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ORIGINAL PAPER

Inheritance of seed condensed tannins and their relationship with seed-coat color and pattern genes in common bean (*Phaseolus vulgaris* L.)

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Abstract Condensed tannins are major flavonoid end products that affect the nutritional quality of many legume seeds. They chelate minerals and interact with proteins, thus reducing their bioavailability. Tannins also contribute to seed coat color and pigment distribution or intensity. The objective of this study was to analyze the relationship between quantitative trait loci (QTL) for seed tannin concentration in common bean and Mendelian genes for seed coat color and pattern. Three populations of recombinant inbred lines, derived from crosses between the Andean and Mesoamerican genepools were used for QTL identification and for mapping STS markers associated with seed color loci. Seed coat condensed tannins were determined with a butanol–HCl method and a total of 12 QTL were identified on separate linkage groups (LGs) in each of the populations with individual QTL explaining from 10 to 64% of the phenotypic variation for this trait. Loci on linkage groups B3 and B10 were associated with the Mendelian genes *Z* and *Bip* for partly colored seed coat pattern, while a QTL on linkage group B7 was associated with the *P* gene which is the primary locus for the control of color expression in beans. In conclusion, this study found that the inheritance of tannin concentration fits an oligogenic model and identifies novel putative alleles at seed coat color and pattern genes that control tannin accumulation. The results will be important for the genetic improvement of nutritionally enhanced or biofortified beans that have health promoting effects from higher polyphenolics or better iron bioavailability.

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Introduction

Condensed tannins (or proanthocyanidins) comprise a group of oligomers and polymers of the type polyhydroxyflavan-3-ol that are synthesized through the flavonoid pathway (Dixon et al. 2005) and that are united by carbon–carbon bonds between flavonol subunits (Schofield et al. 2001). The name proanthocyanidin reflects the fact that when these tannins are subjected to heat in an acid/alcohol solution (acid hydrolysis), they produce colored anthocyanidins (Porter 1989).

Tannins represent a complex range of polyphenolic compounds that vary in size, bond types, monomeric composition, and contents across different plant species, tissues, and stages of development (Marles et al. 2003). These compounds are known to play diverse ecological and nutritional roles (Makkar et al. 1999). For example tannins that accumulate in leaves have an important function in preventing insect herbivory in many plants, while tannins in seed coat are suggested to function against fungal infection (Winkel-Shirley 2001; Kantar et al. 1996). In nutritional terms, the presence of multiple hydroxyl groups in condensed tannins lead to the formation of complexes with proteins (Hagerman et al. 1998; Naczk et al. 1996), metallic ions (Foo et al. 1997; McDonald et al. 1996; Reed 1995; Scalbert 1991), and polysaccharides (Barahona et al. 1997) and this influences the dietary absorption of each of these nutrients.

Studies carried out with animals have shown that a high percentage of tannins in the diet negatively affect the digestibility of proteins and carbohydrates, reducing weight gain and efficiency in nutrient absorption (Barahona et al. 1997; Li et al. 1996; Terrill et al. 1992). Likewise, the anti-nutritional effect of tannins in cereal grains and legumes has been reported as reducing the quality of these foods for human consumption (Cabrera and Martin 1989; Price and Butler 1977; Reddy and Pierson 1985; Strumeyer and Malin 1975). For example, legume seed tannins are known to precipitate proteins and form complexes with iron in the gastrointestinal lumen, thus reducing the bioavailability of the minerals in the grain (Brune et al. 1989; Marles et al. 2003; Mira et al. 2002). Bioavailability is a measure of fractional utilization of an ingested nutrient assayed through in vivo or in vitro approaches, and iron is currently the only micronutrient for which there is a direct measure of bioavailability (Fairweather-Tait 2001). Despite these concerns, tannins may function as anti-carcinogenic compounds and antioxidants (Beninger and Hosfield 1999; Dangles et al. 2000; Takeoka et al. 1997). Therefore, the balance between health benefits and anti-nutritional effects of tannins is important.

Common bean (*Phaseolus vulgaris* L.) is the most important legume for direct human consumption forming the principal source of protein for the diet of people in countries such as Brazil, Mexico, Rwanda, Uganda, and other parts of East Africa and Latin America (Broughton et al. 2003). In common bean, most tannins are located in the seed coat (Reddy and Pierson 1985; Van Der Poel et al. 1991) and as in other legumes their concentration affects mineral and protein bioavailability (Adewusi and Falade 1996). Common bean shows considerable variability for seed traits, and different groups of consumers have developed specific preferences for different combinations of seed size, form, and color (Beninger and Hosfield 2003). Color hue and patterns are the principal criteria, with the pigments responsible for variations in seed color being flavonoids, principally flavonol glycosides, (Beninger and Hosfield 1998), anthocyanins, and condensed tannins (Beninger and Hosfield 1999; Takeoka et al. 1997).

From the early 20th century, research has been carried out on the inheritance of seed color in *P. vulgaris*. According to the currently accepted model (Prakken 1970; Prakken 1972), *C*, *D*, and *J* are genes for seed color and are expressed only in the presence of gene *P*, which is hyperstatic to all seed color and pattern genes and determines color expression in both seed coats and flowers (Emerson 1909). Genes *G*, *B*, *V*, and *Rk* are modifier genes (i.e., they intensify colors or influence their hue). Gene *V* is also called the violet factor and the dominant allele causes bluish or violet to black colors (McClean et al. 2002). Meanwhile other genes control seed color pattern in partly colored seed coats, namely the genes *J*, *L*, *T*, *Z*, and *Bip* (Bassett 1994, 2002).

The *P* locus is particularly important as the basic color gene, has multiple alleles for seed coat and flower pattern and is known as the *ground* factor for all seed coat color genotypes (Bassett 2007). The locus has the previously described alleles, *P*, p^{gri} and *p* with genotypes that when in a homozygous recessive state for the *p* allele produces all white seed with no flavonoids. However, Basset (1996) reported new alleles for the *P* locus (p^{mic}, p^{hbw}, and p^{stp}) with pleiotropic effects in flower and seed coat. In summary, *P* is considered as the controlling factor for the presence or absence of flavonoids in the seed coat and the specific color then depends on the epistatic interactions of the alleles at the other genes described above (Erdmann et al. 2002).

Studies of common bean populations have shown the existence of genetic differences for condensed tannin concentration, suggesting a pattern of quantitative inheritance. For example, Guzmán-Maldonado et al. (2003) identified quantitative trait loci (QTL) for whole grain tannin content using a Prussian blue method, which quantifies total phenolic compounds but is not specific for condensed tannins. However, they did not find an association with seed color or seed color genes. Several studies have attempted to establish relationships between the classical Mendelian genes known for color seed and pigment synthesis in *P. vulgaris* but such relationships are still speculative (Beninger and Hosfield 1999; Beninger et al. 1998; Feenstra 1960). In this study, our goal was to identify QTL associated with tannin concentration in three recombinant inbred line populations using a uniform quantification method for condensed tannins, and to evaluate the relationship between this trait and seed color genes so as to develop a genetic model for the accumulation of condensed tannin in common bean seed coats.

Materials and methods

Mapping populations

A total of 243 recombinant inbred lines (RILs) were evaluated; 87 ($F_{9:11}$) derived from the cross DOR364 × G19833 (D × G); 72 ($F_{8:11}$) derived lines from the cross BAT93 × JALOEEP558 (B × J); and 84 ($F_{5:8}$) derived lines from the cross G2333 × G19839 (G × G). DOR364, BAT93, and G2333 are all of the Mesoamerica race and have small red, small yellow, and medium red seeds, respectively, whereas G19833, JALO, and G19839 of the Andean gene pool have large yellow seeds; G19833 with red stripes, G19839 with brown stripes, and JALO with no pattern. To analyze for condensed tannins, 10 seeds from each line and

500 seeds from each parent were manually peeled after a 48 h treatment with *n*-heptane to facilitate seed coat removal without affecting tannin concentration (this laboratory, unpublished data), after which the seed coats were finely ground in a Retsch mill MM200. The resulting flour was stored at 5°C and 50% relative humidity until analysis.

Phenotypic evaluation

Purification of seed coat tannins from the parents of the RIL populations was carried out as described by Jones et al. (1976). To extract the tannins, 60 ml of an aqueous solution of 70% acetone and 0.1% ascorbic acid was added to the ground seed coat obtained from each parent. The samples were placed on a shaker for 75 min at room temperature and then filtered through Whatman No 1 filter paper until the solutions showed no residues. To clean the samples of non-phenolic compounds, diethyl ether was added to each filtered sample until the initial volume was doubled. In this step, two phases were formed. The ether (upper layer) was discarded through vacuum removal, repeating the procedure twice more with diethyl ether and then three times with ethyl acetate. After this cleaning procedure, the samples were placed in a Precision sample concentrator, equipped with a 37° water bath during 6 h to evaporate the initial solvents to completion. The samples were re-suspended in a double volume of 100% methanol and placed onto Sephadex LH-20 columns previously balanced with methanol. After centrifuging for 5 min at 4500 rpm, the flow through was discarded, a process which was repeated twice. Once the tannins adhered to the column, a double volume of aqueous solution of 70% acetone was added to elute them. The samples were centrifuged for 5 min at 4500 rpm and each supernatant was recovered in 400-ml beakers. The same procedure was carried out three additional times, adding acetone solution each time. Each sample of recovered supernatant was then placed in the sample concentrator for 9 h to eliminate the solvents. The dried samples were then frozen at −20°C and lyophilized. A calibration curve was constructed using a combination of purified tannins from each parent mixed in equal weights. Quantification was carried out using the butanol–HCl method (Porter et al. 1986) and the samples were read at 550 nm on a spectrophotometer (Shimadzu UV-1601).

The extraction of tannins from ground seed coat and their quantification were carried out in triplicates on 10 mg of sample as described by Terrill et al. (1992), with modifications suggested by Barahona et al. (1997). To start, 2.5 ml of 70% acetone:0.1% ascorbic acid was added to each sample followed by 2.5 ml of diethyl ether. At this stage, two phases were formed, with the bottom phase containing the tannins. The samples were vortexed and the upper phase removed from each sample by vacuum removal. The acetone:ascorbic acid solution and the diethyl ether were added again and the upper phase was again removed. The samples were then concentrated during 1 h using the method described for tannin purification. Each sample was then filled to 5 ml with distilled water and centrifuged for 15 min at 3500 rpm. The supernatant was separated from the pellet and used to analyze soluble tannins (ST), whereas the pellet was used to quantify insoluble tannins (IT) by direct analysis of the extraction residue.

For detection of ST, 300 µl of supernatant was transferred to a glass tube with a thread stopper and 1.8 ml of 5% HCl in butanol was added. For IT detection, the pellet was dried for 40 min in an oven at 40°C, after which 0.7 ml of water was added to each dried sample, followed by 4.2 ml of 5% HCl in butanol. All samples were then vortexed and subjected to a 95°C water bath for 75 min to obtain acid hydrolysis (Terrill et al. 1992). The samples (colored by now) were cooled in an ice water bath for 10 min, and read at 550 nm in a spectrophotometer (Shimadzu UV-1601). Blanks consisted in samples treated with 5% water in butanol (Jackson et al. 1996) and total tannins concentration (TT) was estimated from the sum of ST and IT. The results for tannin concentration were expressed as percentage of condensed tannins in seed coats of the genotype analyzed.

Evaluation and mapping of STS markers

The 11 STS markers developed by McClean et al. (2002) for various seed coat color genes as well as the OU3 RAPD marker reported by Erdmann et al. (2002) as linked to the *P* gene, were evaluated in the parents and lines of each RIL population. PCR reaction volumes were 20 µl, containing 10 mM Tris–HCl (pH 7.2), 50 mM KCl, 1.5–2.0 mM Mg (depending on the primer), 0.2 mM dNTPs, 0.2 µM of each primer (0.8 µM in case of the RAPD), 1.0 unit of *Taq* polymerase, and 50 ng of genomic DNA template. DNA amplifications were carried out in a PTC-100 thermal cycler (MJ Research), under the following conditions: 94° for 3 min (initial denaturing), followed by 40 cycles of 92° for 1 min (denaturing), annealing temperature for 1 min, 72° for 2 min (extension), and one cycle of final extension at 72° for 5 min. The PCR products were run on 2% agarose gels to evaluate parental polymorphism and then to determine the segregation of polymorphic markers for the entire population. For some markers, we had to change the original amplification conditions used by McClean et al. (2002), as follows: (1) concentration of MgCl at 1.5 mM for OJ17 and OAP2, and 2.0 mM for OAM10 and OU14; (2) annealing temperature at 62° for OAM10; (3) initial denaturing for 3 min at 94° and the other denaturations at 92° for 1 min in each cycle in all cases.

Data analysis and QTL identification

Analysis of variance, components of variance, and mean separation were conducted for each variable and for each population using SAS version 9.1.3 (SAS Institute Inc, Cary, NC). Descriptive statistics and analysis of correlation for ST, IT, and TT were determined with STATISTIX v.8.0 (Analytical Software, Tallahassee, FL, USA). An additional analysis of variance was conducted to test the association of TT with seed color classes as defined by CIAT (1987) using a Levine test for variance homogeneity. The placement of STS markers on the genetic maps of the RIL populations was determined using the place function in MAPMAKER v. 2.1 (Lander et al. 1987) with genetic distances expressed in Kosambi cM units. QTL analysis was carried out with Windows QTL Cartographer v.2.5 (Wang et al. 2005) using the composite interval mapping method (CIM). QTL significance thresholds for all variables were estimated by permutation tests as recommended by Doerge and Churchill (1996) based on 1000 permutation tests at a 5% level of significance for each trait. Multiple interval mapping (MIM) analysis was conducted using the same parameters as CIM method for all significant QTL using the same software program. The maps used for QTL analysis were those reported by Beebe et al. (2006), Blair et al. (2003), and Ochoa et al. (2006) for D × G, B × J, and G × G RIL populations, respectively.

Results

Quantification and distribution of condensed tannins

Significant differences ($P < 0.01$) were found between the genotypes of the three populations for the three variables evaluated in this study (ST, IT, and TT) (Table 1). The coefficients of variation were higher for insoluble tannins compared to soluble tannins with total tannins having the lowest coefficient of variation across the three populations. The B × J population had higher coefficient of variation for all three values compared to intermediate values for D × G and low values for G × G. Overall, experimental error was not a significant source of variation, meaning that the methodology used was reliable for the measurement of seed coat tannins in common bean. On the other hand, the correlation of ST with TT was highly significant ($r = 0.98$; $P < 0.01$). Thus, ST explained about 85% of the values estimated for TT. The calibration curves generated from the purified tannins had a confidence interval of 0.067 mg/ml, with detection limits at 0.027 mg/ml, and quantification limits at 0.075 mg/ml, the last two being equivalent to about 0.11% and 0.3% tannins on a whole-seed basis and 1.3% and 3.7% on a seed coat basis, respectively. The overall mean value of TT for all individuals analyzed was 24.3% while for ST and IT, the mean values were 21.4% and 2.9%, respectively.

Table 1 Average percentage of soluble, insoluble, and total condensed tannins in seed coat of parents and recombinant inbred line (RILs) genotypes in three populations of common bean

Condensed tannin concentrations[a]			
Generation	Soluble	Insoluble	Total
B × J population			
BAT93 (P1)	6.16 ± 0.15**	1.23 ± 0.07**	7.39 ± 0.13**
JALO (P2)	13.39 ± 0.42**	5.66 ± 0.04**	19.05 ± 0.48**
RILs (mean)	8.74 ± 0.24	2.32 ± 0.11	11.06 ± 0.23
RILs (Range)	0–25.3*	0.5–7.2*	0–29.2*
D × G population			
DOR364 (P1)	24.75 ± 1.06	5.08 ± 0.41**	29.84 ± 1.39
G19833 (P2)	25.15 ± 0.69	2.94 ± 0.16**	28.09 ± 0.84
RILs (mean)	26.03 ± 0.52	2.85 ± 0.09	28.71 ± 0.72
RILs (Range)	13.64–35.20*	1.1–7.6*	15.60–38.50*
G × G population			
G2333 (P1)	16.84 ± 0.78	2.99 ± 0.24	19.83 ± 0.57
G19839 (P2)	14.97 ± 0.09	3.39 ± 0.18	18.36 ± 0.79
RILs (mean)	15.66 ± 0.35	3.00 ± 0.10	18.66 ± 032
RILs (Range)	0–27.4*	0–4.3*	0–31.6*

** Significant differences between the two parents at $P < 0.01$

* Significant differences between RILs at $P < 0.01$

[a] Mean ± standard deviation

When comparing the three populations, D × G had the highest condensed tannin concentration, followed by G × G and B × J (Table 1). Parental differences were significant for IT in the first population and for all three variables in the second population, while the third population presented similar parental means for each tannin measurement. We found that both the D × G and G × G population had tendencies to be normally distributed (Fig. 1), whereas B × J had a non-normal distribution that tended to be binomial and biased toward the parent with the lower condensed tannin concentration for the three variables. Soluble and total tannin ranged from 0% to 30% for B × J and G × G populations and from 13% to 38.5% for D × G population. Interestingly, IT always represented less than 10% of the value of TT, with ranges from 0% to 7%; 0% to 4%, and 1% to 7% for B × J, G × G, and D × G populations, respectively (Table 1).

Seed coat color and tannin concentration

Within the D × G and G × G populations, most individuals had red or yellow primary seed color with some having red or brown stripes; while in the B × J population most individuals had cream or light yellow seed colors.

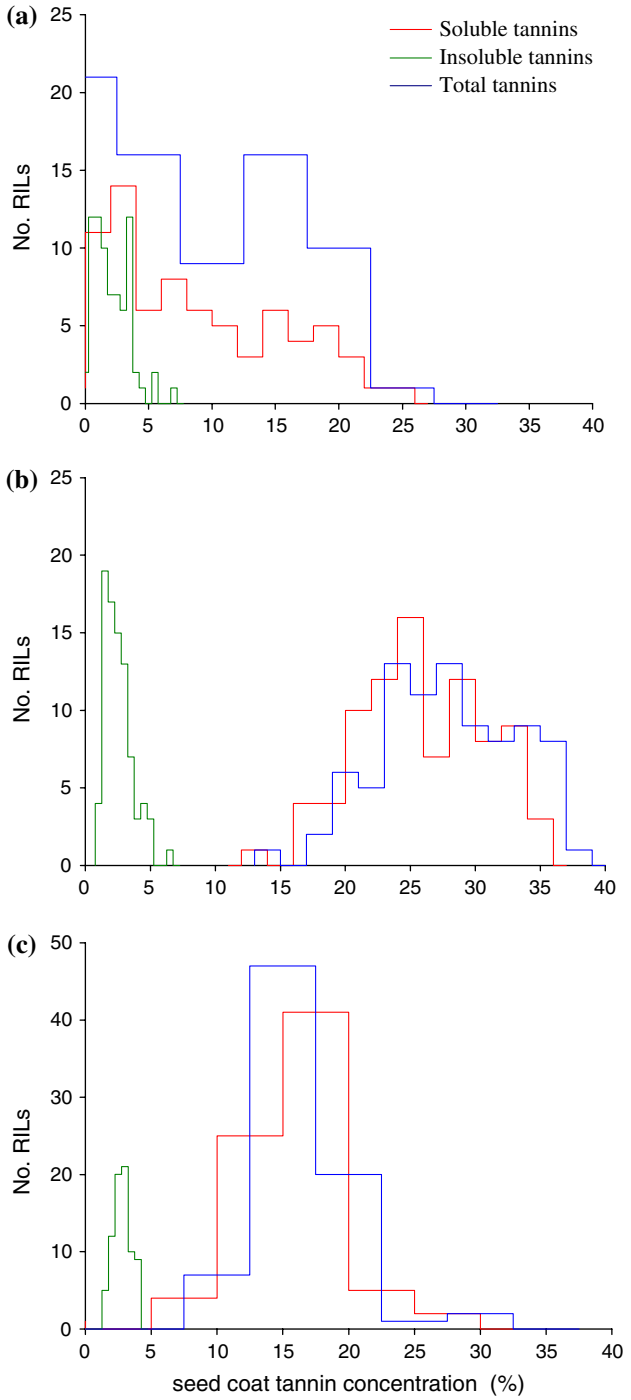

Fig. 1 Frequency distribution of soluble, insoluble, and total tannins concentrations in three populations of common bean (*P. vulgaris*). **a** B × J population, **b** D × G population, and **c** G × G population

However, other genotypes were observed including brown seeds in the case of B × J and dark red seeds in the case of D × G and G × G populations. Overall, yellow seeded genotypes had the lowest tannin percentages and those with darkest seed coats had the highest tannin percentages, although variability within each color class was observed. Using analyses of variance for tannin concentration in each primary seed color class found in the populations, we found significant differences between the darkest (purple, red) and the lightest (yellow) colored genotypes in two of the populations (Table 2). Although cream colored seeds were expected to give low tannin percentages given that they were among the lightest seed type, this color class showed the tendency of having high tannin concentrations in the G × G and D × G populations. Our results suggest that although some correlation exists between tannin concentration and seed coat color class, this correlation was not perfect and therefore, it was important to clarify the genetics of seed tannin accumulation through QTL analysis.

QTL analysis of tannin concentration

Composite interval mapping (CIM) detected 12 QTL in the three populations, which were named according to a convention based on the type of tannin they were detected for, together with the linkage group (LG) and the order of the QTL in that group (Fig. 2, Table 3). For ST concentration, four QTL were identified on LGs B3, B7, B8, and B10 (*Cst3*, *Cst7*, *Cst8*, and *Cst10*), explaining from 11% to 64% of the phenotypic variation. A total of five QTL for TT concentration (*Ctt3a*, *Ctt3b*, *Ctt7*, *Ctt8*, and *Ctt10*) were found on the same linkage groups as QTL for ST and explained similar amounts of variation (R^2 of 10–63%). Three QTL for IT concentration were found, one in each population and each one accounting for over 21% of the variance, located on B6 (*Cit6*, 21%), B7 (*Cit7*, 34%), and B9 (*Cit9*, 22%). Overall, the most significant QTL were observed in the B × J population and the population with the most QTL was D × G. QTL alleles for higher tannins concentration were derived from the higher tannin parental genotype (DOR364, G2333, and JALO) for 10 of the 12 QTL identified. Two QTL for IT were increased by alleles from the low tannin parent, one in the case of G19833 and one in the case of G19839. All QTL detected through CIM were also identified by MIM analysis, but epistatic effects between QTL were not found in any case. The total phenotypic variation TR^2 were high due to the small number of QTL, ranging from 0.50 to 0.58 in D × G, from 0.45 to 0.52 in G × G, and from 0.64 to 0.90 in B × J population. Most of the QTL for ST and TT concentration were associated with the same marker or at least with the same region of the LG. This could have been explained because of the high correlation found between ST and TT whereas this was not the case for IT, for which two QTL were found on different LG.

Genetic mapping of seed coat color gene markers

As part of our dissection of tannin accumulation genetics, we also mapped a set of markers for classically defined

Table 2 Multiple comparisons for condensed tannin concentrations according to seed coat color in genotypes of three common bean populations

Common bean population	B × J		D × G		G × G	
	Genotypes[a]	Mean[b]	Genotypes	Mean	Genotypes	Mean
Black (8)a	–	–	–	–	22	21.34[c]
Brown (4)	24	11.87[c]	11	29.28[c]	10	18.09[a,c]
Cream (2)	–	–	15	30.43[c]	10	18.54[a,c]
Pink (5)	–	–	–	–	11	17.10[a]
Purple (7)	7	19.09[a]	–	–	–	–
Red (6)	–	–	25	29.82[c]	22	18.99[a,c]
Yellow (3)	40	8.64[a]	32	27.61[c]	7	17.45[a,c]
F value		7.05*		1.18		3.25*

* Significant differences between color classes at $P < 0.01$

[a] Number of genotypes by seed coat color class

[b] Average percentage of tannins concentration by seed coat color class

[c] CIAT scale number for seed coat color of common bean

seed coat color and pattern genes (McClean et al. 2002) on the three genetic maps used for QTL analysis. We found that of the 11 STS markers evaluated in the parents, six (OAM10 linked to *Z*, OU14 linked to *G*, OD12 linked to *V*, OAP2 linked to *C*, OL4 linked to *L*, and OJ17 linked to *Bip*) were polymorphic between some of the parents and could be mapped. Additionally, the OU3 RAPD associated with the *P* gene (Erdmann et al. 2002), was polymorphic in the B × J population and was also integrated into the map used for QTL analysis. The markers associated with the genes *Z*, *G*, *V*, and *C*, were located in linkage groups B3, B4, B6, and B8, respectively (Fig. 2). Meanwhile, two markers for the genes *L* and *Bip* were located in linkage group B10 and the OU3 RAPD was found on linkage group B7 with linkage group assignments in agreement with those of McClean et al. (2002). In terms of specific markers in the B × J population, the STS markers OU14, OAP2, OJ17, and OL4 were located 0.12 cM, 0.04 cM, 1.5 cM, and 1.6 cM from their respective phenotypic loci according to McClean et al. (2002). In the D × G population, the OU14 marker was located 1.9 cM from the microsatellite PV-gaat001 and 2.0 cM from PV-at003 while in the G × G population, it was at a comparable distance from PV-at003 (2.3 cM). An additional STS marker, OJ17, was located 0.6 cM from the RAPD marker G161G in the D × G population, while in B × J it was at 2.6 cM from the same marker. The OD12 marker associated with the *V* gene was mapped at 1.7 cM from the AK061D marker in the D × G population and similarly the marker D12SV was located at 3.0 cM from RAPD AK61D in the B × J population. While this marker was not polymorphic in the G × G population, the *V* gene had been mapped in this population by Ochoa et al. (2006) based on flower color phenotyping.

It was notable that several STS markers were very closely linked to QTL for tannin concentration in the three populations. For example, the QTL *Cst3* was located at 0.8 cM from STS OAM10 while the QTL *Ctt3a* overlapped with this marker (Fig. 2). Similarly, the OU3 RAPD marker was closely linked to the QTL *Cst7*, *Cit7*, and *Ctt7* on B7; and the QTL *Cst10* and *Ctt10* were located in the region that included the OJ17 marker on B10. By comparative mapping, meanwhile, the OAP2 marker associated with the C locus on linkage group B8 in the D × G population was in the same region as the QTL *Ctt1c* and *Cst1c* in the G × G population although mapping of this marker in that population was not possible. Likewise the marker for the *V* locus mapped in the D × G population to linkage group B6, which was in the same region as the QTL *Cit1c*. The association of the QTL for tannin concentration and the individual seed color and pattern genes tagged by each of these markers is discussed in the next section.

Discussion

Relationship of tannin QTL with the pattern genes *Z* and *Bip*

Of the 12 QTL found in this study, four were located in regions containing the STS markers associated with genes *Z* and *Bip* for partly colored seed coat pattern, namely OAM10 associated with QTL *Cst3* and *Ctt3* and OJ17 associated with QTL *Cst10* and *Ctt10*. These QTL were found only in the D × G population and were not identified in the B × J or G × G populations. In the case of the B × J population this may be because of the absence of segregation for seed coat pattern. However, it was

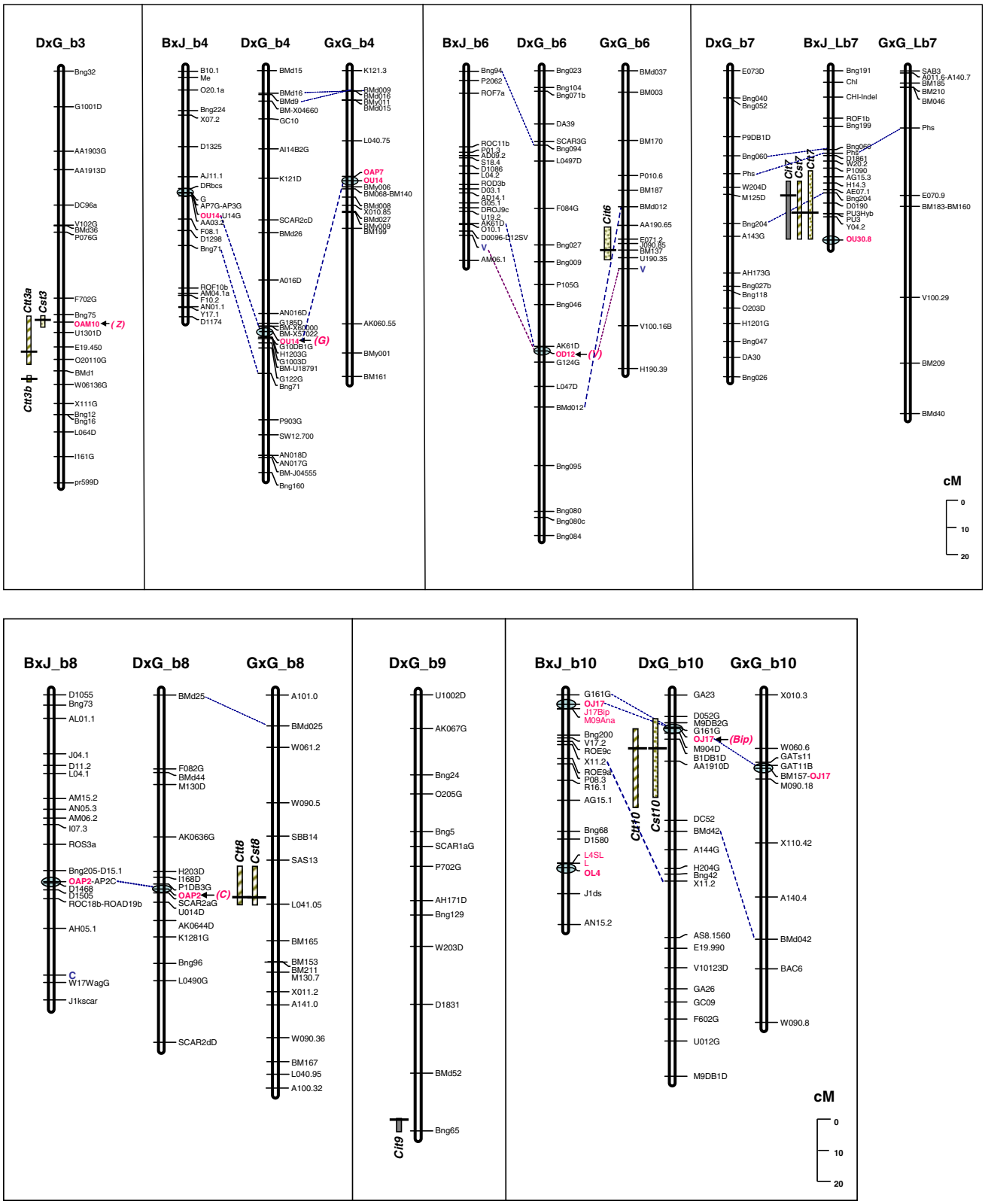

Fig. 2 Positions of quantitative trait loci (QTL) for condensed tannin concentration on three genetic common bean populations. D × G map, adapted from Blair et al. (2003) using high LOD mapping; B × J map, adapted from Freyre et al. (1998); G × G map, adapted from Ochoa et al. (2006). Color genes and STS markers developed by McClean et al. (2002) are indicated by circles within the linkage group. The QTL for composite interval mapping analysis are indicated vertically to the left of the linkage group. A horizontal line across the QTL indicates position of highest LOD peak within the QTL

Table 3 Quantitative Trait Loci (QTL) for condensed tannins in three recombinant inbred line populations of common bean (*P. vulgaris*)

QTL[a]	Linkage group	Nearest marker	Position	LOD[b]	Source	Additive	R^{2c}_{CIM}	R^{2d}_{MIM}	TR^{2e}	Putative gene
B × J population										
Soluble tannins										
Cst1a	7	Y04.2	55.9	23.20	JALO	−5.99	0.64	0.64	0.88	*P*
Insoluble tannins										
Cit1a	7	PU3Hyb	52.1	9.62	JALO	−0.87	0.34	0.52	0.64	*P*
Total tannins										
Ctt1a	7	Y04.2	55.9	24.80	JALO	−6.96	0.63	0.71	0.90	*P*
D × G population										
Soluble tannins										
Cst1b	3	OAM10	92.8	3.93	DOR364	1.83	0.11	0.11	0.50	*Z*
Cst2b	10	B1DB1D	17.1	7.87	DOR364	2.82	0.28	0.26	0.54	*BIP*
Insoluble tannins										
Cit1b	9	Bng65	138.3	3.13	G19833	−0.57	0.22	0.17	0.55	–
Total tannins										
Ctt1b	3	E19.450	102.6	4.07	DOR364	1.96	0.11	0.07	0.58	*Z*
Ctt2b	3	BMd1	113.5	3.27	DOR364	1.83	0.1	0.004	0.57	*Z*
Ctt3b	10	B1DB1D	17.1	10.29	DOR364	3.43	0.37	0.36	0.56	*BIP*
G × G population										
Soluble tannins										
Cst1c	8	L041.05	65.4	5.94	G2333	2.67	0.26	0.23	0.45	*C*
Insoluble tannins										
Cit1c	6	BM137	66	5.60	G19839	−0.38	0.21	0.22	0.52	–
Total tannins										
Ctt1c	8	L041.05	65.4	6.10	G2333	2.96	0.25	0.22	0.48	*C*

[a] QTL names indicate trait and linkage group number

[b] LOD thresholds based on 1000 permutations as per Doerge and Churchill (1996)

[c] Proportion of the phenotypic variance explained by QTL using CIM

[d] Proportion of the phenotypic variance explained by all significant QTL using MIM

[e] Total phenotypic variation

surprising that we did not find QTL close to the *Bip* or *Z* genes in the G × G population as we had in the D × G population, given the similarity in seed color between the parents for the two populations, both being derived from crosses between a red seeded Mesoamerican genotype and a yellow striped Andean genotype (brown striped in the case of G19839 and red striped in the case of G19833). The lack of these QTL might explain why overall the G × G population had lower levels of condensed tannin (15% of seed coat weight) than the D × G population (25% of seed coat weight). It is notable that DOR364, a dark red seeded genotype, had more tannin in its seed coats than G2333, a medium red seeded genotype.

Genes *Bip*, *Z*, *L*, and *J* are genes known to control seed color pattern in partly colored beans (Bassett and McClean 2000). Patterned seed requires a *t*/*t* genotype for expression of partial color, whereas a *T*/− genotype gives seeds that are totally colored. The types (form and extension) of colored patterns are controlled by the interaction of *t* with genes *Z* (zonal), *L* (limiter), *J* (joker), and *Bip* (bipunctata) (Bassett 1994). Bassett (1999) discovered the allelism between gene *Z* and gene *D*, the gene for seed coat color and pattern. Therefore, *Z* participates both in seed coat color and in controlling pattern in partly colored seed coats (Bassett 2007). Additionally, Bassett (2002) found that *L* is synonymous with *j* and *l* with *J*. Meanwhile, Koinange et al. (1996) reported gene *Z* to be located on linkage group B1, whereas McClean et al. (2002) located it on B3. Our results with populations D × G and G × G agreed with those obtained by McClean et al. (2002) for the locations of *Z*, *G*, *V*, *C*, and *Bip*. However, differences existed with respect to the relative positions of the STS markers for loci mentioned above, which could be explained by the saturation of genetic maps in each population. According to the study by McClean et al. (2002), OAM10 and OJ17 were found at a distance of 1.4 cM and 6.0 cM from genes *Z* and

Bip, respectively, in the populations segregating for those genes. Thus, the regions found as QTL for condensed tannins in this study may be directly associated with genes *Z* and *Bip*.

In the case of the *Z* gene, this locus has been determined as being allelic to the *D* gene for hilum color (Bassett 1999). Therefore, our observations that a QTL for tannin was located at the *Z* gene may reflect accumulation of tannin around the hilum. This situation may be equivalent to seed coloration in soybean (*Glycine max*), where the absence of pigmentation is controlled by the dominant allele at the *I* locus, whereas the homozygous recessive *ii* produce a totally pigmented seed coat and where the alternate i^i allele confines pigmentation to the hilum (Tuteja et al. 2004).

Prakken (1970) also suggested that in common beans the color tones at the hilum are influenced by the various genes that control seed coat color. Furthermore, according to the study by Marles and Gruber (2004), 10 species of the *Brassicaceae* carry condensed tannins in the seed hilum, including species where no tannins were found in other regions of the seed coat. The hilum is known to be the point of attachment of the seed to the placental tissue of the ovary, but also the place where seed water uptake begins, a portion of the seed that would need to be protected by tannins which have roles as antifungal metabolites. Notably, white beans which lack any coloration usually have no tannins or very low tannin levels and are more susceptible to root rots and other diseases (de Mejía et al. 2003; Guzmán-Maldonado et al. 1996; Iniestra et al. 2001; Ma and Bliss 1978).

Tannin accumulation in bean is predicted to be influenced by alleles of seed pattern genes that determine pigment distribution such as *Z* and *Bip* as we have identified here. By extension tannin content might be controlled by *J* (Joker), which is the dominant gene for mature color development but also is involved in the expression of color in the hilum ring and by *T* which is involved in the expression of pattern restriction in partly colored seed coats (Bassett 2007). All these genes could control, at least partially, the increase and rate of formation of tannins, that is, they could act as regulator genes, thus affecting both the variation that is observed in seed coat color and the accumulation of the colorless tannins which were analyzed in this study.

Relationship of tannin QTL with the seed color-pattern complex locus [*R–C*]

Another finding based on the association of a seed pattern gene marker and tannin QTL that merits further discussion was the location of the QTL for soluble and total tannins in the G × G population that were found in the middle of linkage group B8 in a position that is syntenic with that of the *C* gene (Bassett 2007). The region containing this QTL in the G × G population was carefully aligned through comparative mapping with the more saturated genetic maps for the D × G and B × J populations (Blair et al. 2003; Freyre et al. 1998).

The *C* gene is known as the primary locus for seed coat pattern, has multiple allele and exists in a complex locus with the *R* gene for dominant red seed coat, hence by convention the genes are represented as [*R–C*] (Bassett 2007). While not necessarily the same set of genes a parallel situation exists in maize, where the *R* gene along with the *B* locus, both regulatory genes, determines the distribution and quantity of anthocyanins and resulting pigmentation of different plant parts (Holton and Cornish 1995). Locus R regulates the enzymes CHS, DFR, and 3GT, and locus B regulates enzymes DFR and 3GT, all enzymes in the flavonoid pathway.

This kind of relationship between classical loci and flavonoid pathway genes has been studied in systems like maize (*Zea mays*), snapdragon (*Anfirrhinum majus*), and petunia (*Petunia*). In snapdragon flowers, three anthocyanin regulatory genes *Delila* (*Del*), *Eluta*, and *Rosea* have been identified. The first two steps, CHS and CHI, show minimal regulation, but subsequent steps have an absolute requirement for the *Del* gene product and show quantitative regulation by *Eluta* and *Rosea* (Holton and Cornish 1995). In petunia, mutations at four loci, *An7*, *An2*, *An4*, and *An77*, have similar regulatory effects on transcription of at least six structural anthocyanin biosynthetic genes, including *DFR*, *ANS*, *An73*, *3RT*, anthocyanin methyltransferase (*AMT*), and *chsJ* (Quattrocchio et al. 1993; Huits et al. 1994).

For common bean, the genes for color pattern may also control structural genes that determine the synthesis of condensed tannins and other flavonoids. Thus, depending on the action of these genes, tannin production could be limited to the hilum region or diffused around the entire seed coat. The patterning of tannin deposition may be important as tannins have a protective role during germination of common bean seedlings in which the seed coat remains as a covering over the cotyledons until these emerge from the soil (Sicard et al. 2005).

Relationship of tannin QTL with the basal seed color gene *P*

Another region of the genome that was important for determining tannin concentration was located at the *P* locus on linkage group B7. The QTL was found for the B × J population but not for the other populations and was associated with the RAPD marker OU3, which was determined by Erdmann et al. (2002) to be linked to the *P* gene.

In this region we found two QTL accounting for more than 60% of variance for condensed tannin in this population. The binomial distribution of tannins for the lines of this population and the high variance of this single QTL at the *P* locus suggest it to be a major gene controlling the inheritance of tannin accumulation in this population. These results support suggestions by Ma and Bliss (1978) for common bean and Cabrera and Martin (1989) for faba bean that tannin content is sometimes controlled by major genes. These authors also found evidence of binomial distribution and in the case of common bean, dominance for low tannin content when analyzing populations derived from crosses between high and low tannin parents.

The *P* gene is considered the primary locus determining both white seed coat color and white flower color in common bean (Emerson 1909); although another locus, *V*, is also known to produce white flowers. Despite these interesting features of the *P* locus, the nature of this gene or the protein it encodes is unknown. Homozygous recessive *pp* genotype expresses white seed coat regardless of the genotype at any other gene in the complex genetic system controlling seed coat color (Bassett 2007) and Erdmann et al. (2002) proposed three possible roles for the locus *P* in color expression; (1) that *P* could encode a transcription factor that activates other genes; (2) that other seed color genes could encode proteins that interact with the *P* protein or locus to regulate the genes encoding specific enzymes of the flavonoid pathway; or (3) that the *P* protein could be involved in stabilizing a biosynthetic complex of enzymes for seed coloration. Although the QTL found to be closely linked to the *P* gene marker was very important in the B × J population, it is not possible to propose which of these explanations might be correct since many aspects of tannin production and accumulation are unknown. However, the QTL we discovered at the *P* locus may represent an alternate allele of the *P* gene itself or a linked gene. A precedent exists for multiple alleles at the *P* locus. In fact, in addition to the allele p^{gri} which encodes an off-white coloration in certain bean genotypes others alleles have been reported (Bassett 2007). Therefore, this QTL would be the second case of an alternate allele ascribed to the *P* locus which was first recognized as controlling white colored seed but not gradation of tannin or seed color accumulation.

Other QTL and overall pattern of inheritance

The QTL for insoluble tannins concentration on linkage group B6 for the G × G population and on B9 for the D × G population were the only QTL that were not associated with seed coat color although the first of these was associated with the *V* gene which controls flower color. All the remaining QTL were related to seed coat color or pattern genes, implying that other genes controlling seed coloration could also control tannin accumulation, a hypothesis we will be further testing through the evaluation of near isogenic lines for other seed coat color and pattern genes. The separate inheritance of insoluble and soluble tannins is another interesting hypothesis to test in additional mapping populations as it appears that of the seed coat color loci associated with tannins in the study, only the *P* locus so far has a pleiotropic effect on both soluble and insoluble tannin accumulation.

Another interesting observation was that all the QTL found were population specific which implies that the parental genotypes each have separate genes controlling tannin concentration and by extension that additional genes for tannins may exist in further genotypes. This would be consistent with the fact that in common bean, seed coat color is determined by the presence and quantity of flavonol glycosides, tannins, and anthocyanins which are created through a complex biochemical pathway that may involve many structural and regulatory genes (Beninger et al. 1998; Takeoka et al. 1997). Authors such as Cabrera and Martin (1989), working with faba bean, concluded that variation in tannin content is a reflection of various major genes with multiple alleles controlling pigmentation in different organs of the plant. Likewise, Beninger et al. (1998) suggested that the existence of genes controlling color may be pleiotropic for tannin content with possible epistatic interaction among them. The oligogenic inheritance observed in this study for condensed tannins in bean agreed with the results obtained by Guzmán-Maldonado et al. (2003). These authors reported four QTL for tannins, explaining 42% of phenotypic variation, however, the map used was unanchored and does not permit comparisons on QTL locations to be made with our study.

The quantitative distribution we observed in all three populations and most especially in D × G and G × G agreed with results from Beninger and Hosfield (2003), de Mejía et al. (2003), and Ma and Bliss (1978), who also indicated considerable variation in tannin content between bean genotypes. While our values for tannin concentration in seed coat may seem high, they were equivalent to whole seed tannin percentages of only 1% to 3.4% for the D × G population and 0% to 2.4% for B × J and G × G populations. Overall, these values for whole seed tannin concentration expressed as catechin equivalents (CE), where 1 CE is equivalent to 1 mg tannins per gram of seeds, fall in the range reported for bean seed tannin concentration of 9.6–45.4 CE or 0.96–4.54% by previous authors (de Mejía et al. 2003; Guzmán-Maldonado et al. 1996; Iniestra et al. 2001; Ma and Bliss 1978).

According to the classification of Ma and Bliss (1978), seeds with values of 6 CE per gram of seed are considered as being low in tannin content and those with 35 CE per gram as being high in tannin content. Hence, the overall

mean value for the D × G and G × G populations in our study corresponded to a medium to high content of condensed tannins while for the B × J population content was low to medium. In contrast, white beans are usually considered to have very low tannins (Welch et al. 2000). The variability for seed coat tannin content within the species, therefore, appears to be at least fivefold which makes common bean a good system in which to study this characteristic especially compared to model legumes or *Arabidopsis* for which seed coat color is not as diverse. Seed size differences are also greater in common bean which facilitates peeling of the seed coat giving more accurate tannin estimates than whole seed evaluation according to Guzmán-Maldonado et al. (1996).

In conclusion, we identified 12 QTLs not previously described for condensed tannins, several of which are of relatively large effect. Therefore, the differences between the number of QTL mapped among populations and their estimated effects may be due to segregation of meaningful genetic variation among natural lineages, making a multiple mapping population approach as performed here, optimal for characterizing the inheritance of tannin accumulation. Furthermore, our results will be important for the genetic improvement of biofortified beans that have either higher tannins for their health benefits or lower tannins to increase iron bioavailability. This latter goal is an important component of germplasm development programs within the Harvest Plus Challenge Program, that aims to combat micronutrient malnutrition through staple crop improvement.

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