The Maize *macrohairless1* Locus Specifically Promotes Leaf Blade Macrohair Initiation and Responds to Factors Regulating Leaf Identity

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ABSTRACT

The leaf surfaces of almost all plant species possess specialized epidermal cell types that form hairs or trichomes. Maize leaves produce three distinct types of hairs, the most prominent being the macrohairs that serve as a marker for adult leaf identity and may contribute to insect resistance. This report describes the maize *macrohairless1* (*mhl1*) locus, which promotes macrohair initiation specifically in the leaf blade. Each of seven recessive *mhl1* mutant alleles significantly reduces or eliminates macrohairs in the leaf blade. The *mhl1* mutations block macrohair initiation rather than interfering with macrohair morphogenesis. Genetic mapping placed *mhl1* within bin 4 on chromosome 9. A second independently segregating locus was found to partially suppress the *mhl1* mutant phenotype in certain genetic backgrounds. Macrohair density was observed to increase during early adult vegetative development and then progressively decline, suggesting macrohair initiation frequency is affected by factors that act throughout shoot development. Genetic analyses demonstrated that *mhl1* acts in the same pathway but downstream of factors that either promote or repress adult leaf identity. Thus, *mhl1* plays a key role in integrating developmental programs that regulate leaf identity during shoot development with those that specify macrohair initiation within the leaf blade.

HAIRS or trichomes are present on the leaf surfaces of nearly all plant species. The density, morphology, and chemical composition of leaf hairs vary widely and these factors contribute to their diverse physiological functions. Leaf hairs may act as physical and chemical deterrents to insect feeding, may provide hydrorepellency and reflective properties to the leaf, and in some xeromorphic species may limit water loss due to transpiration (Esau 1977). Leaf hairs form through the specialized differentiation of epidermal cells and may be unicellular or be composed of many cells. In many plant species, multiple different types of leaf hairs whose production varies during shoot development are observed, with more than one type often present within the same leaf.

Three different types of hairs are found on maize leaves. Macrohairs, prickle hairs, and bicellular microhairs are produced in patterned files of cells within the adaxial leaf epidermis, beginning with the fifth or sixth leaf (Figure 1). The prominent macrohairs (MHs) thus serve as a readily visible marker for adult leaf identity in vegetative development (Poethig 1990) as well as for dorsoventral polarity within the leaf (Nelson *et al.* 2002). Although the function of maize MHs remains unclear, they have been reported to influence oviposition by the insect pests *Heliothis zea* (Widstrom *et al.* 1979) and *Chilo partellus* (Durbey and Sarup 1982).

Glabrous mutants in which leaf hairs or trichomes are greatly reduced or absent have been identified in many plant species. Molecular analyses of such mutants in Arabidopsis have revealed much about the genes that regulate trichome patterning, initiation, and morphogenesis (reviewed in Szymanski *et al.* 2000). However, insights from studies of the molecular mechanisms that regulate trichome development in Arabidopsis have not yet been extended to other plant species. Glabrous mutant varieties have been identified in many cereal crop species, including rice (Foster and Rutger 1978), wheat (Leisle 1974), barley (Sato and Takeda 1992), oats (Sarkarung and Collins 1977), pearl millet (Kumar and Andrews 1993), sugarcane (Jagathesan 1977), and sorghum (Gibson and Maiti 1983). In each of these species, leaf hairs have been implicated as important factors for insect resistance. Surprisingly, similar mutations have not previously been described in maize, the best developed system among the cereals for the molecular genetic analysis of leaf development.

We report here the identification and characterization of mutations that define the maize *macrohairless1* (*mhl1*) locus, which specifically promotes MH initiation in the leaf blade. Mutant alleles of *mhl1* greatly reduce or eliminate MH initiation in the leaf blade without affecting other aspects of leaf or plant development. We mapped *mhl1* to the long arm of maize chromosome 9 and show that the *mhl1* phenotype can be partially suppressed by an independently segregating modifier

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locus in certain genetic backgrounds. *Mhl1* is the first mutation that specifically affects a marker for adult leaf identity in maize and we demonstrate that it functions in the same pathway and downstream of factors that either promote an adult leaf identity (such as gibberellic acids) or repress adult leaf identity (*glossy15*). The *mhl1* mutation thus defines a gene that integrates the regulation of leaf identity during shoot development with the initiation of MH differentiation within the leaf blade.

MATERIALS AND METHODS

Genetic stocks: The *mhl1-R* allele was discovered as a spontaneous mutation in the inbred line K55 obtained from Paul Sisco, which was confirmed in a second K55 accession (Ames 22754) obtained from the North Central Regional Plant Introduction Station (Ames, Iowa). The A632, 4Co63, NC89, and W64A inbred lines were also obtained from Paul Sisco and used in crosses with K55 to generate the F_2 and BC_1 populations from which the phenotypic segregation ratios that are reported in Tables 1 and 2 were obtained. The *wx1 gl15-L,mhl1-R* or *wx1,gl15-m1,mhl1-R* triple-mutant stocks were generated by crossing K55 to either a *wx1,gl15-L* stock initially obtained from the Maize Genetics Cooperation Stock Center or a *wx1,gl15-m1* stock (Moose and Sisco 1994). Mutant *wx1* kernels were selected from F_2 ears, and seedlings that produced glossy leaves without MHs beginning at leaf 3 were self-pollinated.

Four additional *mhl1* alleles (*mhl1-411*, *mhl1-330*, *mhl1-561*, and *mhl1-249*) were recovered from a targeted *Mutator* transposon mutagenesis experiment where *Mutator*-active plants were crossed as males onto *wx1,gl15-L,mhl1-R* females. A total of 47,000 F_1 progeny were screened for the macrohairless phenotype, 22,000 in a 1998 summer nursery and an additional 25,000 in a 2002 summer nursery. Putative *Mutator*-induced *mhl1* alleles resulting in a macrohairless phenotype in combination with the *mhl1-R* allele were propagated by selfing and outcrossing as males to both the W64A inbred and the *wx1,gl15-L,mhl1-R* parental lines. The heritability of these alleles and their allelism with *mhl1-R* was confirmed in the progeny of these crosses.

Six mutant lines with reduced leaf macrohair density were recovered from a screen for leaf epidermal differentiation defects in an M_2 population of EMS-mutagenized plants and were generously provided to us by Laurie Smith (University of California, San Diego). Hence these alleles are designated as *mhl*-LS(number)*. These mutations were tested for allelism with *mhl1* by crossing to both the K55 and the *wx1,gl15-L,mhl1-R* stocks.

The *mhl1-R*, *mhl1-330*, and *mhl1-411* alleles were each backcrossed at least three times into the maize inbred line W64A. Resultant backcross plants were selfed to generate segregating populations that were phenotyped to generate the data in Table 3. For *mhl1-R*, phenotypic selection for the intermediate MH density and macrohairless phenotypes was conducted by selfing heterozygous plants and visually scoring MH phenotypes segregating in the resulting progeny.

Stocks for the *dwarf1* mutation and those harboring the TB-9Sb, TB-9Sd, or TB-9Lc B-A translocations were obtained from the Maize Genetics Cooperation Stock Center. A *dwarfl; gl15-m1* double-mutant stock was generated by crossing *dwarf1* to a *gl15-m1* stock (Moose and Sisco 1994) and selfing double-mutant F_2 progeny with short stature and adult leaf epidermal traits beginning at leaf 3.

Phenotypic analyses: Glue slide leaf impressions were made as suggested by L. Smith (University of California, San Diego) and described in Nelson *et al.* (2002) from odd-numbered leaf blades beginning with leaf three until formation of the tassel. Since proximodistal gradients for MH density occur within leaves, data are reported from glue slides prepared from the same allometric position on all leaves. Impressions were made equidistant from the margin and midrib 2 in. distal to the ligule on the adaxial blade. MH density was counted from at least two different 1.13-cm^2 fields of each glue impression using a Zeiss Stemi-2000C dissecting microscope at ×40 magnification. The number of bulliform cell files across the width of the field of view was also counted to normalize for potential differences in MH initiation due to the relative density of these cell files. However, the number of bulliform cell files per unit leaf width was not observed to vary significantly among leaves of the genotypes examined in this study. Means and standard errors of the mean were calculated for MH density values from each leaf and phenotypic class.

***mhl1* genetic mapping:** *TB-A translocation tests:* K55 and *wx,gl15-L,mhl1-R* stocks were crossed as females by plants carrying TB-9Lc (breakpoint between centromere and *gl15*), TB-9Sd (breakpoint between the centromere and *wx1*), and TB-9Sb (breakpoint distal to *wx1*). All translocation-bearing plants used in crosses were verified to contain the translocation both by the morphological effects associated with hypoploidy (narrow leaves, semisterility in pollen) and by crossing plants to tester stocks containing markers distal to the translocation breakpoints (TB-9Lc, *virescent1* or *gl15*; TB-9Sb, *colorless1*; and TB-9Sd, *wx1*). F_1 progeny from crosses of *mhl1-R* by translocation stocks were scored for the macrohairless phenotype, which was observed only in the crosses with TB-9Lc.

Three-point linkage test: A *wx1,gl15-L,mhl1-R* plant was crossed to the inbred Gaspé Flint, and F_1 plants were backcrossed to the *wx1,gl15-L,mhl1-R* parental line. A total of 181 progeny were scored visually for *waxy1* kernel, *gl15* seedling (loss of juvenile wax), and macrohairless phenotypes.

Molecular marker mapping: Two populations, one segregating for *mhl1-411* in a W64A background and F_2 progeny from the NC89 × K55 cross, were examined for linkage between a macrohairless leaf phenotype and molecular markers for *gl15*, *umc1120*, and *umc95* on the long arm of chromosome 9.

Double-mutant analyses: Families simultaneously segregating for *gl15* and *mhl1* were generated by crossing K55, which carries the *mhl1-R* allele, to the *wx1,gl15-L* stock introgressed into a W64A inbred background (Moose and Sisco 1994). The *gl15* phenotype was scored as the visual loss of juvenile epicuticular wax at leaf 3. The *mhl1* phenotype was scored visually as the absence or significant reduction in MH density on the abaxial leaf surface of fully adult leaves. Glue slide leaf impressions, MH counts, and data analysis were conducted as described above.

Families segregating for *dwarf1* and *mhl1-R* in a *gl15* mutant background were produced by first crossing the *d1; gl15-m1* stock to the *wx1,gl15-m1,mhl1-R* stock. F_1 plants were then backcrossed to the *wx1,gl15-m1,mhl1* stock. Eight backcross progeny were self-pollinated and one family that segregated for *d1* and *mhl1* in a *gl15-m1* background was used for phenotypic analyses. Macrohair frequencies were estimated from glue slide impressions as described above.

RESULTS

Identification and characterization of *macrohairless1*: A macrohairless leaf phenotype was observed in the maize inbred line K55, grown in the greenhouse during the fall of 1994 in Raleigh, North Carolina. The macro-

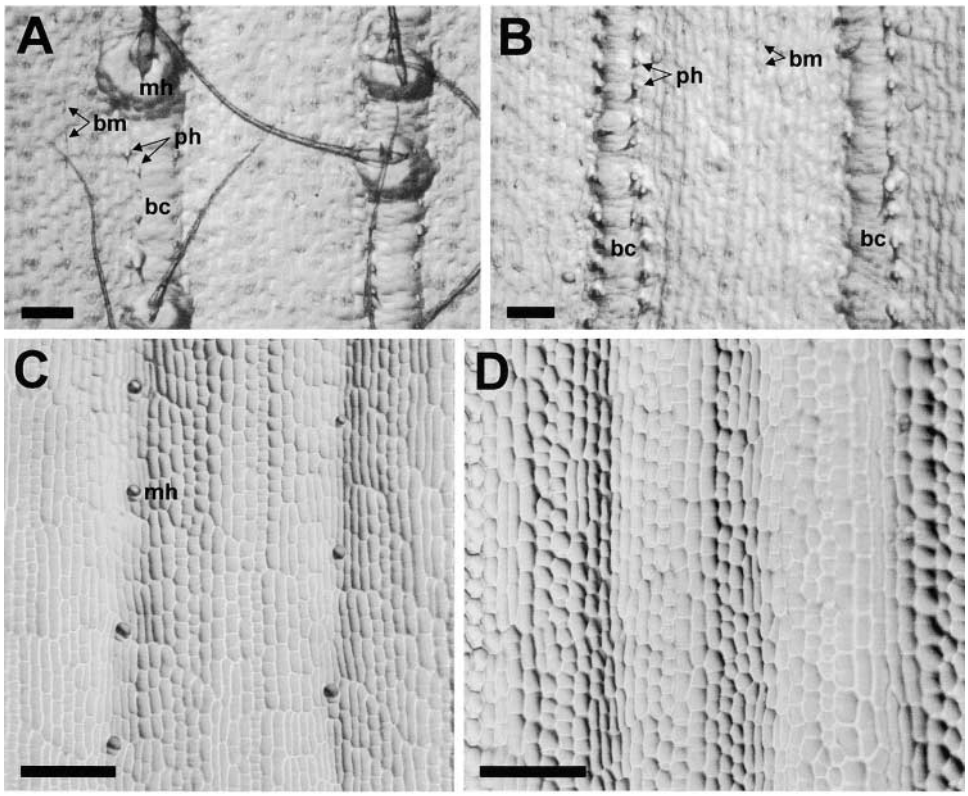

FIGURE 1.—The *macrohairless1* mutant phenotype. Micrographs from normal *Mhl1* (A and C) and *mhl1-R* mutant (B and D) plants are shown. (A and B) Micrographs of glue slide impressions prepared from adaxial surface of mature leaf 9. (C and D) Micrographs of glue slide impressions prepared from the basal 0.5–1.0 cm of leaf 9 early in its development. The region where macrohairs initiate is shown. mh, macrohair; ph, prickle hair; bm, bicellular microhair; bc, bulliform cell files. Bars, 100 μm.

hairless phenotype is environmentally stable, as K55 plants grown in summer nurseries at four locations with different growing conditions (Clayton, North Carolina; San Diego; Mystic, Connecticut; and Urbana, Illinois) have shown the same phenotype. The K55 inbred line was initially released in 1942 and is derived from the open-pollinated Kansas variety "Pride of Saline" (GERDES *et al.* 1993). It is unlikely that the macrohairless phenotype arose recently in our K55 stock, as leaves from plants of a second K55 seed source obtained from the North Central Regional Plant Introduction Station were also macrohairless.

Macrohairs are produced only on adult leaves (POETHIG 1990), raising the possibility that the macrohairless phenotype in K55 was due to a prolonged expression of juvenile leaf identity. However, like most other maize inbred lines, K55 produced juvenile leaf waxes only through leaf 8, demonstrating that the transition from juvenile to adult leaf identity occurred normally in K55, but that adult leaves lacked MHs. Consistent with this conclusion, all K55 leaves beyond leaf 7 possessed each of the other cellular characteristics associated with normal adult leaf identity, including presence of bulliform cells, prickle hairs, and bicellular microhairs, as well as intercostal cells that possessed highly invaginated cell walls (Figure 1, A and B) and stained aqua with toluidine blue (data not shown).

The *macrohairless1* mutation affects MHs only on leaf blades. The macrohairs on the margins and collar of the leaf sheath and the variably sized macrohair-like hairs present on the auricles and at the very edges of the leaf blade, both of which are present in most maize inbred lines, were not affected in K55 macrohairless leaves (data not shown). Further, the *macrohairless1* mutation has no discernible effects on root hairs, which are affected by mutations in some plant hair initiation genes from Arabidopsis (data not shown; WALKER *et al.* 1999; SCHELLMANN *et al.* 2002).

K55 did occasionally produce a few MHs, most often near the leaf tip or margins. The normal morphology of these MHs suggested that the macrohairless phenotype was more likely to be a defect in MH initiation rather than in morphogenesis. This idea is supported by the observation that the group of differentiated cells that normally proliferate at the base of MHs, the pedestal cells (PAYNE 1978), is also absent in K55 leaves, indicating a defect in the entire program of MH differentiation. Confirmation that the macrohairless phenotype is due to a block in MH initiation was obtained by examining the developing leaf blade epidermis at the stage where MH initiation occurs (the basal 0.5–1 cm). Macrohair initiation is the first observable specialized cell type to differentiate in the epidermis of the adult leaf blade and begins as an outgrowth of a single cell perpendicular to the sheet of otherwise undifferentiated epidermal cells (Figure 1C). These cellular outgrowths fail to occur in macrohairless leaves, demonstrating that the macrohairless phenotype is due to a block prior to or at the earliest observable sign of MH initiation (Figure 1D).

TABLE 1

Inheritance of the *mhl1* phenotype

Cross[a]	Total	Normal	Macrohairless	Best-fit ratio	Chi square
(A632 × K55) F_2	227	169	58	3:1	0.04[b]
K55 × (A632 × K55)	190	84	106	1:1	2.55[b]
(4Co63 × K55) F_2	245	185	60	3:1	0.03[b]
K55 × (4Co63 × K55)	279	142	137	1:1	0.09[b]
(NC89 × K55) F_2	536	401	135	3:1	0.01[b]

Plants with <10 MH/cm^2 were considered macrohairless.

[a] The parents and type of segregating progeny (F_2 or backcross) are indicated. K55 is homozygous for the *mhl1-R* allele.

[b] Failed to reject the hypothesized best-fit ratio using $\alpha = 0.05$.

The inheritance of the macrohairless phenotype was examined by crossing K55 plants to each of three inbred lines with normal MHs. The phenotypes of the F_1 progeny from each cross indicated recessive inheritance, because all plants exhibited a normal frequency and distribution of MHs beginning with leaves 5 or 6. F_1 plants were self-pollinated and also backcrossed as males to the K55 parent. The resulting progeny segregated into two distinct classes of plants, those with a normal MH density and macrohairless plants (Table 1). The proportions of the normal and macrohairless classes in both the F_2 and the backcross progenies closely followed those expected if the macrohairless phenotype was conditioned by a single recessive locus, which we have named *macrohairless1* (*mhl1*). Because the *mhl1* allele present in K55 is the first to be described at this locus, we have named this allele *mhl1-reference*, abbreviated as *mhl1-R*.

The NC89 inbred was chosen for crosses to *mhl1-R* because it possesses a relatively high density of leaf MHs, particularly on the medial area of the leaf sheath just below the ligule. This "hairy sheath" phenotype is rare among maize inbred lines and is not present in K55, although it is often observed in the wild relative of maize, teosinte (Lauter 2001). A larger number of F_2 progeny were scored from the NC89 × K55 cross to assess the role of *mhl1-R* in promoting medial leaf sheath MHs. We observed that *mhl1-R* was fully penetrant for the leaf blade MH phenotype (Table 1). The hairy sheath phenotype showed a quantitative mode of inheritance, although a few F_2 individuals that lacked sheath MHs were observed. No clear association was observed between the macrohairless leaf blade phenotype and sheath MH density. Some plants with macrohairless leaf blades still expressed the hairy sheath phenotype, while others exhibited very few MHs on the medial portion of their sheaths.

A dominant suppressor of *mhl1*: Crosses of K55 (homozygous for *mhl1-R*) to the inbred line W64A produced three phenotypic classes in the F_2 progeny: plants with normal macrohair density, completely macrohairless plants, and plants with intermediate MH density (Figure 2; Table 2). Similarly, backcrosses of W64A × K55 F_1 plants to K55 also produced plants with a normal density of MHs, no MHs, or an intermediate density of MHs. Combining the intermediate and no MH phenotypic classes represented ~25% of the F_2 progeny and 50% of the backcross progeny, suggesting that *mhl1* continued to be inherited as a simple recessive factor in these crosses but was partially suppressed by a modifier locus inherited from the W64A inbred parent. Comparisons of the proportions of macrohairless to intermediate MH density plants in both F_2 and backcross progenies suggested that W64A is homozygous for a dominant factor, which we designate in this study as *Suppressor of macrohairless1* (*Smh1*), that segregates independently from *mhl1* and partially suppresses the macrohairless phenotype. In this scenario, F_2 progeny would be expected to segregate into three phenotypic (genotypic) classes with the following proportions: 12/16 normal (*Mhl1/−; Smh1/−*), 3/16 intermediate MH density (*mhl1/mhl1; Smh1/−*), and 1/16 macrohairless (*mhl1/mhl1; smh1/smh1*). Similarly, progeny of F_1 plants (presumed *Mhl1/mhl1; Smh1/smh1* genotype) backcrossed to K55 (*mhl1/mhl1; smh1/smh1*) would be expected to segregate 1/2 normal (*Mhl1/−; Smh1/−*), 1/4 intermediate MH density (*mhl1/mhl1; Smh1/−*), and 1/4 macrohairless (*mhl1/mhl1; smh1/smh1*). The observed proportions of these phenotypic classes closely fit those expected for the hypothesis that W64A is homozygous for *Smh1*, which is unlinked to *mhl1* and partially suppresses the *mhl1* phenotype. Chi-square tests for

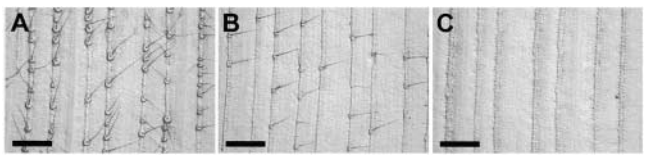

Figure 2.—Variation in the *mhl1-R* phenotype. Macrohair phenotypes on blades of leaf 11 in (A) W64A, (B) a plant with the intermediate macrohair density phenotype associated with *mhl1-R* and *Smh1* alleles, and (C) a plant with the completely macrohairless phenotype associated with *mhl1-R* and *smh1* alleles are shown. Bars, 1.0 mm.

TABLE 2

Segregation of the *suppressor of macrohairless* locus identified in W64A

Cross	Total	Normal	Intermediate	Macrohairless	Best-fit ratio	Chi square
(W64A × K55) F_2	291	217	60	14	12:3:1	1.85[a]
(W64A × K55) × K55	139	63	32	43	2:1:1	2.78[a]
Intermediate plant selfed	164	0	126	38	3:1	0.31[a]
Intermediate plant × K55	405	0	204	201	1:1	0.02[a]
Macrohairless plant selfed	237	0	4	233	0:1	NA
Macrohairless plant × K55	55	0	4	51	0:1	NA

The parents and type of cross are indicated. K55 is homozygous for *mhl1-R*. Normal, MH density >50 MHs/cm^2; intermediate, MH density from 10 to 49 MHs/cm^2; macrohairless, density <10 MHs/cm^2; NA, not applicable.

[a] Failed to reject the hypothesized best-fit ratio using $\alpha = 0.05$.

goodness of fit could not reject this hypothesis at the 0.05 significance level for either F_2 or backcross data (Table 2).

Further support for the above hypothesis was obtained by examining the segregation of MH phenotypes among progeny from crosses involving plants that exhibited either an intermediate MH density or a macrohairless phenotype, which were initially derived from the backcross of W64A × K55 F_1 plants to K55 (Table 2). When plants with an intermediate MH density were selfed, they segregated ~75% intermediate and 25% macrohairless plants. Backcrossing these intermediate plants again to K55 resulted in equal segregation for intermediate and macrohairless phenotypes. When macrohairless plants were similarly selfed and backcrossed, they produced all completely macrohairless progeny. These ratios are consistent with the intermediate parent plants having an *mhl1/mhl1; Smh1/smh1* genotype and the macrohairless parent plants having an *mhl1/mhl1; smh1/smh1* genotype. Chi-square tests for goodness of fit could not reject this hypothesis at the 0.05 significance level (Table 2).

Genetic mapping of *mhl1*: Among 979 F_2 individuals from crosses between K55 and a stock homozygous for mutant alleles at the linked *gl15* and *waxy1* (*wx1*) loci, only 1 plant was observed to exhibit glossy leaves beginning with leaf 3 and no MHs on any leaf blades, the phenotype expected for *gl15, mhl1* double mutants. Repulsion phase linkage, rather than epistasis, appeared to be the most likely explanation because ~25% (239/979) of the F_2 progeny exhibited a macrohairless phenotype and many more macrohairless plants were observed from nonwaxy (203) compared to *wx1* mutant seeds (36). The *mhl1* locus appeared to be more closely associated with *gl15* compared to *wx1* in these crosses, indicating that *mhl1* is nearer to *gl15* on the long arm of chromosome 9 than to *wx1* on the short arm. To further test this hypothesis, K55 was crossed to different B-A translocation stocks with breakpoints on chromosome arms 9S and 9L. The *mhl1* phenotype was observed only among hemizygous plants from crosses of K55 to TB-9Lc, whose breakpoint is located between the centromere and *gl15*. Attempts to map the *smh1* locus using B-A translocation stocks were unsuccessful, perhaps due to the presence of other modifiers of the macrohairless phenotype in the genetic backgrounds harboring the B-A translocations.

The single *wx1,gl15-L,mhl1-R* plant recovered from the F_2 progeny of crosses between *wx1,gl15-L* and *mhl1-R* was selfed and the resulting triple-mutant stock was used to perform a three-point linkage test. The segregation ratios of the different phenotypic classes among 181 testcross progeny indicated that *mhl1* mapped ~16 cM distal to *wx1* (29 recombinants) and 5 cM distal to *gl15* (10 recombinants). No double crossovers involving *gl15* were observed between *wx1* and *mhl1*. Nineteen recombinants were observed between *wx1* and *gl15*, which is consistent with the 10-cM distance previously reported for these loci (Howell *et al.* 1991). A more precise map location and markers distal to *mhl1* were determined by molecular marker mapping in 28 plants with a macrohairless phenotype from the NC89 × K55 F_2 population. These efforts produced the following map order with genetic distances in Haldane centimorgans: *glossy15* [restriction fragment length polymorphism (RFLP)]—6.7 cM—*mhl1*—14.7 cM—*umc1120* (simple sequence repeat)—6.3 cM—*umc95* (RFLP).

Macrohair density varies during both normal and *mhl1* mutant shoot development: One feature of the intermediate MH density phenotype was that it was often difficult to macroscopically distinguish from normal plants in early adult leaves (leaves 7–9), but became readily apparent in later adult leaves. Closer examination of MH densities in W64A and *mhl1* mutant plants provided an explanation. Macrohairs were first observed at leaf 7 and increased in density until leaf 9, after which MH density progressively declined until leaf 13 (Table 3). Macrohair densities in the *mhl1* mutant plants exhibiting either the macrohairless or the intermediate MH density phenotype showed the same pattern of variation during shoot development as W64A plants, although the density of MHs was reduced in all leaves of the

TABLE 3

Leaf blade macrohair density varies during shoot development

Genotype	Leaf 5	Leaf 7	Leaf 9	Leaf 11	Leaf 13	Leaf 15
W64A inbred (15)	None	80.7 ± 4.8	125.9 ± 11.9	94.2 ± 24.1	47.8 ± 3.1	48.4 ± 6.0
mhl1-R/mhl1-R; *Smh/−* (16)	None	39.7 ± 11.3	49.2 ± 8.1	26.9 ± 8.8	3.2 ± 1.4	10.2 ± 3.2
mhl1-R/mhl1-R; *smh/smh* (13)	None	6.1 ± 4.7	8.6 ± 3.7	6.6 ± 5.1	None	None
mhl1-330/mhl1-330 (10)	None	None	None	None	None	None
mhl1-411/mhl1-411 (10)	None	None	None	None	None	None

Each of the *mhl1* mutant alleles was introgressed into the W64A background, which carries the *Smh1* allele. At least three generations of backcrossing were followed by selfing and phenotypic examination of segregating progeny. Values are given as mean ±SE for the number of macrohairs per square centimeter of leaf area, sampled allometrically at the base of each leaf. The number of plants observed for each genotype is in parentheses. None, macroscopic observation found no macrohairs to be present on the leaf blade.

mutants relative to W64A (Table 3). As suggested by macroscopic observations, the differences in MH densities between plants with the intermediate MH density phenotype and wild-type plants were greater after leaf 9 (Figure 2).

The data in Table 3 demonstrate node-to-node variation in macrohair density during shoot development. This same trend is evident in tip-to-base comparisons within single leaf blades (data not shown). For example, MH density within leaf 8 is lower at the tip than at the base. Since the base of a leaf develops later than its tip, the early adult trend toward greater pubescence is manifested within leaf 8. Likewise, MH density is higher at the tip of leaf 12 than at its base, illustrating the late adult tendency toward decreasing hair density. These examples demonstrate the importance of allometric sampling when comparing MH densities. This is particularly important in transition and early adult maize leaves because they have the longest blades, such that the tip of leaf 10 often forms earlier in a plant's life than the base of leaf 8 does (Lauter 2001).

Additional macrohairless mutants were generated by transposon and EMS mutagenesis: The presence of a few MHs on leaf blades of K55 plants suggested that perhaps the *mhl1-R* allele was weakly functional. The spontaneous nature of *mhl1-R* also did not immediately suggest a strategy to molecularly clone the *mhl1* gene. Thus, we generated four additional *mhl1* alleles in a *Mutator* transposon tagging experiment. To date, only two of these putative *Mutator*-induced *mhl1* alleles (*mhl1-330* and *mhl1-411*) have been sufficiently introgressed into the W64A background to permit comparisons with *mhl1-R*. The *mhl1-330* and *mhl1-411* mutant phenotypes are very similar to those previously described for *mhl1-R*, but all leaves are essentially bald throughout shoot development (Table 3). The more severe macrohairless phenotype observed in the *mhl1-330* and *mhl1-411* mutants compared to *mhl1-R* suggests that *mhl1-330* and *mhl1-411* could be null alleles, whereas *mhl1-R* may be a weak allele. This idea is supported by the observation that after introgression of *mhl1-330* or *mhl1-411* into the W64A inbred background, which is homozygous for *Smh1*, intermediate MH density phenotypes are not observed. Thus, *Smh1* appears capable of suppressing the *mhl1-R* allele, but not *mhl1-330* or *mhl1-411*.

Six lines with macrohairless or reduced leaf macrohair density phenotypes were recovered from screens of an EMS-mutagenized population and were kindly provided to us by Laurie Smith. The *mhl*-LS4* and *mhl*-LS14* lines failed to complement *mhl1-R* in allelism tests and their macrohairless phenotypes were linked to *gl15* (data not shown), indicating that they represent additional *mhl1* mutant alleles hereafter designated *mhl1-LS4* and *mhl1-LS8*. Among F_2's derived from crosses of the *mhl-LS4* and *mhl-LS14* lines to W64A, plants that were homozygous for either the *mhl1-LS4* or the *mhl1-LS14* alleles exhibited totally macrohairless leaf phenotype, suggesting that they may both be null *mhl1* alleles. Each of the other four EMS mutant lines (*mhl*-LS8, mhl*-LS9, mhl*-LS10*, and *mhl*-LS11*) showed weak, partially penetrant phenotypes on their own, characterized by near normal macrohair densities in early adult leaves followed by intermediate MH density phenotypes in later leaves. Tests for allelism between these mutants and *mhl1* suggest that these mutants are not allelic to *mhl1* (data not shown). The phenotypic variability of the *mhl*-LS8, mhl*-LS9, mhl*-LS10*, and *mhl*-LS11* mutants may be due in part to genetic background effects; thus these mutations are being introgressed into multiple inbred lines to clarify their phenotypic effects and modes of inheritance.

***mhl1* is epistatic to *glossy15*:** The *gl15* gene encodes a putative transcription factor that both promotes the expression of juvenile leaf epidermal traits (*e.g.*, waxes) and represses adult leaf epidermal cell differentiation, including MHs (Moose and Sisco 1996). Macrohairs are produced precociously within *gl15* mutant shoots, beginning at leaf 2 or 3 instead of leaf 5 or 6 (Evans *et al.* 1994; Moose and Sisco 1994). To investigate whether *mhl1* is required for macrohair production in leaves 3–6 of *gl15* mutant seedlings, MH production was observed in families segregating for *gl15* and *mhl1* (Table 4).

MH density in nonmutant plants shows a pattern simi-

TABLE 4

MH density among a family segregating for *mhl1-R* and *gl15*

Phenotypic class	Leaf 3	Leaf 5	Leaf 7	Leaf 9	Leaf 11	Leaf 13	Leaf 15
Gl15, Mhl1 (12)	None	None	75.3 ± 5.4	139.3 ± 13.4	121.4 ± 27.2	51.3 ± 3.5	41.7 ± 6.8
Gl15, mhl1 (12)	None	None	31.7 ± 8.0	43.4 ± 5.8	20.7 ± 6.2	4.1 ± 1.0	7.9 ± 2.3
gl15, Mhl1 (12)	9.0 ± 19.1	103.2 ± 17.0	136.5 ± 19.6	142.7 ± 18.2	119.1 ± 24.6	94.5 ± 8.6	65.3 ± 4.3
gl15, mhl1 (19)	None	4.5 ± 1.1	8.5 ± 1.2	34.2 ± 3.5	21.2 ± 4.0	2.8 ± 7.4	None

Values are given as mean ±SE for the number of MHs per square centimeter of leaf area, sampled allometrically at the base of each leaf. The number of plants in each phenotypic class is in parentheses. Gl15, normal wax on juvenile leaves; gl15, glossy juvenile leaves; Mhl1, plants with normal MH density; mhl1, plants with greatly reduced MH density; none, macroscopic observation found no macrohairs to be present on the leaf blade.

lar to that previously observed, where MH production begins at leaf 7, peaks at leaf 9, and declines until leaf 13. Macrohair production begins at leaf 3 in *gl15* single mutants, progressively increases until peaking at leaf 9, and then declines in later adult leaves (Table 4). As expected, MH production was increased only in leaves 3–7 of *gl15* mutants relative to nonmutant plants. The *gl15-L* parental stock used to generate this segregating family had been introgressed into W64A and thus had an *Smh1/Smh1* genotype, explaining why the *mhl1* single mutants in this family exhibited MH frequencies throughout shoot development that are typical for intermediate MH density plants (Tables 2 and 4). Plants homozygous for both *gl15-L* and *mhl1-R* did not produce MHs in leaves 3 or 4 as in *gl15* single-mutant siblings. MHs were observed beginning with leaf 5 and continuing through leaf 13, but their frequency was significantly reduced in *gl15; mhl1* double mutants relative to *gl15* single mutants and was instead similar to *mhl1* single-mutant siblings (Table 4).

Results similar to those reported here were also observed either when *mhl1-R* was combined with the *gl15-m1:dSpm* allele or when *mhl1-411* was combined with *gl15-L*, except that the *mhl1-411; gl15-L* double mutants did not produce any MHs (data not shown). The *gl15; mhl1* double-mutant phenotype demonstrates that *mhl1* is required for the early onset of MH production in *gl15* mutants, which indicates that *mhl1* acts in the same pathway as *gl15* and that juvenile leaf identity is determined in part through the repression of *mhl1* by *gl15*.

The gibberellin class of plant growth regulators promote macrohair initiation and act through *mhl1*: The gibberellin class of plant growth regulators (GAs) promote adult leaf identity and, therefore, MH production, in maize (Evans and Poethig 1995). Trichome production in Arabidopsis leaves has also been shown to be sensitive to GAs (Chien and Sussex 1996; Telfer *et al.* 1997). Although GAs clearly influence the production of MH within the context of vegetative phase change, their role in promoting MH initiation within adult leaves has not been directly investigated. To determine if GAs act as positive regulators of MH initiation in maize, we measured MH frequencies in *dwarf1* (*d1*) mutant plants, which synthesize greatly reduced amounts of bioactive GAs (Spray *et al.* 1996). We also investigated the potential requirement for *mhl1* in the response of MH initiation to GA reduction by examining *d1; mhl1* double mutants. Both of the above investigations were carried out in a family segregating for *mhl1* and *d1* in a *gl15* mutant background (Table 5), to separate potential effects of GA on MH initiation from the known role of GAs in promoting vegetative phase change.

Throughout shoot development, MH frequencies in *gl15* single mutants and *gl15, mhl1* double mutants were similar to those previously observed (Table 4), with MH production beginning as expected at leaf 3 but being significantly reduced when *mhl1* is also present. The *d1; gl15* double mutants showed the typical short stature, delayed flowering, and reproductive phenotypes expected for *d1*, as well as glossy leaves beginning with leaf 3. However, MHs were not observed in leaves 3 or 4, and their frequency was significantly reduced in leaves 5–9 relative to *gl15* single mutants (Table 5). The differences in MH frequency between *gl15* and *d1; gl15* plants became progressively less with successive leaves. By leaf 11 and continuing through leaf 15 (the last leaf where comparisons could be made) MH frequencies for *d1; gl15* plants were similar to *gl15* single-mutant siblings. These observations suggest that GAs do have a positive effect on MH initiation, with this effect being mainly limited to early adult leaves. Even in early adult leaves, GAs are not required for MH initiation because MHs are produced in both *d1* and *d1; gl15* plants. As observed previously, MH production was essentially abolished in *gl15, mhl1* double mutants (Table 4). MH production was also nearly eliminated in *d1; gl15, mhl1* triple mutants, indicating that the formation of MHs in *d1; gl15* double mutants would also require a functional allele of *mhl1*.

DISCUSSION

We have described a locus in maize, *macrohairless1*, which functions specifically in MH initiation within the leaf blade. Genetic analyses indicate *mhl1* is a major regulator of macrohair initiation, but is not the only genetic factor associated with this specific cellular differentiation pathway. Because MHs are a marker for adult leaf

TABLE 5

MH density among a family segregating for *mhl1-R* and *dwarf1* in a *gl15* mutant background

Phenotypic class	Leaf 3	Leaf 5	Leaf 7	Leaf 9	Leaf 11	Leaf 13	Leaf 15
D1; gl15, Mhl1 (9)	9.8 ± 2.8	74.3 ± 4.2	121.8 ± 14.8	175.2 ± 12.1	105.4 ± 23.5	83.6 ± 16.4	57.8 ± 11.4
d1; gl15, Mhl1 (6)	None	4.8 ± 3.0	26.5 ± 9.6	113.0 ± 5.6	104.0 ± 16.6	81.5 ± 19.2	39.5 ± 7.1
D1; g115, mhl1 (8)	None	None	None	0.8 ± 0.8	None	None	None
d1; gl15, mhl1 (6)	None	None	None	0.7 ± 0.4	None	None	None

Values are given as mean ±SE for the number of MHs per square centimeter of leaf area, sampled allometrically at the base of each leaf. The number of plants in each phenotypic class is in parentheses. D1, nondwarf; d1, dwarf; gl15, juvenile wax absent starting at leaf 3; Mhl1, normal MH density; mhl1, greatly reduced MH density; none, macroscopic observation found no macrohairs to be present on the leaf blade.

identity, analysis of MH initiation in the context of shoot development and the effects of the *mhl1* mutation on this process provide additional insights into the regulation of leaf identity in maize.

Leaf MH density varies during maize shoot development: One consistent observation in the studies reported here is that MH initiation frequency in maize progressively increases from the onset of adult leaf identity through leaf 9. In contrast, adult leaves produced later in shoot development show a progressive decline in MH density until leaf 13, when MH initiation frequency appears to reach a minimum stable level. In Arabidopsis, trichome density and distribution in leaves also vary quantitatively during shoot development (Martínez-Zapater *et al.* 1995) and are coordinated with the reproductive development of the shoot (Chien and Sussex 1996). Genetic analyses indicate that reduced adaxial trichome production in bracts (leaves produced during inflorescence development) is associated with the onset of inflorescence development (Telfer *et al.* 1997). MH initiation frequency in maize may be similarly reduced by the onset of inflorescence development, as the transition from increasing to decreasing MH initiation frequency appears to be correlated with the vegetative node that produces the uppermost branch terminating in a female inflorescence, which occurs at nodes 9–11 in the genotypes examined here. The trends in MH initiation frequency are also consistent with changes in leaf size during shoot development, which increases until the uppermost ear node and then decreases (Greyson *et al.* 1982). However, the fact that leaf 5 of *gl15* mutants produces the same or greater frequencies of MHs as leaf 13 from the same plants (Table 4), despite leaf 5 being considerably smaller than leaf 13 (data not shown), argues that MH initiation frequency is more closely correlated with inflorescence development than with leaf size *per se*.

Because the same patterns of variation in MH initiation frequency during shoot development were observed in *mhl1-R* plants with an intermediate MH density phenotype (Table 3), the factors that regulate leaf MH initiation frequency during shoot development must act upstream of *mhl1*. Indeed, both GAs and *gl15* act upstream of *mhl1* in their antagonistic regulation of the onset of MH production in transition and early adult leaves (Tables 4 and 5). However, while both of these factors affect the macrohair response to the juvenile-to-adult vegetative phase change, neither of them seems to affect MH initiation during late adult vegetative development (Tables 4 and 5).

These observations suggest that MH initiation in maize is stimulated by at least two pathways, one dependent on GAs and acting in early adult leaves and the other becoming increasingly active during shoot development and operating independently from GAs. One candidate for a gene acting in a second MH-stimulating pathway is *viviparous8* (*vp8*), which like GAs functions to promote the transition from juvenile to adult vegetative development (Evans and Poethig 1997). As observed for *d1; gl15* double mutants (Table 5), the early adult leaves of *vp8; gl15* double mutants also express all other adult traits except MHs (Evans and Poethig 1997). The peak MH frequencies observed near leaf 9 suggest that both of these pathways may converge to maximize MH initiation during early adult vegetative development.

***mhl1* and the specification of adult leaf identity in maize:** The *mhl1* mutation represents the first characterized defect in a cellular differentiation pathway specific to adult leaf identity in maize. The *mhl1* mutant phenotype demonstrates that MH initiation can be uncoupled from the differentiation of other adult leaf epidermal cell types such as bulliform cells and prickle hairs (Figure 1, A and B). Detailed analyses of MH initiation throughout adult vegetative development in normal and *mhl1* mutant plants also shows that even this single cellular differentiation pathway is regulated by multiple factors during shoot development.

Double-mutant analyses (Tables 4 and 5) demonstrate that both GAs and *gl15* regulate adult leaf identity in opposite ways by acting through *mhl1*, which defines the primary pathway through which these factors influence MH initiation. We propose here a model (Figure 3) where GAs cooperate with other factors (possibly defined by the *vp8* and *smh1* loci) to stimulate *mhl1* activity and/or MH initiation in adult leaves, whereas in normal juvenile leaves *gl15* instead acts as a repressor of MHL1 function. The *gl15* gene acts downstream of

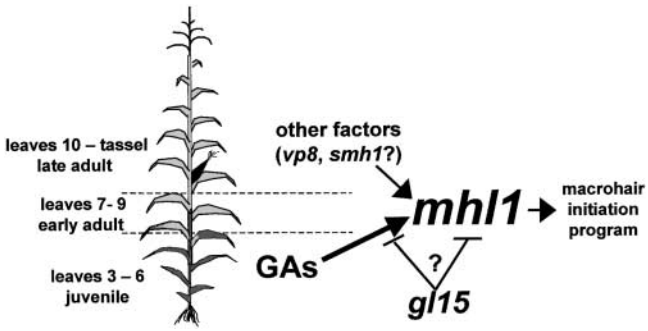

FIGURE 3.—A model for the regulation of MH initiation in maize. Juvenile leaves (dark gray) lack macrohairs because *gl15* activity represses *mhl1*, which is required to initiate MH differentiation. GAs promote the transition to adult vegetative development and also stimulate *mhl1* activity in early adult leaves (light gray). Other factors, possibly defined by the *viviparous8* (*vp8*) or *suppressor of macrohairless1* (*smh1*) loci, also stimulate *mhl1* activity and MH initiation in adult leaves. While *vp8* is known to act at the whole-shoot level, it is likely that *smh1* acts further downstream and possibly at the level of *mhl1*. The activating arrow for GAs is thicker than the repressing T-bars for *gl15* to indicate the greater sensitivity of *mhl1* to GAs compared to *gl15*.

GAs and *vp8* (EVANS and POETHIG 1995, 1997) but upstream of *mhl1* (Table 4). Therefore, *gl15* could suppress MH initiation either by repressing *mhl1* activity or by interfering with the response of *mhl1* to upstream factors. In this model, juvenile leaves are competent to express *mhl1* and produce MHs in response to GAs, but fail to do so because *gl15* represses *mhl1* or some relatively late component of the GA signal transduction pathway leading to adult leaf identity. When *gl15* activity is reduced or lost, either by mutation or by its downregulation in adult leaves (MOOSE and SISCO 1996), the *mhl1* gene is capable of responding to activation by GAs. The antagonistic interactions between GAs and *gl15* appear to regulate MH initiation only in transition leaves that express both juvenile and adult traits (*e.g.*, leaf 7) and the earliest adult leaves, but have little effect on MH initiation in later adult leaves, which are instead promoted by other factors whose identities are currently unknown.

The proposed model predicts that GAs would have their greatest effect on promoting MH initiation in *gl15* mutant leaves, where *mhl1* would be stimulated by GAs in the absence of *gl15*. The drastic reduction in MHs of *d1; gl15* double mutants compared to *gl15* single mutants supports this view (Table 5). The onset of MH production in transition leaves of normal plants prior to the loss of juvenile traits also suggests that *mhl1* activity is more sensitive to GAs than to *gl15*. Consistent with this idea, treatment of *dwarf3; Teopod1* double mutants with exogenously applied GA_3 affected MH production more than the expression of juvenile epicuticular waxes (EVANS and POETHIG 1995).

Possible functions for *mhl1* and *smh1*: The *mhl1* mutations exhibit mutant phenotypes only in the leaf blade, suggesting that either MHL1 function is limited to the leaf blade or there is redundancy for MHL1 function in the initiation of MHs elsewhere in the plant (for example, on leaf sheaths). The *mhl1* locus is likely to encode a regulatory gene that specifically programs MH initiation in the leaf blade epidermis. This conclusion is based on the observations that *mhl1* conditions a very early block in MH initiation, *mhl1* does not affect MH morphogenesis, and other aspects of cellular differentiation are normal in the *mhl1* mutant leaf epidermis, except for the failure to proliferate the multicellular pedestal associated with the base of each MH (Figure 1, A and B).

In Arabidopsis, a complex of interacting proteins from the MYB domain (GLABROUS1), basic helix-loop-helix (GLABRA3), and WD-40 repeat (TRANSPARENT TESTA GLABRA1) families has been shown to program trichome initiation (reviewed in SCHERES 2002). Thus, it is possible that *mhl1* encodes one of the members of an MYB-bHLH-WD-40 protein complex that may program macrohair initiation in the maize leaf blade. A number of similarities between maize and Arabidopsis epidermal hair formation support this hypothesis. In both taxa, hair initiation is promoted by GAs, varies both qualitatively and quantitatively during shoot development, and is influenced by the transition to inflorescence development (CHIEN and SUSSEX 1996; TELFER *et al.* 1997; Tables 3–5). In addition, the placement of both types of hairs within the epidermis is affected by a minimum distance spacing mechanism (LARKIN *et al.* 1996; N. LAUTER and S. MOOSE, unpublished observations). Finally, several cases of apparent coregulation of anthocyanins and macrohairs have been documented in a close wild relative of maize, *Zea mays* ssp. *mexicana* (LAUTER 2001; N. LAUTER, C. GUSTUS, A. WESTERBERGH and J. DOEBLEY, unpublished observations). Since anthocyanins are regulated by MYB, bHLH, and WD-40 repeat family members in both maize and Arabidopsis (CHANDLER *et al.* 1989; CONE *et al.* 1993; SELINGER and CHANDLER 1999; WALKER *et al.* 1999; BOREVITZ *et al.* 2000; NESI *et al.* 2000, 2001), the coregulation observations further support the hypothesis that a similar protein complex may regulate maize MH initiation.

If such a macrohair initiation complex does exist in maize, we believe *mhl1* would be most likely to represent a defect in the MYB domain-containing partner orthologous to Arabidopsis *GLABROUS1* (*GL1*). The phenotypes of *GL1* (KOORNEEF 1982; OPPENHEIMER *et al.* 1991) and *mhl1* mutants (Figure 1) are similar in that both are specifically defective in hair initiation on the medial blade without affecting the hairs at the margins of the blade. In addition, both *GL1* (PERAZZA *et al.* 1998) and *mhl1* (Table 5) activities are required for the positive effects of GA on MH initiation. Finally, the reductions in both trichome size and density conditioned by *GLABRA3* mutations in Arabidopsis are more similar to the weaker phenotypes seen in our macrohairless mutants that are nonallelic to *mhl1* (PAYNE *et al.* 2000). It should

be noted that although many MYB-, bHLH-, and WD-40-encoding DNA sequences are known for *Z. mays*, no clear orthologs of *GL1*, *GL3*, or *TTG1* have emerged in Blast searches using either the expressed sequence tag or the genomic survey sequence database.

The *Smh1* allele present in the W64A, but not the A632, 4Co63, or NC89 inbred backgrounds, acted as a dominant suppressor of *mhl1* that conditioned an intermediate density of leaf macrohairs (Tables 1 and 2). Notably, plants with the intermediate MH density phenotype show the same changes in MH frequencies during shoot development as normal plants do, suggesting that the *smh1* locus functions at the level of MH initiation rather than at the level of shoot developmental programs. Since *Smh1* plants homozygous for the putative null alleles of *mhl1* are glabrous while *Smh1* plants homozygous for *mhl1-Ref* display an intermediate MH density phenotype (Table 3), the *Smh1* allele likely requires some MHL1 protein to cause macrohair initiation. Together, these observations suggest that the *Smh1* allele characterized here could represent a dominant hypermorphic allele of a macrohair initiation regulator that is capable of stimulating MH initiation despite reduced levels of MHL1 protein.

If the *smh1* locus were to encode an ortholog of an Arabidopsis gene affecting trichome initiation, we think it would be a gene like *GL3*, which is directly involved in the initiation process and, when overexpressed, can condition hairs even when other members of the initiation-promoting protein complex are present at reduced levels (Payne *et al.* 2000). Alternatively, *smh1* could encode an ortholog of the *Reduced Trichome Number* (*RTN*) locus, which is known as a quantitative trait locus difference between the Columbia (Col) and Landsberg erecta (Ler) ecotypes of Arabidopsis (Larkin *et al.* 1996). Although the exact action of *RTN* remains unclear, it could be argued that the Col allele of *RTN* is hypermorphic, acting semidominantly to increase trichome density, which is similar to our interpretation of the action of the *Smh1* allele from maize inbred W64A. However, *GL1* and *TTG1* mutations are not suppressed by the Col allele of *RTN*.

In addition to the *mhl1* and *smh1* loci, the *mhl*-LS8*, *mhl*-LS9*, *mhl*-LS10*, and *mhl*-LS11* mutations, which are not allelic to *mhl1*, also appear to regulate MH initiation in maize. Each of these mutations exhibits a weak, variably penetrant macrohairless phenotype. Another mutation with a weak macrohairless leaf phenotype, which we have named *macrohairless2* (*mhl2*), was identified in a *Mutator* transposon-active population and mapped to the long arm of chromosome 4 by B-A translocation tests (J. Nelson and M. Freeling, personal communication). Interestingly, *mhl2* mutants show a reduction in both MH density and size, which is reminiscent of the *GLABRA3* mutant phenotype in Arabidopsis (N. Lauter, J. Nelson, M. Freeling and S. Moose, unpublished observations). Given this similarity and the fact that *smh1* has not yet been mapped, it remains possible that *smh1* and *mhl2* are the same locus. Allelism testing of these macrohairless mutations, genetic mapping, and tests for their interactions with *mhl1* are ongoing.

Prospects: The availability of putative transposon-tagged *mhl1* alleles and candidate genes from Arabidopsis that may be orthologous to *mhl1* in maize should facilitate the eventual molecular cloning of the *mhl1* gene. Once cloned, it should be possible to elucidate the molecular mechanisms by which *mhl1* promotes leaf blade MH initiation and how *mhl1* responds to factors that act upstream of *mhl1* to regulate MH production during shoot development.

In many other cereal crops, studies of glabrous mutant varieties that lack MHs have led to the suggestion that MHs contribute to insect resistance (*e.g.*, Ringlund and Everson 1968; Sosa 1990). In maize, a similar function for leaf MHs has been proposed on the basis of associations between quantitative variation in leaf macrohair density and insect behavior or feeding (Widstrom *et al.* 1979; Durbey and Sarup 1982). Our study has identified *mhl1* mutant alleles that appear to completely eliminate leaf blade MHs, which should permit direct tests of their role in insect resistance and in other physiological functions in maize.

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