Plant Physiol. (1994) 105: 1239–1245

Chemically Induced Cuticle Mutation Affecting Epidermal Conductance to Water Vapor and Disease Susceptibility in *Sorghum bicolor* (L.) Moench.[1]

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Analysis of *Sorghum bicolor* bloomless (*bm*) mutants with altered epicuticular wax (EW) structure uncovered a mutation affecting both EW and cuticle deposition. The cuticle of mutant bm-22 was about 60% thinner and approximately one-fifth the weight of the wild-type parent P954035 (WT-P954035) cuticles. Reduced cuticle deposition was associated with increased epidermal conductance to water vapor. The reduction in EW and cuticle deposition increased susceptibility to the fungal pathogen *Exserohilum turcicum*. Evidence suggests that this recessive mutation occurs at a single locus with pleiotropic effects. The independently occurring gene mutations of bm-2, bm-6, bm-22, and bm-33 are allelic. These chemically induced mutants had essentially identical EW structure, water loss, and cuticle deposition. Furthermore, 138 F_2 plants from a bm-22 × WT-P954035 backcross showed no recombination of these traits. This unique mutation in a near-isogenic background provides a useful biological system to examine plant cuticle biosynthesis, physiology, and function.

EW provides the outermost barrier between plants and their environment. Previous studies have implicated EW layers in tolerance to various kinds of biotic and abiotic environmental stress (Eglinton and Hamilton, 1967; Thomas and Barber, 1974; Blum, 1975; Webster, 1977; Bengston et al., 1978; Jordan et al., 1984; El-Otmani et al., 1989; Jefferson et al., 1989; Percy and Baker, 1990; Stoner, 1990; Bergman et al., 1991). Near-isogenic mutants provide a model system for the dissection of biochemical (Koorneef et al., 1989; Somerville and Browse, 1991) and biophysical (Blum, 1975; Jordan et al., 1984; Saneoka and Ogata, 1987) effects of altered lipid production by plants. Thirty-three independently segregating chemically induced *Sorghum bicolor* mutants with altered visible sheath EW (designated bloomless [*bm*] mutants) were identified. Scanning EM was used to categorize these mutants into 14 unique EW structural classes (Jenks et al., 1992). These near-isogenic *bm* mutants exhibited similar form and stature but varied widely in EW structure and total EW deposition under normal irrigated conditions. Field studies under drought stress and disease pressure in southwestern Mexico suggested that mutants bm-2, bm-6, bm-22, and bm-33, making up an individual allelic group, were more susceptible to drought and more susceptible to leaf blight. Since individuals in this allelic group had EW structures and total EW load similar to other *bm* mutants that were not susceptible to drought and disease, we suspected that these responses were due to alterations in the cuticle proper. To test this hypothesis, cuticle ultrastructures and depositions on the wild-type and *bm* mutants were analyzed and then compared with water loss rates and disease resistance. Our results suggest that allelic mutants bm-2, bm-6, bm-22, and bm-33 possess a mutation affecting cuticle deposition. To our knowledge, a cuticle mutation in plants has not been previously reported.

MATERIALS AND METHODS

Plant Material

The mutagenesis program was initiated using two drought-resistant inbred lines of *Sorghum bicolor* (L.) Moench. These inbred lines, designated P954035 and P898012, were produced in the Purdue Sorghum Improvement Program (Dr. Gebisa Ejeta, Department of Agronomy). Seeds (M_0) were exposed to the chemical mutagens diethyl sulfate (J. T. Baker, Phillipsburg, NJ) or ethyl methanesulfate (Eastman Kodak Co., Rochester, NY). Seeds treated with diethyl sulfate were submerged in either a 5.7, 7.7, or 11.5 mM solution for 3 h at room temperature. Seeds treated with ethyl methanesulfate were submerged in either a 4.7 mM or 9.4 mM solution for 18 h. Treated seeds (designated M_1) were planted at the Purdue Agronomy Research Center, self-pollinated, and advanced to the M_2 generation. Seeds from the wild-type parent P898012 (WT-P898012) were used to generate mutants bm-2 and bm-6, and wild-type parent seeds of P954035 (WT-P954035) were used to generate bm-11, bm-15, bm-21, bm-22, and bm-33 used in this study. Isolines examined had diverse EW phenotypes. WT-P898012 and WT-P954035 possessed approximately 1-μm wide and at least 500-μm long hollow EW filaments, bm-11 possessed sparse long filaments, bm-15

[1] This research was partially supported by the McKnight Foundation Interdisciplinary Research Project in Plant Biology. This is Purdue University Agricultural Experiment Station Article No. 14114.

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Abbreviations: EW, epicuticular wax; g_{cl}, epidermal conductance with stomates closed; g_{op}, epidermal conductance with stomates open; TEM, transmission EM; T_{cl}, transpiration rate with stomates closed; T_{op}, transpiration rate with stomates open.

possessed approximately 1-μm wide globular EW, bm-21 possessed relatively tiny EW globs, and mutants bm-2, bm-6, bm-22, and bm-33 all lacked structural EW (Jenks et al., 1992). Peters (1993) used traditional genetic tests to demonstrate that the mutations designated bm-2, bm-6, bm-22, and bm-33 were allelic. The mutations were also allelic with the bloomless mutant Txbm1. Peterson et al. (1982) designated this locus bm_1. The mutants bm-11, bm-15, and bm-21 contained mutations that were not allelic with each other or with bm_1.

TEM

Leaf tissues were collected from greenhouse-grown plants just prior to panicle emergence. Blade tissues from each isoline were taken at mid-leaf length. Sheath tissues were taken from WT-P954035 and bm-22 at approximately 2 cm below the ligule. The tissue preservation protocol for TEM was similar to protocols used in previous studies of plant cuticle ultrastructure (Chafe and Wardrop, 1973; Reed and Tukey, 1982; Oosterhuis et al., 1991). Excised tissues were cut into 1-mm sections while submerged in 0.05 M potassium phosphate buffer (pH 6.8). Sections were then fixed at room temperature for 3 h in a buffered 4.0% paraformaldehyde and 4.0% glutaraldehyde solution (Karnofsky, 1967). Specimens were rinsed three times in phosphate buffer and post-fixed for 2 h in buffered 2.0% osmium tetroxide. Next, tissues were rinsed in distilled water, soaked for 30 min in 2.0% uranyl acetate, dehydrated in a graded ethanol-propylene oxide series, and embedded in Spurr's resin (Spurr, 1969). Preliminary studies showed that leaf tissues required a 4-d embedding period in a graded series of Spurr's resin-propylene oxide to get satisfactory penetration of resin. Thin sections of 45 to 60 nm were made using a Sorvall Porter Blum MT2-B Ultramicrotome with diamond knife. Sections were mounted on 200-mesh grids lacking support films and then stained for 3 min with 1.0% aqueous lead citrate. Photomicrographs of adaxial and abaxial blade epidermal long cell cuticles and abaxial sheath epidermal long cell cuticles were produced on a Philips EM-200 transmission electron microscope. Average cuticle thickness was determined from 10 measurements each of three to five replicate plants.

Gravimetric Cuticle Measurement

Twenty 1.5-cm^2 blade discs were removed from a 10-cm region at mid-length from the uppermost leaf blades with fully elongated sheaths. Adaxial and abaxial cuticles were separated from blade tissues by a 12-h soak in a 60% (w/v) solution of zinc chloride in hydrochloric acid (Holloway and Baker, 1968). Bulked isolated cuticle discs were placed on glass microscope coverslips, oven dried at 50°C, and then placed in a desiccator containing dried silica gel for at least 12 h. Average cuticle weights were determined from 30 to 40 blade disc cuticles from four replicate plants of each near isoline. Leaf blade discs were collected from greenhouse-grown plants (as described above) of WT-P954035, bm-11, bm-15, bm-21, and bm-22 just prior to panicle emergence. Similar measurements were made of WT-P954035, WT-P898012, bm-2, bm-6, bm-22, and bm-33 grown in field rows (approximate spacing 72 cm between and 14 cm within rows) at the Purdue University Agricultural Research Center (West Lafayette, IN) to compare cuticle deposition of allelic mutants.

Colorimetric Measurement of EW Load

Leaf blade EW deposits were measured on field-grown plants (grown as described above) just after panicle emergence. Preliminary studies showed that EW load varied little during the period just prior to and after panicle emergence. Blade sections (50 cm^2) were removed from either side of the mid-rib of the first and second leaf below the flag leaf. Five sections of each genotype were extracted separately by dipping for 10 s in redistilled chloroform. EW extracts were dried under nitrogen and quantified using the acidic bichromate assay described by Ebercon et al. (1977).

Measurement of Water Loss Rates

T_{op} and T_{cl} were determined by lysimetry in a controlled atmosphere growth room. Measurements were made on five replicates of each near isoline just prior to panicle emergence. Plants used for this study were greenhouse grown in 15-L pots using randomized complete block design. Evaporative water loss from the soil surface was prevented by enclosing pots in double plastic bags that were sealed at the soil-stalk interface before measurements. Plants were held in darkness for 2 h before measurement of T_{cl} and 2 h of light prior to measurement of T_{op}. High-pressure sodium bulbs (1000 W provided photosynthetic photon flux of 900 μmol m^{-2} s^{-1} measured at mid-plant height. An electric fan provided gentle air circulation. g_{op} and g_{cl} were calculated from average plant transpiration and atmospheric conditions during the 1.33-h measurement period using standard diffusion equations (Muchow and Sinclair, 1989). The temperature gradients between the surrounding air and leaves during T_{op} measurements (plants exposed to light) were determined by the average of 60 individual thermocouple measurements made low, mid-height, and high in the canopy. We assumed, as have others (Muchow and Sinclair, 1989; Araus et al., 1991), that leaf and air temperatures during T_{cl} measurements were the same because plants were kept in the dark and water loss rates were extremely small. Leaf vapor pressure was assumed to be at saturation. Vapor pressure and average temperature of the growth room atmosphere were determined from average sensor readings made low and high in the growth room plant canopy using an HTL1 humidity/temperature logger (P. K. Morgan Instruments Inc., Andover, MA). Total plant surface areas were determined using a leaf area meter and calculations of stalk surface areas according to the formula for a right circular cone.

Water loss rates from excised leaf blades were used to examine the segregation of high water loss rate with visible mutation in EW. Approximately 30-cm-long blade sections were excised from the middle of the uppermost leaf blades with fully expanded sheaths of field-grown plants just prior to panicle emergence. Tissues were then submerged in distilled water and soaked for 2 h before initiation of dry down. Excised leaves were suspended and exposed to gentle air

circulation produced by an electric fan. Water loss rates were determined from an individual leaf of each plant in the population. Leaf weight was measured with an electronic balance before and after a 1-h dry-down period, and water loss rates were then expressed as a percentage of turgid leaf weight.

Disease Susceptibility Rating

Three replicate field plots of each of the five near isolines were grown in a randomized complete block design near Valle de Banderas in Nayarit, Mexico. Plants were grown during the winter months and experienced no precipitation. Field plots were ditch irrigated. Northern corn leaf blight was positively identified on plants growing in Valle de Banderas (Frank Loeffel, personal communication). The pathogen *Exserohilum turcicum* was identified via the *Compendium of Sorghum Diseases* (1991). Leaf blight susceptibility ratings were made independently by four investigators and based on visual estimates of surface necrosis within a row. Ratings were based on necrosis of the first leaf below the flag leaf on plants just after panicle emergence. Ratings were scaled 0 to 10 (0 indicated no lesions and 10 indicated greater than 90% leaf area necrosis).

Genetic Analysis

Segregation of water loss and visible EW phenotypes in an F_2 population were used to examine whether more than one gene controlled both the EW mutation and cuticle mutation of bm-22 or whether a mutation in one gene caused these effects by pleiotropy. The mutant bm-22 was backcrossed with the respective wild-type parent P954035 to produce F_1 seed. F_2 seeds (from five self-pollinated F_1 plants) were planted in five individual head rows at the Purdue University Agricultural Research Center as described above. The total F_2 population consisted of 138 plants. The segregation of the EW mutant phenotype and high water loss rate phenotype were determined in the F_2 population. Plants with alterations in the cuticle deposition were identified indirectly as plants having leaf blades with high water loss rate (protocol described above).

RESULTS

Characteristics of the Cuticle and EW Layers

The ultrastructure of the wild-type P954035 (WT-P954035) *S. bicolor* cuticle over epidermal long cells is more complex than that of the mutant bm-22 (Fig. 1). Fibrillae present within the inner secondary cuticle of WT-P954035 are not apparent in the bm-22 mutant. Cuticles of EW mutants bm-11, bm-15, and bm-21 were morphologically similar to WT-P954035 (data not shown). The bm-22 mutant cuticle possessed electron density similar to the resin support, and the image presented was overexposed slightly to enhance visibility of the cuticle ultrastructure (Fig. 1). The cuticle proper of P954035 adaxial and abaxial blades and abaxial sheaths was thicker than the cuticle of bm-22 (Fig. 1; Table I). Cuticles of mutants bm-11, bm-15, and bm-21 were similar in thickness to WT-P954035 (Table I). Remnants of the EW layers are seen above the primary cuticle in these micrographs (Fig. 1).

The weight of WT-P954035 blade cuticles were 4 to 5 times greater than bm-22 blade cuticles on greenhouse-grown plants (Table I). Cuticles of bm-11, bm-15, and bm-21 were similar in weight to WT-P954035 (Table I). EW deposition on field-grown bm-21 and bm-22 were both 3 times lower than EW deposition on WT-P954035 (Table I). The EW loads on bm-11 and bm-15 were similar to WT-P954035 (Table I). EW loads on the allelic mutants bm-2, bm-6, bm-22, and bm-33 were all similar (data not presented).

Water Loss Rates

The mean whole plant g_{cl} of bm-22 was 7.31 mmol m^{-2} s^{-1}, a value approximately 2.5 times higher than that observed in WT-P954035 (Table I). In contrast, g_{cl} values measured in bm-11, bm-15, and bm-21 mutants were not different from those of the WT-P954035 (Table I). A similar result was observed for g_{op}, with bm-22 showing an approximately 35% increase in g_{op} over the WT-P954035. No differences in g_{op} were evident among the bm-11, bm-15, bm-21, and bm-22 mutants. In WT-P954035 plants, g_{op} exceeded g_{cl} by a factor of 7.

Disease Susceptibility

Near isolines differed in their susceptibility to fungal infection by *E. turcicum* (Table I). Mutant bm-22 had a mean leaf blight susceptibility rating that was 3.6 times higher than WT-P954035. The mean susceptibility rating of bm-21 was 2.5 times higher than WT-P954035, whereas disease ratings of bm-11 and bm-15 were similar to WT-P954035 (Table I). Susceptibility to disease appeared to be affected by both cuticle and EW deposition (Table I).

Genetic Analysis

The independently occurring EW mutants bm-2, bm-6, and bm-33 were allelic to bm-22 (Peters, 1993), and all exhibited similar reductions in cuticle deposition compared to their respective wild-type parents (Fig. 2). All four allelic bm_1 mutants also exhibited higher water loss rates than their wild-type parents (Fig. 3). These results suggest that the reduction in cuticle thickness/weight is a pleiotropic effect of the mutation that alters EW structure.

To confirm this hypothesis, we examined segregation of the visible EW phenotype with rate of water loss from excised leaves in a segregating population. The bm-22 EW phenotype acted as a single recessive mutation segregating 3:1 in a population of 138 plants (χ^2 [3:1] = 1.392, $P < 0.05$). In all cases, the bm-22 EW phenotype co-segregated with a high rate of water loss from excised tissues. Figure 4 shows the frequency distribution of water loss rates by the wild-type and mutant EW phenotypes in the segregating population. No parental EW types (wild type or mutant) within the population showed water loss rates that occurred within the range exhibited by the other parental population (Fig. 4). Co-segregation of the EW and high rate of water loss phenotypes indicates that these are caused by the pleiotropic effects of one gene or by the actions of two closely linked genes. The latter possibility cannot be excluded by these studies. How-

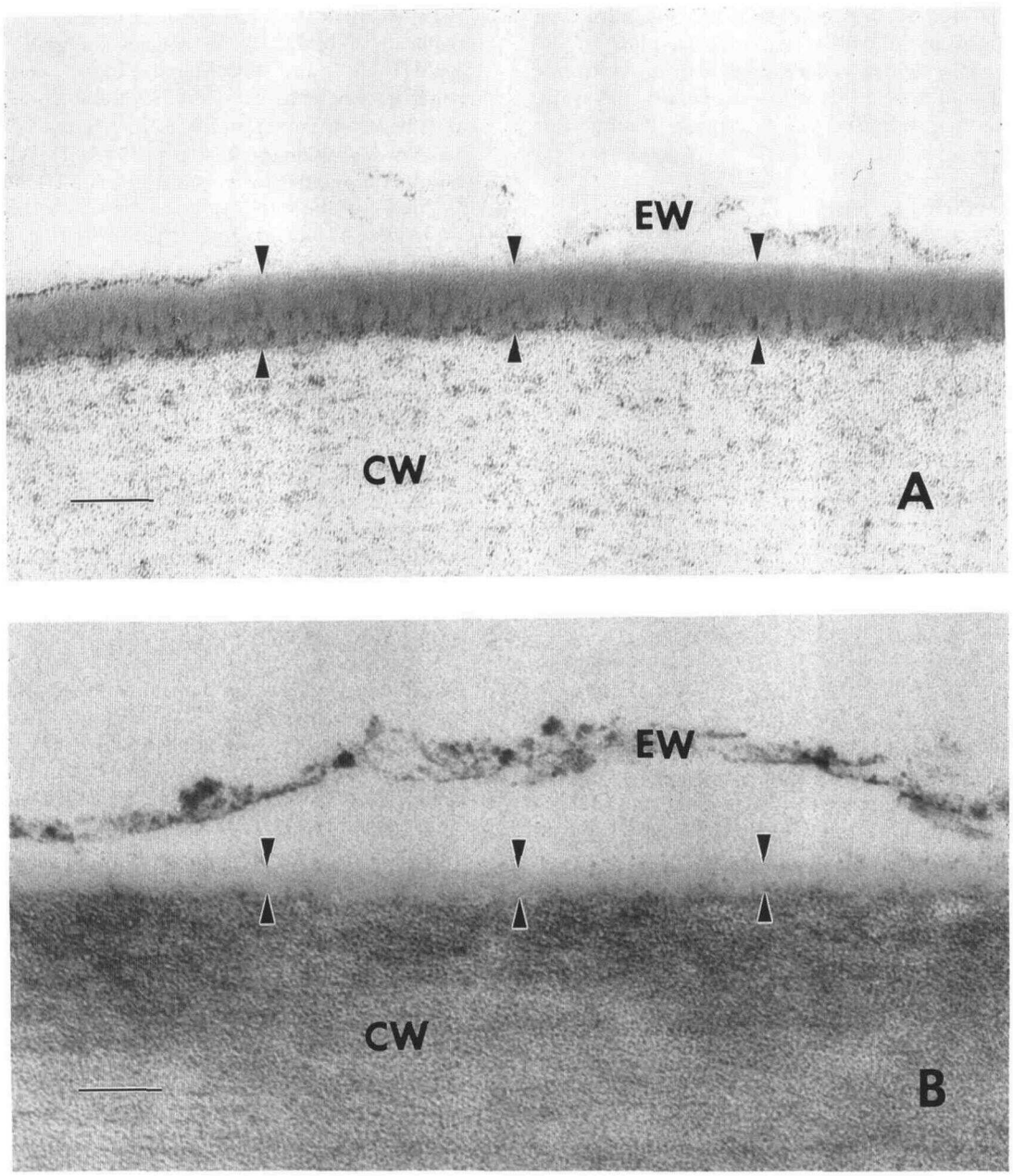

Figure 1. Wild-type (WT-P954035) and mutant (bm-22) epidermal long cell cuticle ultrastructure. Images of WT-P954035 and near-isogenic mutant bm-22 cuticle ultrastructures produced using TEM. A, WT-P954035 abaxial blade cuticle (arrowheads). B, bm-22 abaxial blade cuticle (arrowheads). CW, Cell wall. Bar = 0.1 μm.

ever, the presence of both mutant phenotypes in four independently derived allelic mutations supports the hypothesis that a single-gene mutation affects both EW and cuticle deposition.

DISCUSSION

Cuticle layers cover the outermost surfaces of plants. Normal cuticle membranes on *S. bicolor* are divided into an inner reticulate secondary cuticle, an amorphous primary cuticle, and an outer EW layer. When stomata are closed, as during darkness or drought, plant tissue water loss is controlled primarily by water flow through the cuticle layers and closed stomatal complexes. When stomata are open, water loss by plant tissues is dominated by water flow through the open stomatal aperature. DeLucia and Berlyn (1984) suggested that an increase in *Abies balsamea* cuticle thickness reduced T_{cl}. By comparison, the *S. bicolor* bm-22 mutant with decreased cuticle thickness and weight had higher g_{cl} than the near isolines WT-P954035, bm-11, bm-15, and bm-21 with thicker and heavier cuticles. Because the differences between g_{cl} and g_{op} of various sorghum near isolines were similar and the

Table I. *Physiological and morphological characterization of S. bicolor near isolines*

Cuticle deposition, EW deposition, whole plant g_{op}, whole plant g_{cl}, and susceptibility to northern corn leaf blight (*E. turcicum*) on the wild-type parent P954035 and four nonallelic near-isogenic mutant lines, bm-11, bm-15, bm-21, and bm-22. Values represent means ± SE.

Near Isoline	WT	bm-11	bm-15	bm-21	bm-22
Cuticle thickness (nm)					
Adaxial blade	73 ± 4	75 ± 7	73 ± 4	73 ± 7	28 ± 2
Abaxial blade	90 ± 2	92 ± 4	92 ± 2	90 ± 2	37 ± 2
Abaxial sheath	97 ± 4	N.D.[a]	N.D.	N.D.	39 ± 2
Wt (mg/dm^2)					
Blade cuticle	10.0 ± 1.3	9.9 ± 1.6	10.8 ± 2.7	9.1 ± 0.8	2.2 ± 0.2
Blade EW	1.6 ± 0.2	2.0 ± 0.3	1.9 ± 0.1	0.5 ± 0.0	0.5 ± 0.0
Conductance (mmol m^{-2} s^{-1})					
g_{op}	24.6 ± 1.7	25.6 ± 1.5	28.7 ± 1.1	25.1 ± 1.2	32.9 ± 1.9
g_{cl}	2.9 ± 0.4	3.3 ± 0.4	3.7 ± 0.2	2.5 ± 0.3	7.3 ± 0.3
Blight rating (0–10)					
Leaf necrosis	1.7 ± 0.3	1.7 ± 0.3	2.0 ± 0.0	4.3 ± 0.6	6.2 ± 1.2

[a] Not determined.

thickness and ultrastructure of WT-P954035 and bm-22 cuticles over the surfaces of the stomatal apertures were similar, we suspect that the increased epidermal conductance to water vapor of bm-22 surfaces was primarily due to reduced cuticular resistance as opposed to reduced stomatal resistance.

The amount of EW deposited on the plant surface appeared to have little effect on epidermal conductance to water vapor. Although EW load did not differ between bm-21 and bm-22, g_{cl} of bm-22 exceeded that of bm-21 by 3-fold. By comparison, the isolines WT-P954035, bm-11, bm-15, and bm-21 exhibited a range of EW loads but had similar g_{cl}. Previous studies examining the influence of EW deposition on whole plant T_{cl} and g_{cl} have been inconclusive. Saneoka and Ogata (1987), Jordan et al. (1984), Chatterton et al. (1975), and Blum (1975) showed that near-isogenic *S. bicolor bm* mutants with lower EW load had higher T_{cl} (and/or g_{cl}) than their respective near-isogenic normals. In contrast, Jordan et al. (1984) found no correlation between EW load and T_{cl} in comparisons between different sorghum cultivars with EW loads above 0.67 mg dm^{-2}. Likewise, no differences were found in T_{cl} between nonisogenic lines of *Triticum* species (Johnson et al., 1983; Araus et al., 1991), several western U.S. conifers (Hadley and Smith, 1990), *Medicago sativa* and *Agropyron desertorum* (Jefferson et al., 1989), and *Avena sativa* (Bengston et al., 1978) with differing EW loads. In *S. bicolor* mutants, bm-21 and bm-22 had one-third the blade EW deposition of WT-P954035; however, bm-21 had similar g_{cl} to WT-P954035, and bm-22 had 2 to 3 times higher g_{cl} than WT-P954035. These results suggest that differences in EW at these levels may not significantly alter resistance to water flow.

Near isolines of *S. bicolor* with altered cuticle and EW deposition differed in susceptibility to northern corn leaf blight. Previous investigators have discussed the possible roles of cuticle in disease resistance (Martin, 1964; Kolattukudy et al., 1987; Reuveni et al., 1987). The cuticle may serve

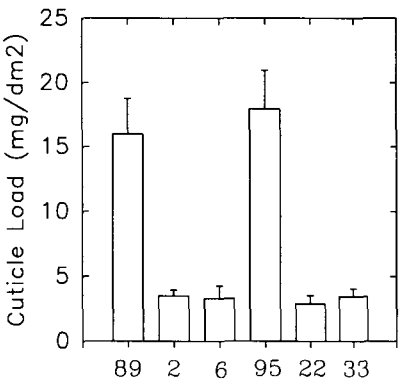

Figure 2. Cuticle deposition on allelic *bm* mutants with reduced EW compared with parental liness. Lines bm-2 (2) and bm-6 (6) are near-isogenic mutant progeny of wild-type parental line P898012 (89). Lines bm-22 (22) and bm-33 (33) are near-isogenic mutant progeny of wild-type parental line P954035 (95). Cuticle load was determined gravimetrically.

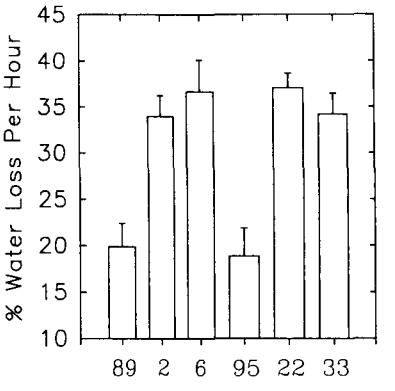

Figure 3. Allelic *bm* mutants with reduced EW and cuticle deposition have lower water loss rates compared to parental lines. Comparisons made between wild-type parent P898012 (89) and near-isogenic mutant progeny bm-2 (2) and bm-6 (6) and between wild-type parent P954035 (95) and mutant progeny bm-22 (22) and bm-33 (33). Near isolines bm-2, bm-6, bm-22, bm-33 were allelic. Water loss determined using excised leaf blades.

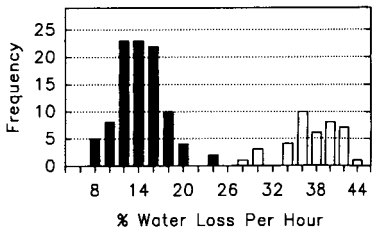

Figure 4. Frequency distribution of bloomless phenotype (lacking any visible EW) and rate of water loss (percentage basis) in a segregating population. The number of individuals of a segregating F_2 population (from backcross bm-22 × WT-P954035) with percentage of water loss per hour falling within intervals of 2% from 7 to 45%. Individuals of the population with the parental WT-P954035 EW phenotype are represented by solid bars. Individuals with the parental bm-22 EW phenotype are represented by open bars. None of the segregates with wild-type EW had water loss rates that fell within the distribution range of the segregates lacking visible EW.

as a physical barrier to fungal hyphae penetration, repel water droplets to prevent spore germination, and/or contain chemicals that inhibit fungal growth. Our results suggest that both EW and the cuticle layers were important barriers to fungal infection. It is unclear whether changes in the chemical composition and/or structure of cuticle and EW layers were responsible for increased disease susceptibility.

Our genetic studies suggest that a single-locus mutation affects both cuticle and EW deposition on bm-22. The probability that four of 33 independently occurring chemically induced allelic mutants with multiple and essentially identical phenotypic alterations would all possess identical double mutations is very low. In addition, we found that 138 plants in a segregating F_2 population from a bm-22 × WT-P954035 backcross showed no apparent recombination of these traits. The combined evidence suggests that the phenotype was the result of a mutation at a single locus with pleiotropic effects. This cuticle mutation in a near-isogenic background provides a unique biological system for further examination of plant cuticle biosynthesis, physiology, and function.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Charles Bracker and Debbie Sherman of the Purdue University EM Center for their cooperation and Dr. Peter Goldsbrough and Dr. Steve Weller for reviewing the manuscript.

Received January 13, 1994; accepted April 18, 1994.

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