Biochemical and Genetic Linkage Analysis of Green Seed Color in Field Pea

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ABSTRACT. Developmental, environmental, and genetic factors affecting seed color were studied in the progeny of a cross between two white-flowered (*aa*) green cotyledon (*ii*) field peas (*Pisum sativum* L.): the pale large-seeded Marrowfat cultivar Primo and the greener small-seeded Prussian Blue OSU442-15. Changes in chlorophyll and carotenoid content during seed development of the parental genotypes were determined by high performance liquid chromatography analysis. Both cultivars accumulated similar pigment quantities per seed, but pigment loss was greater during maturation of 'Primo'. Bleached and unbleached mature seed tissues also were compared for pigment composition. Lutein was the predominant pigment in bleached cotyledons of both cultivars. Only trace amounts of pheophytins were detected in unbleached seed. In both genotypes, chlorophyll A : B ratios were $\approx 1:1$ in seed coats compared to 3:1 in cotyledons. Objective measurements of seed color in terms of luminance (lightness) and chrominance (hue and saturation) were made in YUV color space by video image analysis. Inheritance of seed color was studied in an F_2 population derived from the 'Primo' $\times$ 'OSU442-15' cross and inbred descendants. Quantitative trait loci (QTL) for seed color were localized by interval mapping using a linkage map of 199 molecular markers spanning most of the genome and by bulked segregant analysis and selective genotyping. Four genomic regions affecting seed color were detected. A major gene accounting for 61% of the phenotypic variance in seed lightness (Y luminance component) was identified on linkage group V linked to *r* locus. Another major gene, which accounted for 56% of the phenotypic variance in seed hue (U chrominance component), was mapped to a linkage group containing group III and IV markers. A QTL with smaller effect on seed hue (U and V chrominance components) was detected on linkage group VII. Support for overdominant allelic interaction for a QTL on linkage group I, adjacent to the legumin locus *Lg-J*, was obtained by selective genotyping of the seed lightness distribution extremes.

The visual appearance of grain legume seeds is an important determinant of perceived quality in the marketplace. A uniform green color is desirable in dried field peas; however, wet weather or irrigation during maturation can lead to bleaching and loss of market value (Gubbels and Ali-Khan, 1990; Riehle and Muehlbauer, 1975). Color loss may be manifested as occasional pale seeds ("blonds") or as more general loss of greenness. For human consumption, seed is often dyed during processing to restore acceptable color, although this practice is becoming restricted due to food safety concerns. In dried field peas, the apparent seed color is contributed by the seedcoat and cotyledons. Seedcoat pigmentation in peas is due to photosynthetic pigments (chlorophylls, carotenoids, and xanthophylls) (Marx 1977) as well as phenolic pigments, notably flavonoids.

The biochemical, physiological, and genetic mechanisms underlying legume seed chlorophyll loss are not well understood. Cytoplasmic and nuclear factors have been implicated in senescence of seedcoats and other plant parts of soybean (*Glycine max* L. Merr.) (Thomas and Smart, 1993). Major genes have been described that condition retention of green seed color in *Phaseolus* species (Honma et al., 1968; Tucker, 1965) and soybean (Bernard and Weiss, 1973), but the linkage relationships of these loci have not been determined.

The best known locus affecting chlorophyll retention in pea

seed is *i*-locus, which conditions cotyledon color in the mature seed. Several other loci have been reported to affect seed senescence and color retention in pea, notably *pa*, *gla*, and *vim* (Blixt, 1962; Weeden and Wolko, 1990). Lamprecht (1959) suggested that seedcoat and cotyledon color in dry seed of *aa* (white flowered) pea genotypes are determined by the action of the genes *i*, *o*, and *gla*. In the most detailed study of field pea bleaching to date, Dribnenki (1979) concluded that at least three genes were involved, affecting imbibition rate, green seedcoat color retention, and cotyledon bleaching resistance. Although genetic loci affecting chlorophyll pigmentation of pods, immature seed, and other plant parts in *Pisum* have been studied (Blixt, 1962; Lamprecht, 1959; Marx, 1977), the relevance of these to dry-seed color quality of cultivated types is still unclear. Plant breeders' ability to select for green color quality would be improved by increased understanding of the linkage relationships and interactions of the loci involved and their effect on color phenotype.

Genetic maps saturated with molecular markers have made it feasible to analyze inherited traits that exhibit quantitative variation and to tag the underlying quantitative trait loci (QTL) (Tanksley, 1993). Using marker-saturated genetic maps permits greater precision in the dissection of traits, which are determined by genetic and environmental factors, such as seed color in peas. Suitable populations for mapping QTL include F_2 progenies (Tanksley, 1993). Genetic analyses of color traits usually are based on qualitative visual evaluation and are limited by the subjective nature of human color perception and lack of rigorous quantitation. Now, color can be measured readily, objectively, and quantitatively by instrumental methods (Francis, 1995). In our research, we characterize the photosynthetic pigment composition of seeds of two field pea cultivars, and, by using an F_2 population derived by crossing these two genotypes, we locate genomic regions that affect seed color.

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Materials and Methods

SEED PIGMENT CHARACTERIZATION OF PARENTS PLANT MATERIAL.

Plants of 'OSU442-15' and 'Primo' were grown in a glasshouse in Lincoln, New Zealand, in Spring 1993 under natural lighting in PB5 plastic pots containing a soil-sand-bark mix with Osmocote slow-release fertilizer beads ($3 \text{ kg} \cdot \text{m}^{-3}$). Night time minimum temperature was 12°C , daytime minimum was 18°C , and daytime maximum was up to 22°C .

For high-performance liquid chromatography (HPLC) studies of mature seed tissue pigments, the soil was kept moist as plants were drying off. This process is believed to promote color loss (Riehle and Muehlbauer, 1975). Ordinarily, the soil is permitted to dry out once the pods have begun to dry off. Under continuous watering, 20% to 40% of seeds in any given pod were bleached significantly (i.e., yellow-tan rather than green in color), and seed could be unambiguously classified by eye as "bleached" or "unbleached." Dried seeds were collected from plants that were fully senesced in brown paper seed envelopes. These seeds were stored at ambient temperature and moisture.

For developmental studies of seed pigments, flowers at the second and third flowering nodes were tagged when first fully open. Immature pods were sampled at 14, 21, 35, 42, 49, and 56 d after flowering (DAF). Pods were frozen in liquid N and were stored at -80°C until analysis.

PREPARATION OF PIGMENT STANDARDS. All manipulations were performed in subdued light at 4°C . Immature seeds of 'OSU442-15' were homogenized in ice-cold 1 acetone:petroleum : 1 ether in a homogenizer (Polytron, Kinematica, Luzern, Switzerland),

and the homogenate was clarified by centrifugation at $5000 \times g$ for 10 min. Aliquots were dried under N, redissolved in dichloromethane and streaked onto preparative silica TLC plates (Machery and Nagel Sil-G200 2 mm; Machery & Nagel Co., Duren, Germany). Plates were developed in 70 petroleum ether at 40 to 60°C : 30 dioxan 10 isopropanol (Lichtentaler, 1981) and air-dried in darkness. Colored bands were scraped off and eluted with acetone overnight at 4°C . Pigments in the eluates were quantitated and identified by colors, R_f values, and visible spectra in acetone (Lichtentaler, 1987). Pheophytins A and B were prepared by acid-treatment of their respective chlorophylls (Bazzaz and Rebeiz, 1979).

HPLC ANALYSIS. For developmental studies, two samples were prepared from each of three pods at each sampling date. Pods were thawed on ice. For each extract, two or three seeds were bisected along the longitudinal axis, and half of each seed was used for dry mass determination (80°C , 48 h). Seeds (or dissected tissues) were finely ground in a chilled mortar with 3 mL ice-cold acetone and small quantities of calcium carbonate and acid-washed sand. The debris was allowed to settle, supernatant was decanted, and the debris was ground with further acetone until colorless. The extract was made up to 10 mL and centrifuged for 10 min at $2000 \times g$. Total chlorophyll and carotenoid content of the crude acetone extract was determined spectrophotometrically using the coefficients of Lichtentaler (1987). Supernatant (3 mL) was dried in a rotary test-tube vacuum evaporator (Buchler Instruments, Fort Lee, N.J.) at 40°C . Tubes were chilled on ice, and the dried residue was taken up in 300 μL of dichloromethane for HPLC.

HPLC was conducted on a $100 \times 4.6\text{-mm}$ 5m RP-18 column (Brownlee column; Applied Biosystems, Foster City, Calif.) operating at 30°C and a flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$. Aliquots of 10 μL were analyzed. Detection was conducted at 436 nm, and external standardization was performed using the standards purified and quantitated as previously described. The column was eluted with a linear gradient of 10 ethyl acetate : 72 methanol : 18 water to 60 ethyl acetate : 32 methanol : 8 water over 20 min followed by a 10-min isocratic elution (Eskins and Harris, 1981). Following a return to the initial composition over 2 min, there was 10-min delay before re-injection.

STATISTICAL ANALYSIS. Statistical significance of the quantitative data for each pigment was examined by analysis of variance (ANOVA). For comparison of tissue composition, a one-way ANOVA was performed across all treatments (i.e., all combinations of cultivar, tissue type, and bleaching treatment). Treatments containing no or trace levels were excluded from analysis. For the developmental study, a linear model was fitted with cultivar, DAF, and cultivar $\times$ DAF interaction as factors. Least significant difference (LSD) statistics were calculated from the error mean square in the respective models.

Genetic analysis of seed color

PLANT MATERIAL. The population used for QTL analysis was produced by crossing 'OSU442-15' (Baggett and Hampton, 1977) with 'Primo' (Cebeco, Lelystadt, Netherlands) as female parent. Both parents are green cotyledon (*ii*), white flowered (*aa*) genotypes. The 89 F_1 plants were grown in the glasshouse, and the selfed F_2 seeds from each of these plants (up to 40 per F_1 plant) were sown in randomized single-row plots in the field in Lincoln in Spring 1991, together with five plots of each parent. In Spring 1992, 20 seeds from 180 F_2 plants were sown in single rows in randomized individual plots in the field at Lincoln with 10 plots of each parent. For further inheritance studies, reciprocal crosses were made between the two parents, and in Spring 1993, reciprocal F_1 seeds

Fig. 1. High performance liquid chromatography chromatograms of extracts from unbleached seedcoats of (A) 'OSU442-15' and (B) 'Primo'. The unidentified peak discussed in the text is marked with an arrow.

as well as seed from both parents were sown in the field at Lincoln.

The 1991–92 field-grown F_2 plants were harvested to obtain optimal color discrimination between the two parents. Color was determined by sampling parental seeds during the drying-off period at 3-d intervals and visually examining progression of green color loss. Plots in the 1992–93 trial were harvested early to minimize bird damage, and harvest of the 1993–94 trial was delayed by damp weather. All harvested seeds were stored in brown paper envelopes at ambient temperature and humidity. Total rainfall at Lincoln for Jan. and Feb. 1992, 1993, and 1994 was 52, 89, and 90 mm, respectively, below the 1975–91 mean for this period of 101 mm.

A population of 51 recombinant inbred lines (RILs) produced from the wide cross *P. sativum* ssp. *humile* accession JI1794 by *P. sativum* ssp. *sativum* accession Slow (Weeden et al., 1993b) was used to facilitate linkage map alignment.

VIDEO IMAGE ANALYSIS OF SEED COLOR PHENOTYPE. Tristimulus colorimetry was conducted using the color image analysis system (Video Pro 32; Leading Edge Pty, Adelaide, South Australia (McCallum et al., 1993). Color balance was normalized using 3MM chromatography paper, and lighting was adjusted for densitometry using a Kodak Gray Card (R-27; density = 0.5) (Eastman Kodak Co., Rochester, N.Y.). A gamma value of 1 was used for all densitometry. Camera response was calibrated against a Kodak Gray Scale (Q-14) and was linear over the range of seed density (0.2 to 0.7 optical density). Images were captured from threshed samples of 50 mature seeds per plant. Seed color was measured in YUV color space. Densitometric measurements of seed lightness (i.e., light vs. dark) were expressed on a scale of optical density, which is linearly related to the Y luminance component and L^* of commonly used Commission Internationale de L'Éclairage (CIE) color spaces (Poynton, 1995). For chrominance measurements, U and V values were obtained by transformation of the red, green, and blue components of RGB color space measurements using the following equations: $Y = 0.59 \times G + 0.30 \times R + 0.11 \times B$, where $U = B - Y$ and $V = R - Y$. Further explanation of color terminology and measurement may be found in Poynton (1995).

Seed samples from plants showing symptoms of viral infection (alfalfa mosaic virus, pea seed-borne mosaic virus, cucumber mosaic virus) were excluded from phenotypic analysis to eliminate the confounding effects of seed lesions on color measurement.

DNA MARKER METHODS. Isolation of DNA and analysis of restriction fragment length polymorphisms and random amplified polymorphic DNA (RFLP and RAPD, respectively) were conducted as described by Timmerman et al. (1993). DNA for RAPD analysis was isolated from individual field-grown F_2 plants. DNA was isolated from seedling leaves of five F_3 descendants for amplification fragment length polymorphism (AFLP), RFLP analyses, and for bulked segregant analysis (BSA) using RAPD markers. RAPD primers were obtained from Operon Technologies, Alameda, Calif. AFLPs were analyzed following the method described in the European patent application (Zabeau and Vos, 1993) and according to Pickering et al. (1995). Linker-adaptor and primer sequences used were as described by Zabeau and Vos

Table 1. Concentration of photosynthetic pigments in bleached and unbleached seedcoats and cotyledons of 'Primo' and 'OSU442-15' in micrograms per gram of dry mass.

Compound	Cotyledon				Seedcoat				LSD ^y
	Primo		OSU442-15		Primo		OSU442-15		
	U ^z	B ^z	U	B	U	B	U	B	
Neoxanthin	nd ^x	nd	1.60	nd	nd	nd	nd	nd	---
Violaxthin	1.92	Trace	4.76	0.79	nd	nd	2.32	nd	0.56
Lutein	8.6	2.84	16.0	3.34	4.30	nd	11.7	Trace	1.77
Chlorophyll b	13.4	nd	32.6	Trace	24.6	nd	72.1	Trace	10.4
Chlorophyll a	48.8	nd	109	Trace	22.3	nd	94.9	Trace	12.1
Pheophytin b	nd	nd	nd	nd	0.62	nd	2.01	nd	0.50
β-carotene	0.35	Trace	1.46	Trace	nd	nd	nd	nd	0.26
Optical density ^w	---	---	---	---	0.34	0.23	0.44	0.31	0.03

^zU = unbleached; B = bleached.

^yLSD_{0.05} calculated from one-way analysis of variance, excluding treatments with no or trace detected. All F tests significant at $P < 0.01$.

^xnd = not detected.

(1993) for the *Pst*II/*Mse*I double digestion example. Probes for RFLP analysis were either random cDNAs from a library constructed in our laboratory using poly(A)+ RNA from etiolated seedlings or cloned cDNAs kindly provided by other researchers. The conventions used for naming markers were as follows: names of AFLP loci begin with "AFP," RAPD polymorphism names consist of the primer name (Operon Kit letter and number) and fragment size in base pairs delimited by an underscore, and all other names are RFLP loci.

LINKAGE AND QTL MAPPING. Linkage maps were computed for the 'Primo' x 'OSU442-15' F_2 and 'JI1794' x 'Slow' RIL populations using MAPMAKER ver. 3.0 (Lincoln et al., 1992) with LOD (Log odds ratio) thresholds for marker assignment set at 3.0 and 4.0, respectively, for these populations. Linkage map construction and QTL mapping in the 'Primo' x 'OSU442-15' F_2 population was based on four F_1 families totalling 102 plants, which were a subset of the total F_2 population of 550. Identification of the linkage groups comprising the 'Primo' x 'OSU442-15' map was achieved by mapping anchor loci accepted as group-specific markers (Weeden et al., 1993a; 1993b), by mapping loci from another pea linkage map (Ellis et al., 1993), or by scoring markers from the 'Primo' x 'OSU442-15' map in the 'JI1794' x 'Slow' RILs.

Tests of association between single marker genotypes and trait means and variances were conducted via ANOVA and Levene's test with routines programmed in XLisp-Stat (Tierney, 1990). Standard interval mapping (Lander and Botstein, 1989) was conducted by using Map-QTL software developed at CPRO-DLO Netherlands (J.W. van Ooijen, personal communication), and multiple QTL interval mapping of seed density was conducted using QTL-Cartographer software (Zeng, 1994). Empirical significance levels for QTL detection were determined by permutation tests according to Churchill and Doerge (1994) with 1000 permutations. Data from selective genotyping of high and low seed-color tails were analyzed by χ^2 tests on 2×2 or 2×3 contingency tables.

For BSA (Michelmore et al., 1991) and selective genotyping, DNA was isolated from seedling leaves of each of five F_3 descendants of F_2 plants representing the upper and lower 1.5% tails (18 plants each) of the optical density distribution. BSA was conducted as described by Timmerman et al. (1994). A total of 250 primers from Operon Technologies primer kits A through R that were not previously scored in the mapping population, were screened for RAPD polymorphisms with parental DNAs, high and low optical density DNA bulks.

Results

ANALYSIS OF SEED PIGMENTS IN PARENTAL GENOTYPES. Chromatograms of unbleached green seed extracts showed profiles typical of chloroplast photosynthetic pigments (Fig. 1). HPLC analysis of mature seed grown in the glasshouse confirmed that concentrations (based on dry mass) of all pigments were higher in seedcoat and cotyledon tissues of 'OSU442-15' (Table 1). Lutein was the predominant pigment in bleached cotyledons of both genotypes. Concentrations of other carotenoids were low in bleached and unbleached tissues. When precautions were taken to minimize de-pheophytinization during extraction, there were traces of pheophytins a and b only in samples with relatively high chlorophyll content. Chlorophyll a : b ratios were approximately 3 to 4 in cotyledons of both genotypes but nearer to 1 in the seedcoats. Bleached seed of 'OSU442-15' was darker in color than that of 'Primo', as measured by video densitometry, and still contained some pigments detectable by HPLC. The only qualitative difference noted between seedcoat and cotyledon pigment chromatograms was the appearance of a peak with retention time of 4.0 min (Fig. 1). This peak was present in variable quantities in unbleached and bleached seedcoats of both genotypes. The short retention time of this component on a reversed-phase HPLC column is consistent with a pheophorbide derived from chlorophyll catabolism (Amir-Shapira et al., 1987); however, it was not examined further in this study.

Developmental analysis of total seed pigments revealed differences between genotypes in time courses of pigment accumulation and degradation (Fig. 2). The F-tests for the cultivar $\times$ DAF interaction term in ANOVA were highly significant ($P < 0.001$) for all pigments. Results are presented on a per-seed basis to facilitate comparison, and it should be noted that final mean dry mass of 'OSU442-15' was 0.17 g per seed in contrast to 0.34 g per seed for 'Primo'. In both genotypes, maximum pigment content was attained at about the same time as maximum dry mass. Maximum pigment accumulation on a per-seed basis was similar in the two genotypes, but loss of extractable pigment per seed was greater in 'Primo'.

Genetic analysis of seed color.

OBJECTIVE MEASUREMENT OF SEED COLOR. Video image analysis provided quantitative measurements of seed color. Initially, the available software measured only seed lightness as optical density; however, later versions of the software provided full RGB color space data. For genetic mapping analyses, the RGB data were transformed into Y (density), U, and V color space components by using the equations previously described. The Y component is related to the perceived lightness of the seed, while the U and V components are related to the hue and saturation.

SEGREGATION OF SEED COLOR. Variation in lightness (Y component), U, and V is shown in Table 2 for parental seed and reciprocal F_1 seed from plants grown in the field in 1994. The lightness (Y) of reciprocal F_1 seed was not significantly different from selfed seed harvested from the same plants ($P > 0.05$ for all t tests), although the mean lightness (Y) values were shifted toward the midparent value. The mean U and V values approached the 'OSU442-15' mean.

Initially, only seed lightness (as optical density) was measured on the complete F_2 population as well as both parents. Once upgraded software was available, the remaining seed of the F_2 mapping population ($n = 77$ plants) along with seed from both parents was remeasured to obtain complete RGB color data. Seed lightness did not change significantly between measurements (R^2

Fig. 2. Changes in dry mass, percent moisture, and content per seed of photosynthetic pigments in developing 'Primo' (solid lines with circles) and 'OSU442-15' seeds (dashed lines with squares).

= 0.94). The raw RGB color data were not used to examine segregation of seed color in the F_2 generation since R, G, and B values were highly correlated ($R > 0.8$ for all pairwise comparisons). The YUV transformation of the RGB values provided more independent variables for QTL mapping purposes. The correlation coefficients for the three comparisons were $R = 0.26$ ($P < 0.05$) for Y vs. U, $R = -0.75$ ($P < 0.01$) for Y vs. V and $R = 0.64$ ($P < 0.01$) for U vs. V. The frequency distributions of the Y, U, and V color space components in the F_2 population ($n = 77$) are shown in Fig. 3. The Y and U distributions were significantly skewed ($g_1 = -1.57$ and 0.70 , respectively; $P < 0.01$) toward the means of 'Primo' and 'OSU442-15', respectively. The F_1 and F_2 distributions of the U-chrominance component were consistent with recessiveness of 'Primo' allele(s). However there was no overlap between the U-chrominance distributions of the 1992 and 1994 data, indicating that significant year-to-year variation in chrominance may occur. There were significant differences in plot mean lightness (Y) of F_2 plants in 1992 ($P < 0.001$ by ANOVA). However, correction of data did not alter QTL mapping results and uncorrected data were used in subsequent analysis.

LINKAGE MAP CONSTRUCTION. The genetic map constructed in the 'Primo' $\times$ 'OSU442-15' F_2 population ($n = 102$) was based on 199 molecular markers (RAPDs, RFLPs, and AFLPs), spans 1510 cM (Haldane units), and, therefore, has an average saturation of 7.5 cM per marker. This map consists of 14 linkage groups, including three small unassigned groups. There were 11 unlinked loci. Markers displaying skewed segregation ratios were included and mapped to two regions: the region of linkage group V containing *r*-locus (scored as an RFLP) and the region of linkage group VI in the vicinity of *Gs-p*. The skewed segregation in the latter region resulted from elimination of F_2 plants exhibiting obvious symptoms of infection by pea seed-borne mosaic virus (PSbMV). The 'Primo' $\times$ 'OSU442-15' population segregated for the *sbm-1* locus, which conditions resistance to PSbMV and was previously mapped to this region of group VI by Timmerman et al. (1993).

Linkage groups corresponding to virtually all of the *Pisum* linkage map (Weeden et al., 1993a) were identified in the 'Primo' $\times$ 'OSU442-15' map, but some genomic regions are not represented. Notably, the 'Primo' $\times$ 'OSU442-15' map is missing a segment of the linkage group containing *i*-locus, a segment of linkage group VI between *sbm-1* and *Fed-1*, and the distal end of the arm of linkage group VII containing *rDNA2*. The linkage group designated III/IV contains markers that have been assigned to groups III and IV of the "classical" linkage map.

QTL MAPPING. Interval mapping of genes affecting seed color as measured using the Y, U, and V color space components suggested that seed color in the F_2 population was controlled by two major

Table 2. Means and SD (in parentheses) of YUV color space components for seed from parental lines 'Primo' and 'OSU442-15' and reciprocal F_1 seed grown in the field at Lincoln, New Zealand, 1993–94.

Color space component	OSU442-15	Primo	OSU442-15 (female) $\times$ Primo (male) F_1	Primo (male) $\times$ OSU442-15 F_1 (female)
Density (Y)	0.334 (0.025)	0.250 (0.024)	0.291 (0.015)	0.277 (0.025)
U	-21.3 (2.03)	-27.1 (2.65)	-22.2 (1.39)	-22.7 (1.83)
V	11.1 (3.27)	24.0 (5.36)	12.6 (2.05)	15.9 (4.43)

Fig. 3. Frequency distributions of color space components Y (density), U, and V in the F_2 population used for QTL mapping. Means for the parents 'Primo' (Pr) and 'OSU442-15' (OSU) grown in the same year are indicated.

genes (Fig. 4 and Table 3) and two other QTL (Table 3). The loci detected had effects of varying direction and magnitude in color space. According to the ANOVA, no unlinked markers exhibited significant association with seed color components.

Two genomic regions affecting seed lightness (Y) were detected by standard interval mapping. Single-marker ANOVA indicated a significant association between Y and P108, an RFLP locus. In the 'J11794' $\times$ 'Slow' RILs, P108 cosegregated with linkage group V loci, including *tl*. The linkage group V assignment was subsequently confirmed by mapping *r*-locus in the 'Primo' $\times$ 'OSU442-15' population using a clone encoding starch branching enzyme I (clone pJAM425; Genbank Accession no. X80009) (Burton et al., 1995). Interval mapping indicated strong support for a major dominant (for the 'Primo' phenotype) gene affecting Y and was in the 35 cM interval flanked by P108 and *r*-locus (Fig. 4). The same genomic region also exhibited a significant effect on the V color space component (Table 3). A putative QTL with a smaller effect on lightness (Y) also was detected on a separate linkage group (designated "C"), whose relationship to the classical pea map is not known. However analysis of the Y data by multiple QTL interval mapping suggested that there was only support for the major gene on group V (data not presented).

Effects of the P108 genomic region on seed lightness were confirmed in $F_{2.3}$ families grown in the field in 1992–93. The $F_{2.3}$ families that were grown from seed of F_2 plants homozygous for the 'OSU442-15' allele of P108 exhibited significantly higher plot mean seed color density than those homozygous for the 'Primo' allele ($t = 2.90$, 18 df, $P < 0.005$).

Interval mapping indicated that the U chrominance component of the seed color phenotype was controlled by a major gene mapping to linkage group III/IV (Fig. 4 and Table 3). In contrast

to the linkage group V locus, this locus had no effect in the Y (luminance) dimension but had a significant effect in the chrominance plane (i.e., it affected the hue). Assignment of this locus to the pea map is complicated by the unclear linkage relationships of group III and IV markers. While markers M75 and LI09 co-segregated with group IV loci including *le*, *tpi*, and *Lox-10* (lipoxygenase) in the 'J11794' x 'Slow' RILs, markers on the other side of the QTL, M27, B08_1250, and L01_1600, co-segregated with group III loci (cMH95, *M* and *Acp-3*; data not presented).

QTL with smaller effects on the U and V color space components also were detected on linkage groups I (V only) and VII (U and V; Table 3). Multiple QTL interval mapping could not be performed on the U and V data due to the smaller sample size.

MODES OF INHERITANCE. The four QTL detected using the 'Primo' x 'OSU442-15' F_2 population showed various modes of inheritance (Table 3). The mode of inheritance for the major QTL on linkage group V, affecting the Y and V color space components, best fitted a dominant model (for the 'Primo' phenotype), while the major QTL on linkage group III/IV, affecting the U chrominance component, displayed a mode of inheritance that was best explained by a dominant model (for the 'OSU442-15' phenotype). These results account for the distributions for the Y and U color space components in F_1 and F_2 populations (Fig. 2). The QTL detected on linkage group I, linked to locus P482, displayed over-dominant allelic interaction. The individual F_2 plants that were heterozygous at locus P482 had higher mean values of U ($\mu_{\text{heterozygote}} = 19.6$) than either homozygote

class ($\mu_{\text{Primo}} = 16.5$, $\mu_{\text{OSU442-15}} = 16.8$). A plausible genetic basis for this mode of inheritance is two tightly linked dominant genes in repulsion (Tanksley, 1993). The remaining two QTL on linkage group VII and C displayed additive inheritance.

SELECTIVE GENOTYPING AND BULKED SEGREGANT ANALYSIS. The primer G18 produced two RAPDs of opposite phase that were associated significantly with seed lightness by single marker analysis and selective genotyping of extreme phenotype individuals (Table 4). The two RAPD products were not linked. The 1.6-kb fragment was linked to *r*-locus, the 880-bp fragment was linked to *Lg-J* on linkage group I. Interval mapping detected a QTL in this region of linkage group I (Table 3).

Although >200 DNA markers were scored in the 'Primo' x 'OSU442-15' cross, no polymorphic loci were mapped in the 35-cM interval bounded by P108 and *r*-locus, and containing the major QTL affecting the Y (lightness) component. Bulk segregant analysis was applied in an attempt to increase the marker saturation in this region. More than 250 RAPD primers were screened against pooled samples of DNA isolated from the top and bottom 1.5% extremes of the seed lightness distribution. This yielded three RAPD polymorphisms that mapped adjacent to, but not in, the interval (data not presented).

Fig. 4. LOD plots showing localization by standard interval mapping of major genes affecting seed color to linkage groups V and III/IV. The horizontal dashed line represents the empirical 95% experimentwise threshold estimated from 1000 permutations of trait data.

Discussion

The pigments found in extracts of green pea seeds were

typical of chloroplast photosynthetic pigments found in other legume seeds (Eskins and Harris, 1981; Lopez-Hernandez et al., 1993). The time course of chlorophyll turnover in 'OSU442-15' resembled that previously reported for the pea cultivar 'Burpeeana' (Storey and Beevers, 1978). The low concentrations of β -carotene in the mature seed of 'Primo' and 'OSU442-15' were within the range reported in previous studies in pea and other dried legume seeds (Gross, 1991). Lutein was the major pigment found in bleached seed. In addition, pheophytins did not contribute significantly to dry seed pigmentation in these genotypes. In contrast, studies of frozen peas (Forni et al., 1988) and other green vegetables showed that pheophytins commonly were present and imparted an olive color (Gross, 1991). The characteristic blue-green color of dried peas may result from the low chlorophyll a : b ratio in seedcoats of both genotypes. Decline in chlorophyll a : b ratio is a common feature of leaf senescence and reflects the lower stability of chlorophyll a (Gross, 1991).

Our HPLC data indicated that the most significant difference between the parental genotypes was that developing and mature seed of 'OSU442-15' contained more photosynthetic pigments on a dry mass basis than 'Primo' due to greater pigment accumulation during development and less breakdown during maturation. In a companion study (Timmerman-Vaughan et al., 1996), QTL for seed mass were mapped using the 'Primo' x 'OSU442-15' population. There was no significant correlation between seed color lightness (Y) and seed mass ($R = 0.04$; $P > 0.05$), and no genomic regions affecting seed mass and color were detected. Therefore, the genes controlling seed color may affect pigment concentration rather than total amounts per organ.

Measuring a color involves specifying that color in terms of coordinates in a three-dimensional color space (Francis, 1995). The power of interval mapping with individual color space components, therefore, is limited because information from the correlated components is not used. By contrast, multi-trait QTL mapping (Jiang and Zeng, 1995) permits simultaneous analysis of several characters, providing greater statistical power and the capability to dissect correlated traits, such as color. When suitable software becomes available, this technique should facilitate color QTL mapping and encourage a more quantitative genetic analysis of color.

Several mutations have been described in pea that affect seed color and plant senescence (Blixt, 1962; Weeden and Wolko, 1990), but the interactions and practical significance of these in cultivated genotypes is poorly understood. Green color appearance also may be influenced by variation in seedcoat structure or in other seedcoat pigments. Variation in seedcoat translucency could influence appearance by affecting visibility of the underlying cotyledon color. Lamprecht (1959) described *gla*, a gene that conditions colorless vs. green seedcoats in white-flowered pea genotypes. Since the linkage relationships of this locus were not determined, it is not possible at this time to speculate on its relationship to the genetic loci affecting seed color in the 'Primo' x 'OSU442-15' cross.

The major gene identified on linkage group V may control the quantitative difference in photosynthetic pigment composition between 'Primo' and 'OSU442-15' seed. The effects of this region

Table 3. Locations and effects of putative loci affecting seed color in the 'Primo' x 'OSU442-15' field pea F_2 population detected by standard interval mapping.^z

Color space component	Linkage group	Associated marker(s) ^y	LOD support	% Variance explained	d/a ratio ^x
V	I	<i>Lg-J-P482</i>	2.55*	17	-19.7
U	III/IV	105_530-K02_1700	4.70**	56	-1.26
Y	V	P108- <i>r</i>	13.5	61	0.76
V	V	P108- <i>r</i>	3.81*	27	1.09
U	VII	P694	2.43*	16	0.16

^zWhere loci exhibited significant effects in two dimensions, results for both are presented.

^yLocation is given as map interval in which putative QTL lies or marker name if LOD maximum fell on marker location.

^xParameters estimated with unconstrained genetics.

*,**Exceeds empirical comparisonwise or experimentwise threshold, respectively, at 0.05.

Table 4. Support for QTL affecting seed lightness on linkage groups I and V based on selective genotyping of 1.5% extremes of an F_2 population (n = 550) from 'Primo' x 'OSU442-15' field peas.

Locus	N ^a	df	χ^2	p value	Linkage group	Linked anchor locus
PID5 ^y	43	2	11.4	0.0034	I	<i>Lg-1</i>
G18_880	36	1	12.5	0.0004	I	<i>Lg-1</i>
111_2000	36	1	8.9	0.003	I	<i>Lg-1</i>
G18_1600	36	1	18.0	0.00002	V	<i>r</i>

^aNumber of plants genotyped.

^yGranule-bound starch synthase I.

on seed color, expressed in the YUV color space, are predominantly in the luminance or lightness (Y) dimension. This finding is consistent with a quantitative, rather than qualitative influence on pigment concentration. The locus *pa*, which conditions bright green foliage and pod and fresh seed color, was mapped to linkage group V, close to *tl* (Lamprecht, 1957) and in the same region as this major gene. This raises the possibility that the major gene we have detected in the interval between P108 and *r*-locus may be *pa*. Foliage and pod color were not noticeably different between 'Primo' and 'OSU442-15'. Crosses have been made between 'OSU442-15' and *pa* genotypes to determine whether the gene we detected is an allele of *pa*. A major gene affecting the level of green pigmentation in dry pea seed is consistent with findings in other grain legumes. A major gene controls green vs. tan color in dry seed of *Phaseolus* species (Honma et al., 1968; Tucker, 1965). A dominant nuclear gene, *G*, conferred green seedcoat color in soybean in the presence of a recessive nuclear gene *y3* (Bernard and Weiss, 1973).

Nomination of candidate genes corresponding to the major gene affecting the U chrominance component, which we mapped to linkage group III/IV, is confounded by differences between the classical and molecular marker maps in these genomic regions. Three candidate loci have been mapped in the linkage group IV region associated with this gene. *Lox-10*, which catalyzes co-oxidation of chlorophylls and carotenoids (Arens et al., 1973), is one of two loci containing structural genes for lipoyxygenase (Ellis et al., 1993). Two other relevant loci also have been assigned to this region in experiments using other germplasm sources: *vim*, which has a phenotype similar to *pa* (Lamprecht, 1957), and *olv*, which gives greenish olive gray seedcoat (Blixt, 1962).

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