I–*): 4 green (*Orc– ii* and *orc orc ii*). The data were large enough to conclude that this trait is under digenic 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 x^2=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* (x^2=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* (Swiecicki, 1989).

Table 3. Joint F_2 segregation and recombination of genes controlling three morphological traits in Pea.

No.	Cross	Phase	F_2 phenotype DD	DR	RD	RR	Joint segregation Chi-square	Recombination frequency	Standard Error
4	P1743(*r*,*orc*) × P1546-7(*R*,*Orc*)	C	486	175	149	45	0.852^{ns}	-	-
8	P1404(*Ans*,*pur*)× P1935 (*ans*,*Pur*)	R	207	109	94	1	41.75^{****}	9.90	4.8
9	P1546-7(*Orc*,*idh*)×P1744 (*orc*,*Idh*)	R	105	41	50	5	8.36^{**}	31.34	6.2
10	P1546-7-2(*Orc*,*ans*)×P1404 (*orc*,*Ans*)	R	182	85	74	7	17.18^{****}	28.5	4.8
11	P1546-7-2(*Orc*,*pur*)× P1935(*orc*,*Pur*)	R	473	180	192	38	17.37	38.00	2.8

** 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 *Orc* and *Pur* was obtained from the cross P 1546-7-2× P1935 (x^2=17.37; P< 0.0001). The distance between these two genes was estimated as 38 ± 2.8 crossover units. This linkage is also reported for the first time. Finally, the distance between *Ans* and *Pur* was estimated from the cross P 1404× P1935. The linkage between these two genes was highly significant and the recombination fraction for this gene pair was 9.9 ± 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.

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مطالعه بيشتر بر روي لپه‌هاي نارنجي در نخودفرنگي (*Pisum sativum* L.)

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چكيده

نخودفرنگي (*Pisum sativum* L.) يك گياه مدل براي تحقيقات ژنتيكي، بيوشيميايي و فيزيولوژيكي است. نقشه ژنتيكي اشباع شده‌اي از نخودفرنگي شامل نشانگرهاي متعدد مورفولوژيكي، بيوشيميايي و مولكولي تهيه شده است، ولي هنوز ژنهاي متعددي وجود دارند كه نحوه توارث و مكان ژني آنها شناخته شده نيست. رنگ نارنجي لپه در بذور نخود زنگي صفت جالب توجهي است كه ماهيت دقيق اثرات متقابل ژني درمورد آن مشخص نيست. تجزيه ژنتيكي با استفاده از تلاقي بين لاين‌هاي داراي لپه نارنجي و لاين‌هاي با لپه زرد يا سبز نشان داد كه صفت رنگ نارنجي لپه تحت كنترل يك ژن غالب است. همچنين مشخص شد كه ژن *I* (توليدكننده رنگ سبز لپه) داراي اثر اپيستاتيك بر روي ژن *Orc* (رنگ نارنجي لپه) مي‌باشد. غالبيت ناقص و غالبيت به ترتيب در مكان‌هاي ژني *Orc* و *I* شناخته شد. تجزيه براي مكان‌يابي ژني نشان داد كه ژن *Orc* بر روي گروه لينكاژي ۱ و با فاصله ۲۸/۵ واحد كراسينگ‌اور از ژن *Ans* و ۳۱/۳ واحد كراسينگ‌اور از ژن *Idh* قرار دارد. بعلاوه يك لينكاژ معني‌دار بين دو ژن *Pur* و *Ans* با فاصله ۹/۹ واحد نقشه بدست آمد. فاصله بين *Orc* و *Pur* برابر ۳۸ واحد نقشه برآورد گرديد.