Cuticle Layer as a Determining Factor for the Formation of Mature Appressoria of Erysiphe graminis on Wheat and Barley

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ABSTRACT

Conidia of *Erysiphe graminis* produced high percentages of normal-appearing, mature appressoria on host leaves, the upper surface of epidermal strips, and on the upper surface of enzymatically isolated cuticles. Few normal-appearing, mature appressoria, or none, were formed when spores germinated on the lower surface of epidermal strips, on the lower surface of isolated cuticles, on reconstructed wax layers, or on a number of different artificial surfaces. Malformed appressoria were observed on plants that possessed eceriferum (*cer*) mutations which affect chemical components and the physical structure of wax layers.

The *Ml* and *Pm* genes in barley and wheat, respectively, did not appear to interact with the corresponding *P* genes in the parasites to affect the morphological development of mature appressoria.

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Additional key words: Hordeum vulgare, Triticum aestivum.

The powdery mildew diseases of barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) are caused by *Erysiphe graminis* f. sp. *hordei* and *Erysiphe graminis* f. sp. *tritici*, respectively. Primary infection of wheat and barley by *E. graminis* consists of a number of morphologically identifiable stages of development:

spore germination, formation of appressoria, penetration into host cells, formation of haustoria in host cells, and formation of elongating secondary hyphae which are capable of initiating secondary and tertiary infections. A procedure for examining the early infection process of *E. graminis* on wheat and barley has been described (10, 11, 13, 16, 20). Elongating secondary hyphae (ESH) have been used as an indication of the establishment of compatible, functional host-parasite relationships because the formation of ESH is apparently completely dependent on the formation of haustoria in the host epidermal cells (10). With appropriate environmental conditions, the synchrony of the various stages in the infection process can be increased and the percentage of successful infections can be maximized.

Prior to the infection of host tissues, many parasitic fungi develop characteristic structures such as appressoria, and it is generally assumed that these are a prerequisite for infection. Some fungi produce them readily when growing on a wide variety of living and nonliving structures (1, 2). Others do so only, or much more frequently and characteristically, on plants that can function as hosts (3, 9). In the first group, contact with a sufficiently firm surface appears to initiate appressorial formation. In the second group, the response may be conditioned by properties of the surface other than hardness.

Particular chemical stimuli may be involved in the formation of appressoria (3, 5, 6, 18), and the formation of appressoria may also be dependent on the pathogenicity of the pathogen to the host (3, 5, 6). The formation of appressoria in only certain areas of leaf surfaces (4, 14) suggests that leaf surfaces are not uniform.

If the chemical composition and the physical structure of the wax layer is important for the normal differentiation of appressoria of the parasite, mutations which affect the chemical composition and physical structure of the wax layer may differ in their effects upon the parasite. Mutations which give a glossy appearance to the leaves have been induced in barley by a variety of different mutagens (8), and are called eceriferum mutants because they affect the wax layer on the leaves.

The objective of this study was to determine the role of the host cuticle on the formation of mature appressoria by *E. graminis*.

MATERIALS AND METHODS.—Cultures of powdery mildew: *Erysiphe graminis* (DC.) Merat f. sp. *hordei* Em. Marchal strain CR-3 was maintained on barley (*Hordeum vulgare* L. 'Manchuria'). *Erysiphe graminis* (DC.) Merat f. sp. *tritici* Em. Marchal strain MS-1 was maintained on wheat (*Triticum aestivum* L. 'Little Club'). Wheat and barley plants were grown in 4-inch pots, and were inoculated when they were 6 to 7 days old. A new set of 6- to 7-day-old susceptible wheat and barley plants was inoculated each day by dusting conidia produced 7 days after inoculation onto the leaves. Inoculated plants were maintained in a controlled environment chamber with a 15-hr light period (650-750 ft-c from white VHO-fluorescent tubes and 50 ft-c from 25 w incandescent bulbs) at 18 ± 1 C and 65 ± 5% relative humidity and a 9-hr dark period and 16 ± 1 C and 95 ± 5% relative humidity. Mycelial growth of the culture was macroscopically evident 3 to 4 days after inoculation.

TABLE 1. Genotypes and abbreviated designations of host lines

Plant genotypes				Abbreviated designations
Barley				
mla mla	*mlg mlg*	*mlp mlp*	*mlk mlk*	Manchuria or *mlx*
Mla Mla	*mlg mlg*	*mlp mlp*	*mlk mlk*	*Mla*
mla mla	*Mlg Mlg*	*mlp mlp*	*mlk mlk*	*Mlg*
mla mla	*mlg mlg*	*Mlp Mlp*	*mlk mlk*	*Mlp*
mla mla	*mlg mlg*	*mlp mlp*	*Mlk Mlk*	*Mlk*
Wheat				
pm1 pm1	*pm2 pm2*	*pm3 pm3*	*pm4 pm4*	Chancellor or *pmx*
Pm1 Pm1	*pm2 pm2*	*pm3 pm3*	*pm4 pm4*	*Pm1*
pm1 pm1	*Pm2 Pm2*	*pm3 pm3*	*pm4 pm4*	*Pm2*
pm1 pm1	*pm2 pm2*	*Pm3 Pm3*	*pm4 pm4*	*Pm3*
pm1 pm1	*pm2 pm2*	*pm3 pm3*	*Pm4 Pm4*	*Pm4*

Lines of wheat and barley.—Chancellor wheat, which contains no known major genes affecting mildew development, was used as the standard to which mildew development on other lines was compared. Four backcross-derived lines of wheat which contained *Pm* genes affecting mildew development were obtained from L. W. Briggle, Beltsville, Md. The lines were designated as follows: *Pm1* (Axminister x^8 Cc), *Pm2* (Ulka x^8 Cc), *Pm3* (Asosan x^8 Cc), *Pm4* (Khapli x^8 Cc). The symbol, x^8 Cc, indicates an original cross plus seven backcrosses to the cultivar Chancellor. Manchuria barley was used as the standard to which other lines were compared. Four barley lines backcrossed to the cultivar Manchuria, each possessing a different gene affecting mildew development, were supplied by J. G. Moseman, Beltsville, Md. The four near-isogenic lines and their derivations were as follows: *M1a* (Algerian C.I. 1179 x_7^4 Manchuria C.I. 2330), *Mlg* (Goldfoil C.I. 928 x_9^4 Manchuria C.I. 2330), *Mlp* (Psaknon C.I. 1016 x_8^4 Manchuria C.I. 2330). The symbol x_7^4 refers to the original cross with Manchuria, three generations of backcrossing to Manchuria, and then selfing of the heterozygous progeny in each of seven generations. Five lines with eceriferum mutations, *cer-J*^{59}, *cer-J*^{71}, *cer-zd*^{67}, *cer-ze*^{81}, and *cer-zj*^{78}, which were induced by either ionizing radiations or chemical mutagens, as well as wild type cultivar Bonus, were supplied by P. von Wettstein-Knowles, Copenhagen, Denmark. Eceriferum loci control the synthesis and/or excretion of the organic specific wax components. Bonus is not known to contain any dominant *M1* genes effective against CR-3.

Designation of genotypes.—The barley cultivar Manchuria contains no known major genes affecting development of *Erysiphe graminis*. By definition, therefore, it contains the recessive alleles at the four

loci that are known to affect mildew development. The genotypes of the host lines and abbreviations used are presented in Table 1. The genotype of the MS-1 strain of *E. graminis* f. sp. *tritici* used in this study is *P1 P2 P3 P4* (designations according to Loegering) (7, 20). The genotype of the CR-3 strain of *E. graminis* f. sp. *hordei* used in this study is *Pa Pg Pp Pk*.

Method of controlled inoculation.—Single 5- to 6-day-old plants grown in 2-inch pots were inoculated by the rolling method (15) in all experiments where the development of the powdery mildew fungus during primary infection was studied. Conidia were dusted onto a clean glass slide and transferred to a single wheat or barley plant, or to a prepared surface, with a cotton swab. Only the lower (abaxial) leaf surface of each plant was inoculated. A uniform distribution of conidia ranging from 80 to 120/cm^2 of leaf area was obtained by this method. Only single, well-separated parasite units were counted at each observation to eliminate the possibility of inhibition due to crowding (19).

Environmental conditions for experiments.—All experiments were carried out in Scherer-Gillett (Model CEL 512-37 and Model CEL 25-7) growth chambers. The conditions for synchronous growth of each developmental stage of primary infection were as follows: The first hour after inoculation, plants were kept in darkness at 18 ± 1 C and ca. 100% relative humidity. The temperature was maintained at 22 ± 1 C and the relative humidity at 65 ± 5% for the remainder of the experiment. Low light intensity (200 ft-c from white VHO fluorescent tubes and 60 ft-c from incandescent bulbs) was given for the periods 1 to 6 and after 20 hr after inoculation.

Examination of fungal development.—The percentage of the parasite population in each stage of development was determined by direct microscopic observations at a magnification of 150 times with a B & L light microscope equipped with apochromatic objectives. Observations of the development of the parasitic units on 1-cm leaf sections (excluding the 1-cm tip section) were made at various times after inoculation. Each leaf section was observed once and discarded; observations at subsequent hours were made on other inoculated plants. Approximately 80-150 parasite units were counted at each observation. A minimum of five replications for each hour was used with each replication obtained on different days.

Preparation of enzyme solutions for the isolation of intact natural cuticles.—Cultures of *Myrothecium verrucaria* 460 obtained from the Quartermaster Laboratory, Natick, Mass., were grown on potato-dextrose agar slants until spores were produced, usually about 8 days. Sterile distilled water was added to the tubes, the mycelium chopped into small pieces, and the contents of the tubes put into 500 ml of Whitaker's salt liquid medium (17) containing 0.5 g glucose and 5 g ashless cellulose powder (product of W. & R. Balston Ltd., England). Vigorous reciprocal shaking at 22 C for 14 days resulted in nearly maximal amounts of cellulase activity in the medium (the term "cellulase" is used to denote the material that digests the cell walls of wheat and barley) (22). Cultural filtrates were evaporated under reduced pressure to 35 to 40 ml at 43 C in ca. 2 hr. This condensed crude solution was used as stock for the preparation of natural intact cuticle layers.

Preparation of natural intact cuticles.—Sections, ca. 3.5 cm long, of the lower (abaxial) epidermal layer of 6-day-old seedlings were peeled off and quickly transferred to an enzyme solution diluted 1:2 in a petri dish. The epidermal strips floated cuticle-side-up on the enzyme solution until the epidermal cells were loosened from the cuticle and sedimented to the bottom of the solution. Approximately 12 hr was needed to prepare cell-free cuticles. Isolated cuticles were washed and then transferred to a 2% agar surface and subjected to various experiments.

Reconstruction of wax layer.—Chloroform was used to extract the waxy substances from isolated intact cuticles or from fresh leaves of Chancellor wheat. Extraction was performed by dipping cuticles or leaves 3 times, 10 sec each time, into chloroform. To reconstruct a wax layer with approximately the same thickness as natural wax layers, the total surface area of extracted cuticles or leaves was calculated, and the chloroform solution was condensed so that the reconstructed film was of equal area. After the chloroform was completely evaporated, sterile water was gently poured onto agar surface. The reconstructed wax layers floated on the water surface were washed, then transferred to the surface of 2% agar and used.

RESULTS.—*Development of infection structures on compatible hosts.*—Over 90% of the conidia applied to the host leaves germinated and developed mature appressoria by 8 hr after inoculation. Approximately 75 to 80% of the applied conidia

TABLE 2. Formation of mature appressoria of *Erysiphe graminis* on isolated natural cuticles from near-isogenic barley and wheat lines

Near-isogenic lines	Appressoria formed by *Erysiphe graminis*[a]	
	Barley mildew (f. sp. *hordei*)	Wheat mildew (f. sp. *tritici*)
	%	%
Barley		
Manchuria (*mlx*)	88	85
Mla	92	
Mlg	89	
Mlk	86	
Mlp	90	
Wheat		
Chancellor (*pmx*)	83	86
Pm1a		94
Pm2a		90
Pm3a		91
Pm4a		86

[a] Eight hours after inoculation.

TABLE 3. Germination and formation of appressoria of *Erysiphe graminis* f. sp. *tritici* on upper and lower surfaces of isolated cuticle and epidermal strips of wheat 8 hr after inoculation

Surface	Germination	Appressoria	
		Malformed	Mature
	%	%	%
Isolated cuticle			
Upper face (with wax)	93	3	84
Lower face (without wax)	91	42	1
Epidermal strip			
Upper face (with wax)	93	2	89
Lower face (without wax)	90	45	2
Isolated cuticle grafted onto lower surface of the epidermal strip	89	5	82

produced ESH by 26 hr after inoculation.

Formation of mature appressoria on isolated natural cuticles from near-isogenic barley and wheat lines.—No significant differences were found in the formation of mature appressoria of either *E. graminis* f. sp. *hordei* or *E. graminis* f. sp. *tritici* among cuticles isolated from plants with different genes for reaction (Table 2). The formation of mature appressoria is not species-specific, since *E. graminis* f. sp. *hordei* produced nearly the same per cent of mature appressoria on isolated wheat cuticles as on barley cuticles. Similar results were also observed with *E. graminis* f. sp. *tritici* inoculated onto barley cuticles. The results obtained with isolated cuticles were consistent with results of other experiments in which intact plants were used (S. S. Masri, R. S. Slesinski, S. L. Yang, S. Hsu, R. E. Stuckey, and A. H. Ellingboe, *unpublished data*).

Formation of appressoria of Erysiphe graminis f. sp. tritici on various surfaces of isolated cuticles and epidermal strips.—About 85% mature appressoria were formed on the upper surfaces (with wax layers) of both isolated cuticles and epidermal strips 8 hr after inoculation (Table 3). Less than 5% mature appressoria were formed on the lower surface (without the wax layer), and over 40% of the appressoria formed were malformed (Fig. 1). The per cent of mature appressoria on isolated cuticles grafted onto the lower surface of epidermal strips was essentially the same as on the upper surface of intact epidermal strips.

Formation of mature appressoria of E. graminis f. sp. tritici on various artificial and reconstructed surfaces.—The percentages of mature appressoria formed ranged from 0 to 17% on the artificial surfaces tested (Table 4). Eleven per cent of the conidia produced mature appressoria on paraffin-coated cellulose paper, and 90% mature appressoria were formed on the isolated cuticles. Only 9 to 17% of the conidia formed mature appressoria on the reconstructed wax layers. Similar results were obtained whether the wax used for the reconstructed layers was extracted from fresh leaves or from isolated cuticles.

TABLE 4. Germination and formation of appressoria of *Erysiphe graminis* f. sp. *tritici* on various artificial and reconstructed surfaces 8 hr after inoculation

Surface	Germination	Appressoria	
		Malformed	Mature
	%	%	%
Isolated cuticle	98	2	90
Water agar (2%)	96	32	0
Glass slide			
Plain	30	0	0
Paraffin-coated	83	10	0
Cellulose paper			
Plain	93	15	0
Paraffin-coated	96	42	11
Reconstructed wax layer			
Wax from fresh leaf blade	62	43	9
Wax from isolated cuticle	90	54	17

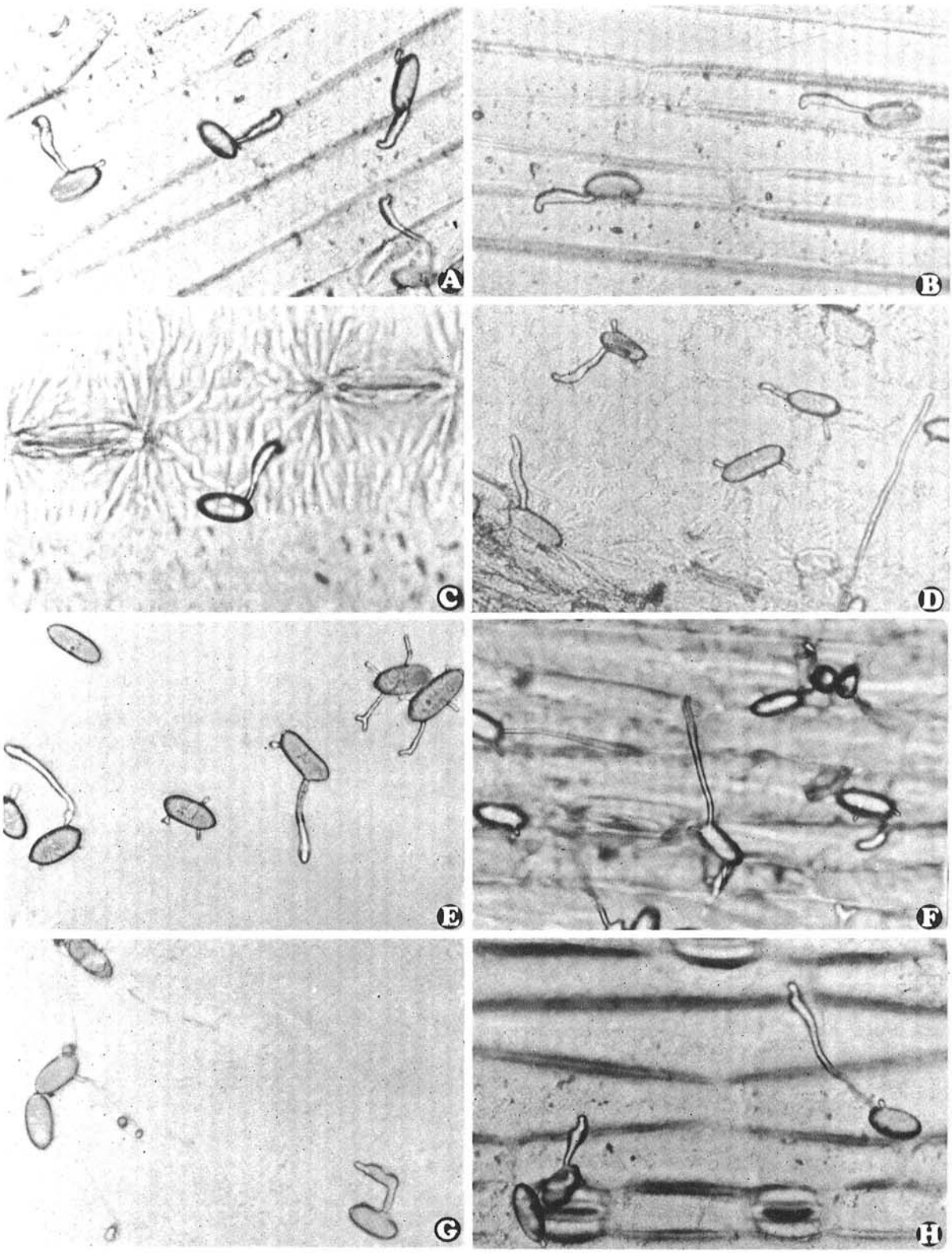
Formation of mature appressoria and elongating secondary hyphae by E. graminis f. sp. hordei on mutants affecting the wax layer of barley.—Eight hours after inoculation, the percentage of malformed appressoria produced on barley plants with the various mutations ranged from 14 to 27% (Table 5). The percentage of malformed appressoria depended upon which mutation was involved, and less than 1% of malformed appressoria were produced on Bonus and Manchuria. Reduction in the percentage of ESH 28 hr after inoculation ranged from basically no differences to 40%.

Formation of mature appressoria of E. graminis f. sp. tritici on isolated natural cuticles after washing with organic solvents.—The hypothesis that the wax layer is a determining factor for the formation of mature appressoria was also tested by inoculating isolated cuticles that had been washed thoroughly with chloroform and ether. Approximately 2 liters of

TABLE 5. Germination, formation of appressoria, and formation of elongating secondary hyphae (ESH) of *Erysiphe graminis* f. sp. *hordei* on barley seedlings with wax mutations (*cer*)

Host lines	Germination[a]	Appressoria[a]		ESH[b]
		Malformed	Mature	
	%	%	%	%
Manchuria	99	1	97	77
Bonus	97	1	96	71
Eceriferum mutations				
cer-J[59]	96	15	80	68
cer-J[71]	92	19	72	62
cer-Zd[67]	97	14	82	59
cer-Ze[81]	97	14	83	55
cer-Zj[78]	93	27	60	30

[a] Eight hours after inoculation.
[b] Twenty-eight hours after inoculation.


A
B
C
D
E
F
G
H

an organic solvent were flushed over a single 2.5-cm-long isolated cuticle during a period of 6 hr. Cuticles washed by the same method but with distilled water were used as control. The spore germination percentage was not significantly reduced, but the percentage of mature appressoria 8 hr after inoculation was ca. 15% less on the cuticles washed with either chloroform or ether.

DISCUSSION.—In the primary infection process of *E. graminis* on wheat and barley, the formation of mature appressoria appears to depend upon properties of the host surface. High percentages of mature appressoria were formed on the upper surface of epidermal strips (with cuticle), whereas few mature appressoria were observed on the lower surface of the epidermal strips (without cuticle) (Table 2). The cuticle, therefore, is the major factor that stimulated the formation of mature appressoria. The possibility that chemical substances are diffusing from epidermal cells and are responsible for the stimulation of the formation of mature appressoria is remote, since mature appressoria were observed to form on the upper surface of the enzymatically isolated cuticle, but few mature appressoria formed when the lower surface was inoculated. This suggested that the wax layer of the cuticle surfaces is a major factor for the maturation of appressoria. Malformed appressoria were prevalent on artificial surfaces which are believed to closely simulate the chemical and/or physical nature of the cuticle surface, including reconstruction of the wax layer with wax extracted from either fresh seedlings or the isolated cuticles. The appressoria were termed malformed by the fact that their irregular shapes were easily distinguishable from those of regular-shaped "normal" appressoria by microscopic observation. The malformed appressoria also gave no evidence of an attempt to penetrate the epidermis.

Under favorable conditions, over 90% of the inoculated conidia of either *E. graminis* f. sp. *tritici* or *E. graminis* f. sp. *hordei* produced mature appressoria on the leaf surface of wheat and barley, respectively, 8 hr after inoculation. Essentially the same percentages of mature appressoria were observed on the isolated intact cuticles from near-isogenic host lines with different dominant *Ml* or *Pm* genes. These results suggest that all the various parasite/host genotypes for incompatibility do not act to affect the development of the parasite before penetration. The *Ml* or *Pm* host genes tested must not have affected the cuticle structure, nor the diffusion of substances from the host epidermal cells. Moreover, conidia of both fungi produced approximately the same percentages of mature appressoria on cuticles isolated from either wheat or barley lines. The formation of mature appressoria is therefore not host-specific, at least in two species tested.

The hypothesis that the wax layer is a determining factor for the formation of mature appressoria was checked by inoculating plants with eceriferum (*cer*) mutations. The *cer* loci control the synthesis and/or excretion of the organ-specific wax components (8). Five eceriferum (wax) mutations, *cer-J*^{59}, *cer-J*^{71}, *cer-Zd*^{67}, *cer-Ze*^{81}, *cer-Zj*^{78}, were available for use. These mutations have been partially characterized both by chemical analysis and with a scanning electron microscope (21). Primary leaves of barley with the mutations *cer-J*^{59} and *cer-J*^{71} produce per unit area 30 and 56% less wax, respectively, than the wild type Bonus barley. The wax mutations at the *cer-J* locus give a much smaller percentage of primary alcohols and a somewhat larger percentage of esters than that of wild type Bonus (8, 21). Approximately a 20% reduction in the amount of wax was found on the seedling leaves of barley with mutations *cer-Zd*^{67} and *cer-Ze*^{81} (21). Morphologically quite different wax coats were found on plants with either *cer-Zd*^{67} or *cer-Ze*^{81}, in addition to the wax bodies similar to those found on wild type bonus. No obvious difference in the proportion of lipid classes composing the wax could be recognized with the latter two mutations, as compared to those of Bonus, the wild type barley (8, 21). Approximately 40% less wax was found on the primary leaves of plants with mutation *cer-Zj*^{78} than on wild type Bonus. The wax bodies were smaller, and were irregularly distributed over the surfaces of the leaf (21). A higher percentage of aldehydes was found in plants with *cer-Zj*^{78}, but the structural arrangements of the fibrils in the wax coating were less obvious.

Malformed appressoria were formed on the primary leaf surface of all five mutants. Much lower percentages of ESH were observed on plants with mutations *cer-Zd*^{67}, *cer-Ze*^{81}, and *cer-Zj*^{78} than was expected, based on the percentages of mature appressoria formed. This suggested that although some appressoria appeared normal, they did not function to give haustoria and ESH. Slightly malformed appressoria may be indistinguishable from mature appressoria by microscopic observation. The distribution of wax bodies may be an important factor for the formation of mature appressoria. The more homogenous the distribution of the wax bodies, the better may be the chance for each parasite unit to be stimulated to differentiate.

Though the eceriferum mutations cause a reduction in the percentage of mature appressoria, the final infection types were the same as with Manchuria and wild type Bonus (S. L. Yang and A. H. Ellingboe, *unpublished data*). Even though part of the

Fig. 1. Formation of mature (A, B, C) and malformed (D, E, F, G, H) appressoria on different natural and synthetic surfaces. *Erysiphe graminis* f. sp. *tritici* **A)** on wheat epidermis; **C)** on the upper surface of isolated wheat cuticle; **D)** on the lower surface of isolated wheat cuticle; **E)** on 2% water agar; **F)** on lower surface of wheat epidermal strip; and **G)** on reconstructed wax layer. *Erysiphe graminis* f. sp. *hordei* **B)** on wheat epidermis; and **H)** on wax mutant *cer-Zj*^{78}.

population of applied parasite units was stopped at the stage of malformed appressoria, some of the remaining parasite units did establish a compatible host-parasite relationship and produce pustules 6 days after inoculation. Since the formation and excretion of wax bodies were affected tremendously by the environmental factors (12), the wax layer could play a role in "field resistance".

The large percentage of mature appressoria formed on the isolated cuticles leached by the organic solvents is possibly due to wax molecules firmly impregnated into the cuticle structure (12).

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