

The Inheritance and Evolution of Leaf Pigmentation and Pubescence in Teosinte

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ABSTRACT

To investigate the genetic mechanisms that underlie morphological evolution in natural populations, we employed QTL mapping to dissect the inheritance of leaf sheath characters that distinguish Chalco from Balsas teosinte. Abundant macrohairs (trichomes) and intense anthocyanin accumulation are found in Chalco teosinte sheaths whereas Balsas teosinte leaf sheaths are green and glabrous. These character states may represent adaptations to the cooler highland (Chalco) *vs.* warmer middle-elevation (Balsas) climates. QTL mapping in multiple populations revealed a mix of major- and minor-effect QTL affecting both sheath color (anthocyanin) and macrohair abundance. The major QTL for macrohairs accounts for 52% of the parental difference. Epistatic interactions were detected between the major-effect QTL and multiple other QTL for both traits, accounting for substantial portions of phenotypic variance. Developmental analyses suggest that regulatory program changes underlie the phenotypic differences. Sheath anthocyanin QTL are clearly associated with *b1* and *a3*, both of which are regulators of anthocyanin biosynthesis. Our findings suggest that changes in a small number of QTL can lead to morphological evolution by modulating existing developmental programs.

A fundamental challenge for evolutionary biologists is to elucidate the genetic mechanisms that underlie phenotypic evolution in natural populations. One approach to addressing this issue is to employ quantitative trait locus (QTL) mapping to dissect the genetic basis of morphological differences between natural populations of cross-compatible taxa. Since QTL mapping can be used to estimate both the number of genetic loci that control trait differences and the relative magnitudes of their effects (MACKAY 2001), it is possible to establish whether morphological evolution has proceeded via a change in a single gene, via changes of large effect in a few genes, or via changes of small effect in numerous genes. Beyond characterizing the mode of inheritance for trait differences, QTL mapping also provides information about the modes of action for the individual QTL. Additive, dominance, and epistatic effects can all be estimated so that their relative contributions to the evolution of traits can be evaluated.

We have analyzed differences in leaf traits between two closely related subspecies of *Zea mays* that grow in separate regions of Mexico. Chalco teosinte (*Z. mays* ssp. *mexicana*) grows at high elevations in central Mexico

and has red, hairy leaf sheaths, while Balsas teosinte (*Z. mays* ssp. *parviglumis*) grows at middle elevations in southwestern Mexico and has green, glabrous leaf sheaths (Figure 1, a and b). The red and hairy sheath phenotypes are thought to be adaptations to the cooler climate where Chalco teosinte grows (DOEBLEY 1984; LAUTER 2001). Increased absorption of radiant energy due to dark pigmentation may significantly improve metabolism and translocation in thermophilic C4 plants growing in cool climates (GALINAT 1967). Improved heat retention would have a similar benefit, concordant with the view that plant hairs may create a boundary layer that reduces heat loss (DAUBENMIRE 1947; CARLQUIST 1974). The large trichomes (macrohairs) of *Zea* may be especially well suited for this, since they become indurated with silica at maturity, which gives them a high heat capacity (LAUTER 2001). Leaf sheath pubescence and pigmentation are also excellent characters to examine because the genetics of both trichome formation in Arabidopsis and anthocyanin biosynthesis in maize have been extensively studied (reviews by HOLTON and CORNISH 1995; SZYMANSKI *et al.* 2000).

Here we report the results of multiple QTL mapping analyses of the genetic basis for the sheath pubescence and pigmentation differences between Chalco and Balsas teosintes. In addition, the developmental underpinnings of these phenotypic differences are investigated. Consistent with some other recent studies of morphological traits in other natural systems, we found that the trait differences are inherited oligogenically and that epistatic interactions among QTL make significant

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contributions to the phenotypes. We show that both major and minor QTL for anthocyanin pigmentation map near known genes in the anthocyanin pathway and that the major QTL for macrohairs maps near the maize gene, *macrohairless1*. Results from developmental analyses suggest that the trait differences are regulatory in nature.

MATERIALS AND METHODS

Plant materials and breeding: Several populations were created for mapping leaf trait QTL. First, pollen from a Balsas × Chalco teosinte F₁ hybrid was used to pollinate the maize inbred A158 to generate a testcross (TC₁) population of 295 plants. The Balsas and Chalco teosinte parents of the F₁ were grown from seed collected near Teloloapán, Mexico (Iltis and Cochrane, collection 81), and Chalco, Mexico (Iltis *et al.*, collection 28622), respectively. Second, a Balsas × Chalco F₁ plant was self-pollinated to generate an F₂ population of 165 plants. The Balsas and Chalco teosinte parents of the F₁ were grown from seed collected near Teloloapán, Mexico (Iltis and Cochrane, collection 81), and Texcoco, Mexico (Iltis *et al.*, collection 28620), respectively. Third, we developed nearly isogenic lines (NILs) for four QTL by introgressing QTL-containing Chalco teosinte chromosome segments into maize inbred A158. This was accomplished by marker-assisted breeding with no attention paid to phenotype. For QTL on chromosomes 2, 5, and 10, fourth-generation backcross plants were selfed to create BC₄F₂ families (116 plants each) for QTL analysis. For the chromosome 9 QTL, a BC₃F₂ family (116 plants) was used. The TC₁, F₂, and chromosome 9 BC₃F₂ populations were grown in winter nurseries on Molokai, Hawaii. All three BC₄F₂ populations were grown in a summer nursery at the University of Wisconsin at Madison.

Sheath macrohair counting: Macrohair counts were obtained in the same way from the sheath of the second leaf from the top of every plant in our six experimental populations. Since Balsas teosinte sheaths have macrohairs along their edges, macrohairs were counted in an area of the sheath where only Chalco teosinte is hairy (centered between midrib and leaf margin, one-third of the way down the sheath from its junction with the blade). Before macrohairs were counted, this allometric location was measured out and marked with a small ink dot, upon which the microscope field of view was centered, thereby preventing field choice bias and reducing counting error due to field wandering. Only macrohairs whose bases were inside the microscope's ×50 field of view (14.8 mm²) were counted. To establish the mean parental difference for sheath pubescence, macrohairs were also counted in this way for 26 plants of each teosinte accession from which the F₂ population was derived.

Sheath color scoring: Two distinct color classes of plants existed in the TC₁ population, allowing plants to be scored in a Mendelian fashion according to whether they were red sheathed or green sheathed. Among red-sheathed plants, variation in both anthocyanin intensity and coverage was observed. Intensity varied from light to dark, while coverage varied according to how far up from the bottom of the sheath the pigment extended. To measure these subtleties, quantitative scores ranging from 1 to 4 were given to each of the 164 red-sheathed plants for both the intensity and the coverage traits. The scores were based collectively on the uppermost two sheaths of each plant and were replicated and averaged.

The F₂ population displayed a continuous range of pigment phenotypes that was wider and more complex than that of the TC₁ population. F₂ plants were given pigment intensity

scores on a seven-unit scale ranging from 0 to 6. The scores were based collectively on the uppermost two sheaths of each plant and were replicated and averaged. F₂ individuals scoring 0 were equivalent to the green-sheathed individuals in the TC₁ population. F₂ individuals scoring 1's and 2's were lighter than any of the red-sheathed TC₁ individuals, and those scoring 6's were darker than any TC₁ individuals. Thus, the four-unit pigment intensity scale for the red-sheathed TC₁ individuals falls in the range of 3–5 on the F₂ scale. Pigment coverage was not scored in the F₂ population. To establish the mean parental difference for anthocyanin intensity, 55 and 53 plants of the respective Chalco and Balsas teosinte accessions from which the F₂ population was derived were scored using the seven-unit scale.

Genetic markers: Molecular markers with full genome coverage in the TC₁ population were obtained by using the maize core RFLP marker set as described in LAUTER and DOEBLEY (2002). We also used probes for several candidate genes in the anthocyanin biosynthesis pathway (*b1*, *r1*, *pl1*, *c1*, *c2*, and *whp1*). The F₂ population was probed with a subset of 24 RFLP markers that covered areas of the genome where *bona fide* QTL and nearly significant LOD peaks for sheath macrohairs and anthocyanins mapped in the TC₁ study (Figure 2). Each of the four sets of linked markers used for making and analyzing the NILs contained at least three markers (Figure 2).

QTL mapping and statistical analyses: The genetic distances between markers were calculated using Mapmaker v2.0 (LANDER *et al.* 1987). QTL Cartographer v1.14 (BASTEN *et al.* 2000) was used for all QTL mapping procedures as described in LAUTER and DOEBLEY (2002). For each statistically significant QTL detected by composite interval mapping, the chromosomal location, magnitude, and direction of both additive and dominance effects and percentage of the phenotypic variance explained (PVE) are reported. A QTL was considered statistically significant only if its LOD score exceeded the corresponding experiment-wise significance threshold ($P = 0.05$) determined using 1000 permutations of the data (CHURCHILL and DOERGE 1994). Correlations, *t*-tests, and ANOVAs were performed using JMP v3.0 (SAS Institute).

Scanning electron microscopy: Since macrohairs tend to sustain damage during standard SEM specimen preparation, we used low-temperature, low-voltage SEM to obtain images of developing macrohairs (AHLSTRAND 1996). Fresh sheath tissue was affixed to aluminum stubs with carbon paint and cryofixed in liquid nitrogen. No metal coating was needed to obtain secondary electron images at up to ×1250 magnification (LAUTER 2001).

RESULTS

Parental difference for macrohair development: To facilitate interpretation of the effects and functions of the QTL, the parental difference in sheath macrohair abundance was quantified and its developmental basis was investigated. Both Chalco and Balsas teosinte plants produce macrohairs in the upper (adaxial) epidermis of the blade and at the edges of the outer (abaxial) epidermis of the sheath. Away from the edges, Balsas teosinte sheaths are completely glabrous while equivalent Chalco teosinte sheaths have 284 macrohairs/cm² (Figure 1, a and b; Table 1). Since Balsas teosinte plants are able to initiate and develop macrohairs in some parts of their leaves, we considered whether macrohairs never initiate or initiate but fail to develop on the medial portion of Balsas teosinte sheaths. To address this ques-

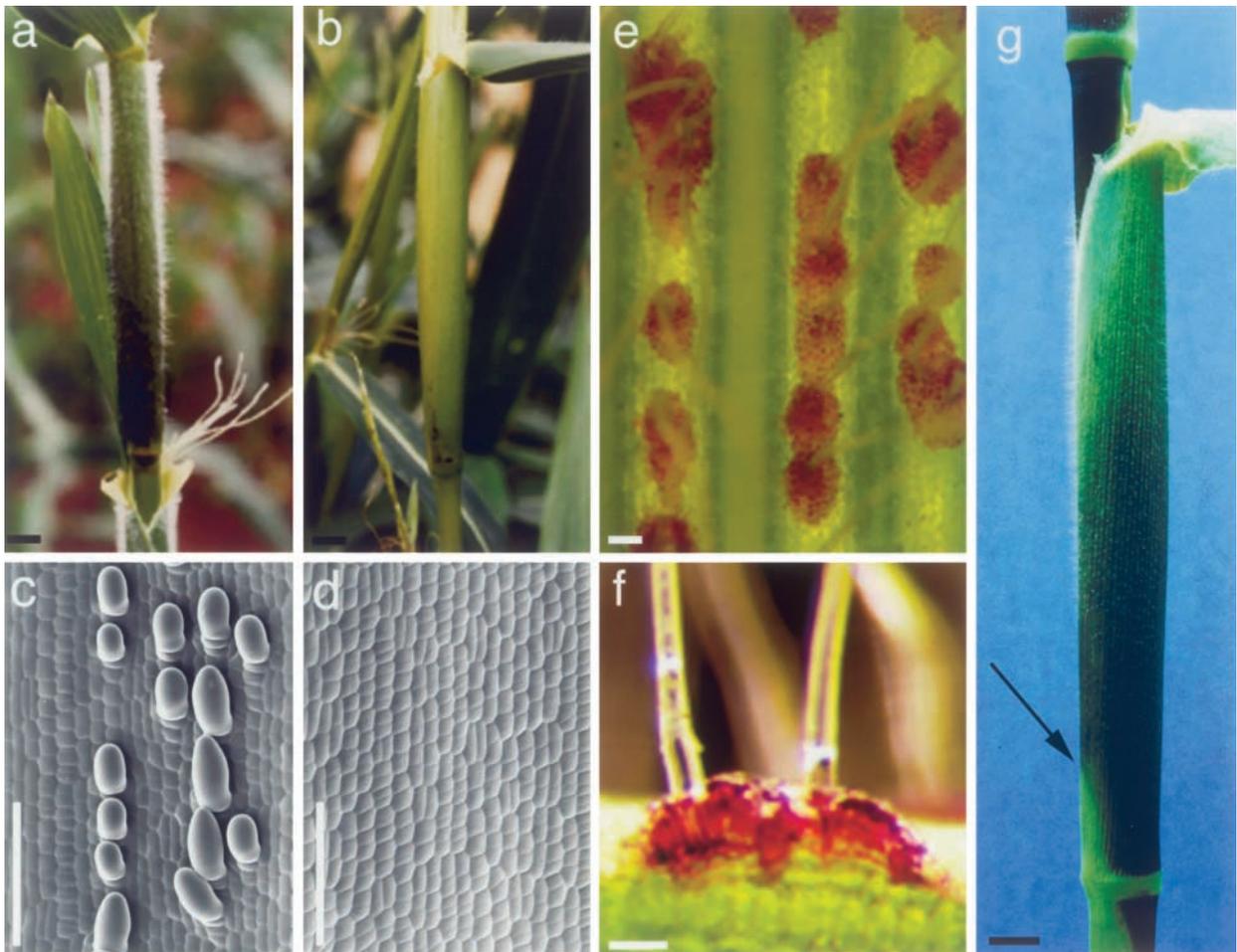


FIGURE 1.—Leaf sheath pubescence and pigmentation phenotypes of Chalco and Balsas teosintes. (a) The sheath of a mature Chalco teosinte plant with dense macrohairs and dark anthocyanin pigmentation. (b) The sheath of a mature Balsas teosinte plant with the glabrous and green phenotypes. Note that macrohairs are present on the adaxial surface of the leaf blade and that small patches of pigmented cells can be seen near the base of the sheath. (c) SEM of macrohair emergence on the ninth sheath of a 30-day-old Chalco teosinte plant. (d) SEM of a ninth sheath of a 30-day-old Balsas teosinte plant showing the characteristic absence of macrohair initials. (e) In Chalco teosinte plants as well as in progeny of their crosses with Balsas teosinte and maize, the subsidiary cells that form the macrohair pedestals are often pigmented with anthocyanin, despite the fact that surrounding epidermal cells remain green. (f) Macrohairs with a pigmented pedestal are shown from the side. (g) This mature Chalco teosinte plant shows a typical intensity and distribution of pigmentation in plants carrying *a3* and *B1* alleles. Multiple vertically oriented organs are pigmented, depending on genotype and exposure to light, as seen where a sheath has been removed (arrow). Black scale bars, 1 cm; white scale bars, 100 μ m.

tion, we used SEM to examine multiple developmental stages of the sheath epidermis using the eighth, ninth, and tenth leaves of 30-day-old Chalco and Balsas teosinte plants. We found that macrohair initials expand out of the epidermis on the central portion of Chalco teosinte sheaths just prior to the first signs of stomatal differentiation when the sheath is ~ 3 mm tall (Figure 1c). Balsas teosinte plants never produced any macrohair initials in this region of their sheaths in any of the developmental stages examined, including those surrounding the first signs of stomatal differentiation (Figure 1d).

Parental difference for sheath pigmentation: Similar to the macrohair case, Balsas teosinte plants are capable of making anthocyanins, but simply produce them in lower amounts (Table 1) and more restricted regions

of the plant (Figure 1). Whereas Chalco teosinte plants accumulate high levels of anthocyanins in nearly all vertically oriented surfaces of the plant, most Balsas teosinte plants are almost totally green (Figure 1, a, b, and g). Some Balsas teosinte plants do produce anthocyanins in their leaf sheaths, although the darkest of them are not as dark as the darkest Chalco teosinte plants. We repeatedly observed that Balsas teosinte plants had less total anthocyanin coverage than Chalco teosinte plants. This is in part attributable to the fact that Balsas teosinte plants do not produce anthocyanins on the uppermost portion of their sheaths, as most Chalco teosinte plants do.

Sheath macrohair QTL: The TC₁ study identified one major- and four minor-effect QTL for sheath macrohairs

TABLE 1
Average phenotypes of TC₁ progeny, F₂ progeny, and the parental accessions

Trait	TC ₁ progeny (<i>n</i> = 295)	F ₂ progeny (<i>n</i> = 164)	Chalco teosinte ^a	Balsas teosinte ^b	Maize A158 ^c
Macrohairs	11.54 ± 0.78	22.50 ± 1.22	41.65 ± 3.47	0.0 ± 0.00	0.0 ± 0.0
Pigment intensity	1.26 ± 0.07 ^d	2.79 ± 0.16	3.37 ± 0.16	1.51 ± 0.12	0.0 ± 0.0

Average phenotypes ±SE are reported as the number of macrohairs counted per 14.8 mm² at the same allometric place on each plant and quantitative score units for pigmentation on a scale from 0 to 6.

^a *n* = 26 for macrohair counts; *n* = 55 for anthocyanin scoring.

^b *n* = 26 for macrohair counts; *n* = 53 for anthocyanin scoring.

^c *n* = 20 for macrohair counts and for anthocyanin scoring.

^d For the purpose of comparison, the TC₁ scores were converted to the F₂ scale as described in MATERIALS AND METHODS.

(Table 2; Figure 2). A single Chalco allele at the major effect QTL on the long arm of chromosome 9 (9L QTL) adds an average of 18.5 macrohairs to the count area of TC₁ progeny carrying it, accounting for 46% of the PVE and approximately half of the mean difference between the parental taxa (Tables 1 and 2). In comparison, the Chalco alleles at the minor-effect QTL explain only small amounts of phenotypic variance. Their combined effect, however, is not insignificant; TC₁ individuals carrying Chalco alleles at the 9L, 5S (S, short arm), 10L and 2C (C, centromeric) QTL had 30 more macrohairs per count area than individuals carrying Balsas alleles at these loci. Thus, collectively the minor QTL contribute nearly as much to the phenotype as the major QTL on 9L.

The map positions and magnitudes of effect of the 9L, 2C, and 10L QTL for sheath macrohairs were reaffirmed by the F₂ study (Tables 2 and 3; Figure 2). The 5S and transgressive 4L QTL identified in the TC₁ study were not detected in the F₂, perhaps because different sets of parents were used in the crosses or sampling effects. Consistent with their detection as QTL in the TC₁ experi-

ment, the 9L, 2C, and 10L QTL showed additive to dominant modes of gene action in the F₂ (Table 3). The 9L QTL had the largest additive effect (11 macrohairs) in the F₂, with the first Chalco allele contributing 13 macrohairs to the count area of progeny and the second only 9, hence the dominance deviation of 2 macrohairs (Table 3). While the single-copy effect (*a* + *d*) of this QTL was lower in the F₂ as compared to the TC₁ (13 *vs.* 18.5), the single-copy effects of the Chalco alleles at the 2C and 10L QTL were correspondingly higher (Tables 2 and 3).

Sheath pigmentation QTL: The TC₁ population segregated into two discrete groups of 164 red- and 131 green-sheathed plants, which is not statistically different from a 1:1 expectation that a single-factor model would predict. Membership in red-sheathed and green-sheathed classes cosegregated perfectly with the respective Chalco and Balsas alleles of the *bl* gene, a transcriptional regulator of anthocyanin synthesis in vegetative tissues of *Zea* (CHANDLER *et al.* 1989). We infer that our Chalco parent carried a functional *Bl* allele and that our Balsas parent carried a recessive, nonfunctional (or weakly func-

TABLE 2
QTL detected in the testcross experiment

Trait	Chromosome	Flanking markers	LOD	<i>a</i>	Allele	PVE
Macrohairs	2C	<i>bl</i> <u>umc259b</u>	5.07	5.4	Chalco	3.9
	4L	umc126b <u>umc66a</u>	2.69	3.9	Balsas	2.0
	5S	umc107b <u>bnl4.36</u>	8.11	7.3	Chalco	7.2
	9L	<u>umc192</u> umc95	43.89	18.5	Chalco	46.0
	10L	umc64 <u>umc259a</u>	4.78	5.5	Chalco	4.0
Pigment coverage	2C	umc259b <u>umc255</u>	5.71	0.93	Chalco	12.6
	3L	<u>umc63</u> csu25a	10.05	0.88	Chalco	25.2
	10L	<u>bnl7.49</u> — ^a	3.20	0.52	Chalco	8.9
Pigment intensity	3L	<u>umc63</u> csu25a	4.4	0.61	Chalco	13.7
	4L	umc126b <u>umc66a</u>	6.3	0.60	Chalco	14.0

QTL positions are reported by chromosome with S and L indicating cytologically defined short and long arms and C denoting centromeric, as well as by the markers on each side of the LOD peak with the closer marker underlined. The peak LOD score, additive effect estimate listed as a trait unit contribution, parental source of the allele, and percentage of the phenotypic variance explained are listed for each QTL. The threshold LOD values for the three traits were 2.64, 2.62, and 2.53, respectively.

^a The LOD curve is still rising at the terminal marker (see Figure 2).

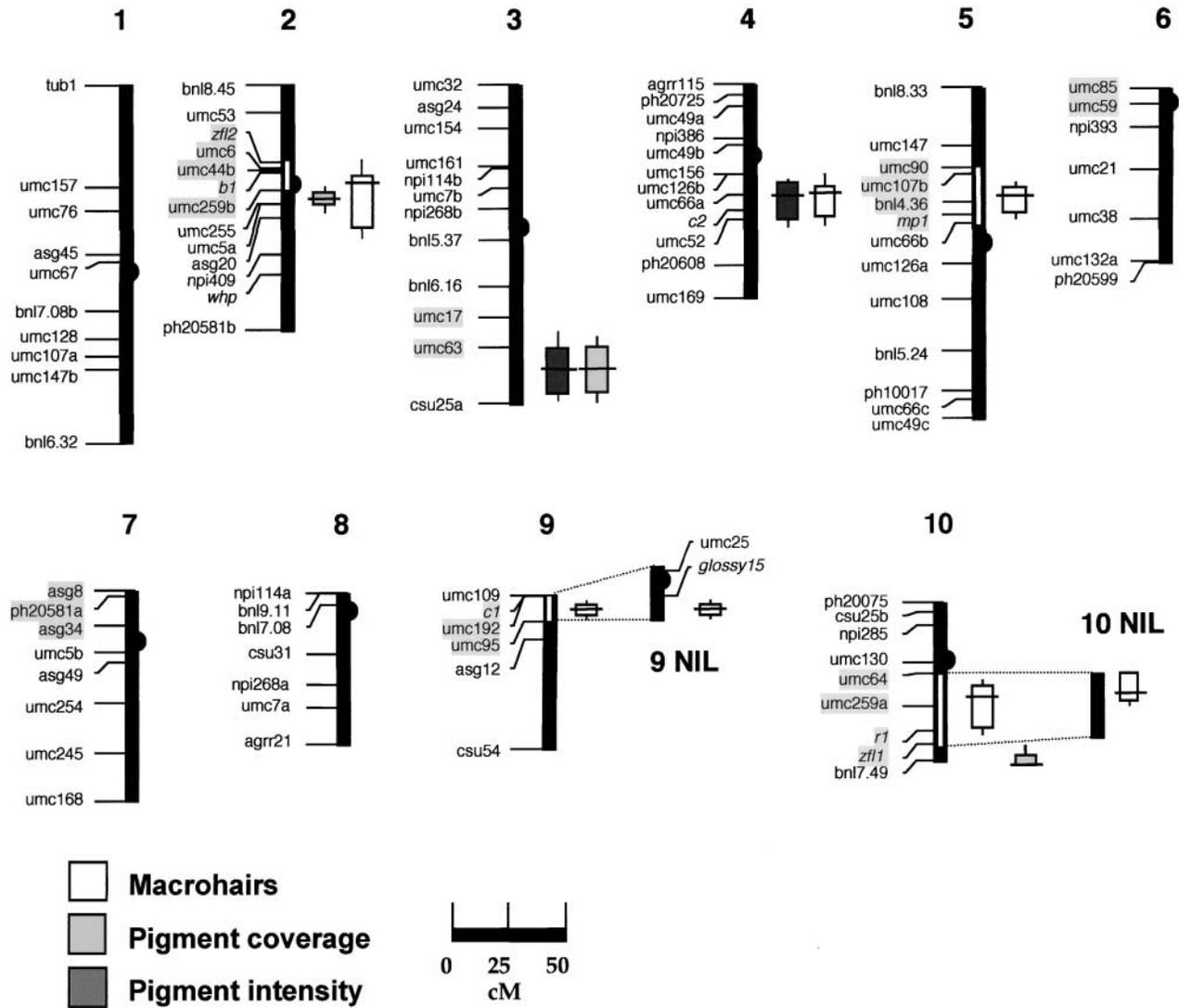


FIGURE 2.—Genetic linkage map of the 10 gametic maize-teosinte chromosomes of the TC₁ population, shown together with two chromosomal segments of NILs carrying macrohair QTL alleles that can exert effects in a maize genetic background. Estimates of positions for statistically significant QTL affecting macrohair density, pigment coverage, and pigment intensity on the leaf sheaths of TC₁ plants are shown as box and whisker plots. A box vertically delineates the region of a chromosome for which the LOD score of the QTL is within 1.0 LOD unit of its peak, the peak LOD score is indicated by a horizontal line through the box, and the whiskers of each box vertically delineate the region of a chromosome for which the LOD score is between 1.0 and 2.0 LOD units of its peak. The positions of RFLP markers are indicated by horizontal lines along the chromosomes. Each chromosome is depicted with its cytologically defined short arm on top. Bulges on the chromosomes indicate centromere position except on chromosome 9, where the entire short arm is collapsed in the TC₁ population but not in the NIL for the 9L QTL. Markers included in the F₂ study have their names highlighted in gray. The introgression segments of the NILs are shown as white lines along the TC₁ chromosomes. The NIL chromosome segments are drawn to the same centimorgan scale and have their terminal markers indicated by dotted lines.

tional) *b1* allele. To test for QTL that modify the sheath pigmentation phenotype in plants with *BI*, subtle variation for both anthocyanin intensity and coverage was scored and analyzed among the 164 carriers of the Chalco *BI* allele. Several QTL were detected for each trait (Table 2), indicating that the phenotypic variation seen in the TC₁ population is controlled by a single gene (*b1*) that acts to “switch on” anthocyanin production plus minor QTL or “modifiers.”

In the TC₁ study, we observed QTL for both anthocyanin intensity and coverage on chromosome 3L (Table

2; Figure 2). The LOD by position plots for the intensity and coverage QTL on chromosome 3L were highly congruent (data not shown), suggesting that the same QTL may affect both aspects of sheath pigmentation. Notably, this 3L QTL maps to the same position as *anthocyanin3* (*a3*), a regulatory locus known to affect anthocyanin biosynthesis (STYLES and COE 1986; ROBINETT *et al.* 1995). Moreover, RNase protection assays have shown that dominant *A3* alleles lower transcript levels of *b1* as well as those of enzymatic genes involved in anthocyanin biosynthesis (C. CAREY, W. VAN HEECKEREN, S. BELCHER, D. BROWN

TABLE 3
QTL detected in the F₂ experiment

Trait	Chromosome	Flanking markers	LOD	<i>a</i>	<i>d</i>	Gene action	Allele	PVE	PMSSDD
Macrohairs	2C	<i>b1</i> <u>umc259b</u>	2.61	5.8	2.2	DA	Chalco	6.0	27.8
	9L	<u>umc192</u> umc95	10.34	10.8	2.1	A	Chalco	23.0	51.8
	10L	umc64 <u>umc259a</u>	4.25	6.6	0.8	A	Chalco	8.6	31.6
Pigment intensity	2C	<u>b1</u> umc259b	10.70	0.88	-0.02	A	Chalco	17.9	94.6
	3L	umc63 — ^a	19.10	1.14	-0.39	RA	Chalco	32.8	122.6
	4L	<i>c2</i> — ^b	4.35	0.49	-0.16	A	Chalco	6.4	52.6

QTL positions are reported by chromosome with S and L indicating cytologically defined short and long arms and C denoting centromeric, as well as by the markers on each side of the LOD peak with the closer marker underlined. The peak LOD score under a hypothesis that allows both additive (*a*) and dominance (*d*) effects is given and those effect estimates are listed as trait unit contributions. Gene action is inferred from *d/a*: recessive (R) is for $d/a < -0.67$, recessive to additive (RA) for $-0.67 > d/a > -0.33$, additive (A) for $-0.33 > d/a > 0.33$, dominant to additive (DA) for $0.33 > d/a > 0.67$, and dominant (D) for $d/a > 0.67$. The parental source of the allele, percentage of the phenotypic variance explained, and the percentage mean subspecies difference (PMSSD) are also listed for each QTL. $PMSSD = 100 \times [2a/MSDD]$. The threshold LOD values for the two traits were 2.87 and 2.62, respectively.

^a The LOD curve is still rising at the terminal marker (see Figure 2).

^b No markers flanking *c2* were mapped (see Figure 2).

and V. CHANDLER, personal communication). Thus, we would expect an allelic difference at this candidate locus to affect both aspects of sheath pigmentation pleiotropically.

The F₂ study reaffirmed the presence of all three pigmentation intensity QTL that were observed in the TC₁ study on chromosomes 2C (*b1*), 3L, and 4L (Tables 2 and 3). Nevertheless, the inheritance of pigmentation was not identical in the two populations. In the F₂, the 3L QTL had the strongest additive effect and highest PVE (33%), while the *b1* gene had a much smaller effect and a PVE value of only 18%. By contrast, *b1* had the strongest additive effect in the TC₁, behaving like a Mendelian locus, while the 3L QTL had only a modest effect that we observed among plants carrying *B1* (Table 2). The 4L QTL had a similarly small effect in both the F₂ and the TC₁. Thus, although *b1* behaved like a Mendelian locus or switch in the TC₁, it behaved like an intermediate effect QTL in the F₂.

A QTL complementation test for *a3*: Our chromosome 3L QTL maps near the *a3* locus, which acts as either a recessive intensifier or a dominant inhibitor of anthocyanin production (STYLES and COE 1986; ROBINETT *et al.* 1995). The coincident map positions suggest that *a3* and our 3L QTL may be allelic. We used an *a3* tester stock to investigate this hypothesis. This test revealed that Balsas teosinte × tester stock and A158 × tester stock F₁'s were all green sheathed, indicating that Balsas teosinte and the A158 inbred line carry the dominant *A3* allele, while Chalco teosinte × tester stock F₁'s were all red sheathed, indicating that Chalco teosinte carries the recessive *a3* allele (LAUTER 2001). These data offer a simple explanation for why the 3L QTL behaved so differently in the TC₁ vs. the F₂ population. In the TC₁, the dominant *A3* allele of the A158 inbred largely obscures the difference in effect between the Chalco

and Balsas alleles such that the 3L QTL has only a small effect. However, the effects of this QTL are fully discernible in the F₂ since both homozygous classes are present. In fact, the Chalco allele of the 3L pigmentation QTL has a strong and negative dominance deviation in the F₂ population, meaning that it behaves recessively relative to the Balsas allele (Table 3). This is consistent with our inference that the *a3* locus is allelic to this QTL.

Epistatic interactions among QTL: We used two-way ANOVAs to test for interaction effects between the major (9L) and minor (2C and 10L) macrohair QTL from the TC₁ study using the genotypes from markers closest to the three QTL. We observed interaction effects of five and six macrohairs for the 2C × 9L and 10L × 9L combinations, respectively ($P = 0.01$, $P = 0.03$). Since the 2C and 10L QTL have similar synergistic interactions with the 9L QTL and map between paralogous marker pairs in duplicated segments of the *Zea* genome (Table 2; Figure 2; HELENTJARIS *et al.* 1988), it is quite possible that a pair of duplicate genes may underlie these QTL. Therefore, a full factorial three-way ANOVA for macrohairs with three QTL was performed. Analyzed in this way, the 9L × 2C and 9L × 10L interaction effects were both about eight macrohairs (Table 4), hinting that the 2C and 10L QTL may have overlapping functions. The three-way ANOVA also shows that the epistatic effects are nearly equivalent to the additive effect of the 9L QTL among individuals that do not carry Chalco alleles at either of these partner loci (Table 4). These interaction effects are quite strong in the context of the mean difference between parental taxa (Table 1).

Epistatic interactions for macrohair QTL were also suggested by the F₂ study. The difference between zero and two copies of the Chalco allele at the 2C QTL for macrohairs has a negligible effect among individuals carrying zero copies of the Chalco teosinte allele for the

TABLE 4
Macrohair QTL interact synergistically in the TC₁ population

Genotype at QTL			Observed phenotype \pm SE	Expected phenotype based on additive QTL interactions	Nonadditive interaction effect	ANOVA effect test <i>P</i> -value
10L	2C	9L				
B	B	B	1.2 \pm 1.7			
C	B	B	3.9 \pm 1.6			<0.001
B	C	B	3.5 \pm 1.7			<0.001
C	C	B	5.5 \pm 1.4	6.2	-0.7	0.171
B	B	C	10.9 \pm 1.8			<0.001
C	B	C	21.9 \pm 1.9	13.6	+8.3	0.017
B	C	C	21.7 \pm 1.6	13.2	+8.5	0.014
C	C	C	26.8 \pm 1.7	15.9	+11.9	0.267

Full factorial three-way ANOVA was used to tease apart the Chalco teosinte allelic effects. The genotypes are from the nearest flanking markers to the QTL, with B and C representing the Balsas teosinte/maize and Chalco teosinte/maize TC₁ genotypes, respectively. The observed phenotypes for the eight genotypic classes are ANOVA least-squares means of the number of macrohairs per count area. The expected phenotypes are computed by adding the single locus contributions to the baseline number of hairs. For example, the 10L + 9L expectation is $(3.9 - 1.2) + (10.9 - 1.2) + 1.2 = 13.6$ macrohairs. A positive number from (observed - expected) indicates a synergistic or more-than-additive interaction.

9L QTL (t -test $P = 0.48$), but accounts for a difference of 15 macrohairs among individuals carrying two copies of the Chalco allele at the 9L QTL (t -test $P = 0.03$). Interestingly, this apparent case of epistasis was not statistically significant in a two-way ANOVA, likely due to the small numbers of F₂ plants in the most informative marker-genotype classes.

Epistatic interactions for sheath anthocyanin QTL were also suggested by the F₂ study. Among plants carrying two Balsas alleles at the 3L QTL and thereby two *A3* alleles, the phenotypes of individuals carrying zero, one, and two copies of the Chalco *B1* allele are statistically indistinguishable (Table 5). In contrast, the genotype at the *b1* locus has a significant effect when even one *a3* allele from the Chalco parent is present, demonstrating an interaction effect (Table 5). Again, this apparent case of epistasis was not statistically significant in a two-way ANOVA, likely due to the small numbers of F₂ plants in the most informative marker-genotype classes.

Macrohair QTL in NILs: Chalco teosinte alleles of the sheath macrohair QTL on chromosomes 2, 5, 9,

and 10 were introgressed into maize A158 genetic background so that their independent effects could be assessed and their positions more finely mapped. Both the 9L and 10L QTL were able to evince statistically detectable phenotypic effects in A158 maize genetic background (Table 6; Figure 2). The positions of the LOD peaks were consistent with the TC₁ and F₂ studies; however, the use of additional RFLP markers (*umc25* and *glossy15*) in the 9L NIL improved the localization of the 9L QTL (Tables 2, 3, and 6; Figure 2). Interestingly, both the 9L and 10L QTL act in a more recessive manner in the NIL compared to the F₂ (Tables 3 and 6). The fact that no effect was detected in either the 2C or the 5S NIL may result from an inability to exert phenotypic effects without other Chalco teosinte QTL alleles or from a failure of the introgressed segment to encompass the QTL (Figure 2).

Correlation of pigmentation and pubescence: Despite the existence of TC₁ and F₂ plants with glabrous, pigmented sheaths as well as hairy, unpigmented sheaths, two lines of evidence suggest that sheath pubescence and pigmentation may not be entirely independently

TABLE 5
The 3L QTL affects pigmentation response to *b1* genotype in the F₂ experiment

3L QTL genotype	<i>b1/b1</i>	<i>B1/b1</i>	<i>B1/B1</i>	<i>P</i> -value
CC (<i>a3/a3</i>)	2.79 \pm 0.32	4.31 \pm 0.25	4.95 \pm 0.30	<0.001
CB (<i>a3/A3</i>)	1.76 \pm 0.34	2.38 \pm 0.21	3.53 \pm 0.23	<0.001
BB (<i>A3/A3</i>)	1.41 \pm 0.37	1.98 \pm 0.27	2.08 \pm 0.37	0.393

Chromosome 3L genotypes at markers flanking the QTL are listed with C for Chalco and B for Balsas teosinte alleles. Within each of the three genotypic classes for the 3L QTL, a one-way ANOVA of pigment intensity by *b1* genotype was performed to see whether or not the background makes a difference. ANOVA means with standard errors are reported in trait units.

TABLE 6
Macrohair QTL detected in the NIL experiments

NIL	Flanking markers	LOD	<i>a</i>	<i>d</i>	Gene action	PVE	PMSSD
9L	<u><i>glossy15</i></u> <u>umc95</u>	6.51	19.4	-11.7	RA	49.7	93.2
10L	umc64 <u>umc259a</u>	5.23	18.2	-14.5	R	27.0	87.4

The QTL position for each NIL is reported by the markers that flank the LOD peak, with the closer marker underlined. The peak LOD score under a hypothesis that allows both additive (*a*) and dominance (*d*) effects is given and those effect estimates are listed as trait unit contributions. Gene action is inferred from *d/a*: recessive (R) is for $d/a < -0.67$, recessive to additive (RA) for $-0.67 > d/a > -0.33$, additive (A) for $-0.33 > d/a > 0.33$, dominant to additive (DA) for $0.33 > d/a > 0.67$, and dominant (D) for $d/a > 0.67$. The parental source of the allele, percentage of the phenotypic variance explained, and the percentage mean subspecies difference (PMSSD) are also listed for each QTL. $PMSSD = 100 \times [2a/MSSD]$. The threshold LOD values for the two studies were 1.99 and 1.90, respectively.

regulated. First, anthocyanin accumulation in the subsidiary cells of macrohairs was commonly seen in otherwise unpigmented upper portions of TC₁, F₂, and Chalco teosinte sheaths (Figure 1, e–g). Since subsidiary cells are likely recruited by each macrohair via a non-cell-autonomous mechanism (LAUTER 2001), the unique presence of anthocyanins in these cells is suggestive of an overlap between the regulation of anthocyanin biosynthesis and macrohair formation. Second, sheath pubescence in the TC₁ population was significantly, although weakly, correlated with anthocyanin coverage ($r = 0.16$, $P = 0.006$), but was not correlated with pigment intensity ($r = -0.08$, $P = 0.313$), despite the fact that the two anthocyanin traits were well correlated ($r = 0.47$, $P < 0.001$). If the basis for the correlation between macrohairs and pigment coverage in the TC₁ study were genetic, one might expect to detect QTL that pleiotropically affect these traits without affecting anthocyanin intensity. Consistent with this possibility, the LOD peak of the minor effect QTL on 2C that affects pigment coverage, but not pigment intensity, is contained within the broad LOD peak of the sheath macrohair QTL on 2C (Table 3; Figure 2).

DISCUSSION

Previous studies in multiple plant species have shown an association of both pigmentation and hairiness with cool temperatures (DAUBENMIRE 1947; GALINAT 1967; CARLQUIST 1974). Similarly, we note that Chalco teosinte plants of the relatively cool Mexican highlands have increased pigmentation and pubescence on vertically oriented organs as compared to their lowland Balsas teosinte counterparts. We suggest that these character states arose in Chalco teosinte because they improve metabolism and translocation by helping the plants absorb and retain radiant energy, particularly at dawn and dusk when the sun is low in the sky (see also GALINAT 1967; DOEBLEY 1984). Testing this hypothesis would be facilitated by the identification of the genes and molecular polymorphisms that control the pheno-

types. Once these are identified, one could construct transgenic lines that differ for the relevant alleles and assay their fitness in warm *vs.* cool environments. A similar approach has proven informative for pollinator attraction QTL in *Mimulus* (SCHEMSKE and BRADSHAW 1999). Our identification of the major QTL for these traits is the first step toward this goal.

Inheritance of quantitative trait differences: Our results suggest that genetic control of the difference between the red and hairy sheaths of Chalco teosinte and green and glabrous sheaths of Balsas teosinte involves a relatively small number of genes, a few of which have large phenotypic effects. These results as well as those of some other investigators who have used QTL mapping to study morphologically divergent cross-compatible taxa cannot be reconciled with a strict interpretation of the infinitesimal model for quantitative trait evolution (FISHER 1930). Oligogenic inheritance including some QTL of large effect has been inferred in multiple crosses between crop plants and their wild relatives (DOEBLEY and STEC 1993; CAMARGO and OSBORN 1996; GRANDILLO and TANKSLEY 1996), as well as in crosses between natural species pairs (VLOT *et al.* 1992; BRADSHAW *et al.* 1995; HOMBERGEN and BACHMANN 1995; GALING *et al.* 1999), although more polygenic inheritance has also been observed in other studies (BURKE *et al.* 2002; FISHMAN *et al.* 2002; WESTERBERGH and DOEBLEY 2002).

Although purely recessive QTL would have gone undetected in our genome-wide TC₁ study, it is unlikely that our results have substantially underestimated the number of QTL while simultaneously overestimating their effect magnitudes, as has been shown to be a potential bias of standard interval mapping (BEAVIS 1994). Our TC₁ study used composite interval mapping and analyzed contributions of dominance and epistasis. In addition, the large effects of the major QTL detected for each trait were also seen in subsequent experimental populations (Tables 2, 3, and 6).

Epistatic contributions to morphological differences: The synergistic epistatic interactions among TC₁ QTL for sheath pubescence were substantial in the context

of the mean difference between parental taxa, demonstrating how epistasis could make a significant contribution to morphological evolution (Tables 1, 4, and 5). Together with other QTL studies of morphological differences that revealed epistatic interactions among QTL (DOEBLEY and STEC 1993; BRADSHAW *et al.* 1998; GAILING *et al.* 1999), our results are congruent with models that attribute a prominent role to epistasis in the control of quantitative traits (LYNCH and WALSH 1998).

We observed more and stronger epistatic interactions in the TC₁ than in the F₂ population. This difference may partly reflect the statistical limitations associated with having fewer individuals spread over more genotypic classes in the F₂ population. However, epistatic interactions typically seem to be more prominent when examined in isogenic and foreign genetic backgrounds (DOEBLEY *et al.* 1995; LONG *et al.* 1995; ESHED and ZAMIR 1996; LUKENS and DOEBLEY 1999; KIM and RIESEBERG 2001). An implication of this observation is that epistasis may more strongly affect traits for which fewer genetic differences are segregating. Consistent with this inference, epistasis has not been detected in studies of heterosis, a trait that has many contributing genetic factors (STUBER *et al.* 1992; XIAO *et al.* 1995).

It has also been suggested that the role of epistasis is reduced as the genes in question become less directly linked to the phenotype, such that epistasis is easier to detect for traits with fewer developmental pathways feeding into them (CLARK and WANG 1997). This would account for the difference in the levels of epistasis seen between our studies of anthocyanins and epidermal hairs and studies of more complex traits like plant height and yield (EDWARDS *et al.* 1992; STUBER 1994).

Dominance and the creation of novel phenotypes: Taken together, the F₂ and NIL populations offer an opportunity to compare dominance effects of the Chalco alleles in maize-teosinte *vs.* maize genetic backgrounds. The 9L and 10L QTL were detected in the TC₁ population, suggesting that the Chalco alleles have additive or dominant modes of gene action (Table 2). The F₂ results affirmed this hypothesis, showing large additive effects and a positive dominance deviation for both of these QTL (Table 3). Nevertheless, the NIL experiments indicated that Chalco alleles at both of these QTL have more recessive modes of action in maize genetic background as indicated by negative dominance deviations (Table 6).

Genetic background has been shown to affect the degree of dominance of QTL in other maize-teosinte experiments. DOEBLEY *et al.* (1995) demonstrated for two QTL affecting plant and inflorescence architecture that the teosinte alleles behaved more dominantly in teosinte genetic background and more recessively in maize genetic background. QTL \times QTL interactions such as those detected in our TC₁ experiment represent a mechanism that could explain such a shift in mode of gene action. When the epistatic and additive compo-

nents are separated either statistically by ANOVA (Table 4) or genetically by using NILs (Table 6), the single-copy effect of the Chalco 9L QTL allele is equivalent in the TC₁ and NIL studies and is considerably smaller than its single-copy effect in the TC₁ *per se* (Table 2), demonstrating how the presence of QTL alleles at other loci alters the degree of dominance for the 9L QTL allele.

An evolutionary implication of this phenomenon is that novel alleles may arise with comparatively subtle or cryptic phenotypic effects that can escape removal by purifying selection, giving them a chance to acquire much stronger effects when combined with partner alleles at other loci. Thus, synergistic epistatic interactions may be important for generating novel phenotypes using alleles that already exist within a population, alleviating the need for the perfectly timed appearance of new alleles that significantly alter phenotypes on their own (LAUTER and DOEBLEY 2002).

Candidate genes and QTL functions: Our two largest-effect QTL for sheath pigmentation appear to represent allelic differences at *b1* (2C) and *a3* (3L), both regulators of anthocyanin biosynthesis in maize (STYLES and COE 1986; CHANDLER *et al.* 1989; ROBINETT *et al.* 1995). We associate *b1* with the 2C QTL since they cosegregate and since *b1* is known to control sheath pigmentation in maize (RADICELLA *et al.* 1992). We associate *a3* with the 3L QTL on the basis of our complementation tests. Since both Balsas and Chalco teosinte are capable of producing anthocyanin, the allelic differences at *b1* are likely *cis*-regulatory differences for the spatial distribution of pigment rather than differences in protein function. We make this inference since *b1* is known to harbor sheath-specific *cis*-regulatory variants that confer distinct spatial patterns of anthocyanin expression (RADICELLA *et al.* 1992; SELINGER and CHANDLER 1999). Because *a3* has not yet been cloned and characterized, there is no adequate foundation from which to predict the nature of the functional difference between the Balsas and Chalco alleles.

One minor-effect QTL (4L) for sheath pigmentation in teosinte may represent allelic differences at *colorless2* (*c2*), which encodes an enzymatic step (chalcone synthase) in anthocyanin biosynthesis (WIENAND *et al.* 1986). We make this inference since our QTL maps near *c2* (Figure 2). Since the 4L QTL affects pigment intensity but not coverage (spatial distribution), an allelic difference at this enzymatic gene might involve either protein function or concentration, rather than its spatial expression pattern.

For macrohair QTL, we associate our major QTL on 9L with the maize mutant *macrohairless1* (*mh1*; MOOSE *et al.* 2004). We make this inference principally because *mh1* is located within the narrowest interval to which the 9L QTL was localized (Table 6). However, the nature of the phenotypic differences controlled by this QTL and a knowledge of trichome development in Arabidopsis

enable us to make a more explicit prediction about the underlying gene. Specifically, both maize *mh1* and Arabidopsis *GLABROUS1* (*GLI*) mutants have initiation-specific defects (variants) that block medial laminar, but not marginal laminar trichome formation (HERMAN and MARKS 1989; MOOSE *et al.* 2004). Similarly, our 9L QTL affects medial but not marginal trichome formation. Thus, it seems plausible that the 9L QTL is a maize homolog of *GLI*.

Knowledge of the molecular genetic basis of trichome development in Arabidopsis may also help explain the epistatic interactions among QTL that we observed. Trichome initiation in Arabidopsis is regulated by MYB (*GLI*), bHLH (*GL3*), and WD40 (*TTG*) repeat containing proteins (OPPENHEIMER *et al.* 1991; WALKER *et al.* 1999; PAYNE *et al.* 2000). *GL1* and *GL3* proteins form a MYB-bHLH complex that requires a WD40 repeat protein to confer wild-type trichome densities (PAYNE *et al.* 2000). The dependence of high trichome density on the formation of a multimeric protein complex could form the basis for synergistic genetic interactions among loci that promote trichome initiation, similar to what was seen between sheath macrohair QTL. Given their dominant modes of action in teosinte genetic background and their capabilities to interact more than additively, it would not be surprising if the 9L, 2C, and 10L QTL encoded macrohair initiation regulators homologous to Arabidopsis trichome regulatory genes.

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