Polypeptide Mapping by Two-Dimensional Electrophoresis and Pathogenic Variation in Field Isolates and Induced Mutants of *Erysiphe graminis* f. sp. *tritici*

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ABSTRACT

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Methods were developed to extract proteins from the mycelium and conidia of the obligate parasite *Erysiphe graminis* f. sp. *tritici* and the facultative parasite *Colletotrichum lindemuthianum*. These methods resulted in preparations suitable for separation by two-dimensional electrophoresis. Over 600 polypeptides were routinely visualized when stained with silver. Proteins were extracted from *C. lindemuthianum* grown in shake culture. Polypeptide maps of a *C. lindemuthianum* mutant and the wild type showed differences in the positions of as many as 10% of the polypeptides. Proteins were extracted from *E. graminis* f. sp. *tritici* grown on wheat, without any detectable extraction of the wheat leaf proteins. Polypeptide maps of three *E. graminis* f. sp. *tritici* mutants with increased virulence were indistinguishable from each other and from wild-type culture MS-1. The two field isolates, MS-1 and MO-10, had unique polypeptide maps that differed by five polypeptides. Independent segregations of the polypeptide differences and three known *P* gene differences were observed in progeny of a cross between MS-1 and MO-10.

The inheritance of disease resistance has been studied in almost every commercially grown crop because host plant resistance is an effective means of disease control. The inheritance of variability in the pathogen as well as in the host has been made in a smaller number of host-parasite combinations (5). These latter studies have demonstrated that each resistance gene (*R* gene) is expressed only when the pathogen has a corresponding avirulence gene (*P* gene). Although the genetics of resistance and avirulence in some host-parasite interactions are well-known, the molecular basis of specific resistance and avirulence in the restriction of development of a pathogen is unknown. We hypothesize that a *P* gene, its mRNA or its protein product specifically recognizes an *R* gene, its mRNA, or its protein product to form a protein-protein or a protein-nucleic acid complex, and that this molecular recognition determines the fate of the interaction. Certain characteristics of the interactions, particularly temperature sensitivity, support an hypothesis that the active products of *P* genes in a pathogen and/or *R* genes in a host are proteins (6).

Two-dimensional (2-d) electrophoresis is a very powerful analytical tool for the separation of proteins. Proteins are separated by isoelectric focusing in one dimension, and by molecular sieving in polyacrylamide gels in the second dimension. Small variations in charge or in molecular weight are detectable with this technique. Mutations that lead to an amino acid substitution can lead to a change in the net charge of a polypeptide. Such variants will lead to a displacement of the polypeptide in the isoelectric focusing dimension. Mutations of codons affecting chain termination can lead to a polypeptide of different size. Such variants will lead to a displacement of the polypeptide in the molecular sieving dimension. Mutations of *P* genes in the pathogen, whether artificially induced or naturally occurring, should be accompanied by detectable changes in the electrophoretic mobilities of the proteins responsible for the observed phenotypic changes.

This paper reports the extraction, separation, and visualization of total protein from the surface mycelium and conidia of the obligate parasite *E. graminis* f. sp. *tritici* and from the mycelium of *C. lindemuthianum*. The objective was to determine whether any of the polypeptides could be associated with particular *P* genes.

MATERIALS AND METHODS

Isolates of *Erysiphe graminis* f. sp. *tritici*. Two isolates of *Erysiphe graminis* f. sp. *tritici*, MS-1 and MO-10, were maintained on *Triticum aestivum* L. 'Little Club' or 'Chancellor' in separate growth chambers under standard conditions as described previously (7). MS-1 and MO-10 differ by at least three *P* genes and are of opposite mating type. They have been crossed and offspring were recovered with all eight possible combinations of the three *P* genes. (Offspring were provided by Dr. C. Bronson, Iowa State University, Ames 50011.) All isolates of *E. graminis* were either kept in separate growth chambers or, if more than one isolate was kept in a chamber, each was maintained on a wheat line susceptible to only one of the isolates in the chamber.

All wheat lines used were congenic. Each line contained a known *Pm* gene obtained by eight backcrosses to the recurrent parent Chancellor. A host line containing, for example, the *Pm*1a gene will be referred to as the *Pm*1a isoline. (Seed of the *Pm* isolines and cultivar Chancellor were supplied by Dr. J. G. Moseman, USDA, Beltsville, MD 20705.)

Growth of *E. graminis* on wheat is limited by an incompatibility system which exhibits genetic behavior of the usual gene-for-gene pattern (11). Therefore, strains found to be avirulent on the *Pm*1a isoline were assumed to possess the *P*1a gene and isolates virulent on the *Pm*1a isoline were assumed to possess a *P*1a allele.

Three isolates with increased virulence (from infection type 0 to infection type 4 on a 0–4 scale [9] against single *Pm* genes were derived from the wild-type culture MS-1 by mutagenesis with *N-methyl-N'*-nitro-*N*-nitrosoguanidine (NTG) as previously described (7). The mutations recovered in these isolates rendered the corresponding host genes *Pm*1a or *Pm*4a ineffective in promoting resistance. Mutant isolate lpl gave an infection rating of

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4 on the *Pm*1a isoline, whereas MS-1 gave an infection type 0. Mutant isolates 2p4 and 3p4 gave an infection rating of 4 on the *Pm*4a isoline, whereas MS-1 gave an infection type 0. We believe that isolate 1p1 had a mutation at the *P*1 locus (from *P*1 to *p*1), and that isolates 2p4 and 3p4 had independent mutations at the *P*4 locus (from *P*4 to *p*4). No other phenotypic changes between the mutants and the wild type were detected (7).

Protein extraction from *Colletotrichum lindemuthianum*. Proteins were extracted from a temperature-sensitive mutant of *C. lindemuthianum* and compared with those extracted from the wild type culture. The mutant was induced with NTG and was temperature-sensitive on agar media but not temperature-sensitive on the host (4). The mutant was not auxotrophic (*unpublished*). Both the mutant and the wild type were grown at 20 C in shake culture in a complete medium containing, per liter: 20 g glucose, 2 g Bacto-peptone, 0.46 g KH_2PO_4, 1 g K_2HPO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$. The mycelium of each isolate was collected in cheesecloth, rinsed twice with distilled water, frozen, and lyophilized. The lyophilized samples were ground to a dry powder with glass beads. About 0.5 g of ground mycelium was added to 10 ml of ice-cold SDS extraction buffer (SEB) containing 0.05 M tris-HCl, pH 6.8; 10 mM 1,4-dithio-L-threitol (DTT); 2% SDS; 0.4 mM $MgCl_2$; and 1 mM EDTA. The samples were held in a boiling water bath for 5 min with constant stirring, then cooled on ice and centrifuged at 4 C for 1 hr at 100,000 *g*. A volume of supernatant containing approximately 400 μg of protein was precipitated in 9 volumes of ice-cold acetone. The pellet was resuspended in 100 μl of SEB and sonicated until the pellet was dissolved. Solid urea (ultrapure from Schwarz-Mann) was added to a 9 M concentration, 200 μl of sample dilution buffer (SDB) (9.5 M ultrapure urea; 2% ampholytes from LKB Instruments; 10 mM DTT; 1 mM EDTA; 8% (w/v) Nonidet P40 from Particle Data Labs) was added, and the solution was vortexed until clear.

Protein extraction from *Erysiphe graminis* f. sp. *tritici*. Extractions were made from very dense growths of *E. graminis* f. sp. *tritici* seven days after inoculation on wheat seedlings. The *E. graminis*-covered leaves of 50 to 100 seedlings were immersed in 10 ml of degassed, ice-cold mildew extraction buffer (MEB) (0.05 M tris-HCl, pH 6.8; 0.04% SDS; 1 mM DTT; 0.5 mM $MgCl_2$; 1 mM EDTA; and 2% (w/v) Nonidet P-40). A No. 2 artist's brush was used to remove the surface mycelium and conidia from the immersed leaves, with minimal damage to the leaves. Buffer containing the fungus was then centrifuged for 30 min at 120,000 *g* at 4 C. The supernatant was assayed for protein content (2) and then precipitated in nine volumes of ice-cold acetone. The precipitate was dried under purified N_2, resuspended in SDB, and sonicated. It was crucial to perform all steps of the extraction quickly to avoid loss of resolution.

Electrophoresis and staining. The conditions used for 2-d electrophoresis and gel staining with Coomassie blue and silver have been described (6). At least ten replicate analyses were made of extracts from isolates MS-1, MO-10, 1p1, and 2p2. At least five replicate analyses were made of extracts from isolate 3p4 and of each of the progeny of isolates MS-1 and MO-10. Gels were compared visually and composite sketches were made using all replications.

RESULTS

The preliminary experiments with *C. lindemuthianum* grown in shake culture demonstrated the feasibility of extracting fungal mycelium in preparations suitable for separation by 2-d electrophoresis. Over 300 spots were seen when the gels were stained with Coomassie blue. When preparations of the wild-type culture were compared with preparations from the temperature-sensitive mutant grown at permissive temperatures, changes in the electrophoretic mobilities of at least 30 polypeptides were observed. This demonstrated that mutagenesis can affect a great many polypeptides, even when only one phenotypic change is obvious. All growth of this temperature-sensitive mutant on agar ceased at 28 C, although growth at 20 C was normal. Other than the polypeptide pattern, temperature-sensitivity was the only observed phenotypic change.

Since *E. graminis* f. sp. *tritici* is an obligate parasite, the mycelium was removed from the leaf surface of its natural host, and plant proteins might have been mixed with fungal proteins in the extracts. Uninoculated leaves were treated the same as the infected leaves, and the extracts were electrophoresed as a control to determine if any plant proteins were extracted from the leaf by this method. Ten very faint spots and two streaks appeared in these gels, when stained with Coomassie blue. When extracts were made from seedlings grown under sterile conditions (6), the spots were not visible, but the two faint streaks remained. The streaking was eliminated by using less brush pressure in removing the mycelium from the leaf. When the extracts from infected leaves were electrophoresed and stained with Coomassie blue, at least 300 peptides of *E. graminis* f. sp. *tritici* were routinely observed, with a maximum of about 400 on certain gels (Fig. 1, a and c). When silver stain was used, at least 600 spots were always visible, with a maximum of around 900 on the best gels (Fig. 1, b and d). Thus, the MEB effectively lysed and extracted the fungal mycelium, but it did not appear to penetrate the waxy cuticle of fresh, undamaged leaves and extract the leaf proteins.

The protein extracts from isolates MS-1 and the three mutants derived from MS-1 were compared following electrophoresis. No differences in electrophoretic mobility of any of the polypeptides could be found in 10 separate experiments. The polypeptide maps were essentially identical for any given pH range. Although apparent differences could occasionally be found among isolates, espeeially when the isolates were run in different experiments, such differences were always found to be either quantitative or not reproducible.

Proteins were also extracted and separated from another field isolate of *E. graminis* f. sp. *tritici*, MO-10. MO-10 differs from MS-1 by at least three *P* genes. Five qualitative differences in polypeptide positions were found in comparisons of the polypeptide maps of the two isolates. The polypeptide maps were unique to each isolate and highly reproducible. The positions of four of the five polypeptides that differentiate MS-1 from MO-10 are indicated by numbered arrows in the electrophoretograms shown in Fig. 1. The position of polypeptide no. 4 cannot be clearly distinguished on the gels shown and so is not indicated in Fig. 1. The alternate positions of polypeptide variants 1, 3, and 4 represent a change in the isoelectric focusing dimension, with no apparent change in molecular weight. These three changes are consistent with the hypothesis that the alternate alleles present in MS-1 and MO-10 code for one or a small number of amino acid substitutions that result in a net change in charge of the polypeptide but not in a size change. A polypeptide corresponding to polypeptide #2 could not be found in MS-1, and a polypeptide corresponding to polypeptide #5 could not be found in MO-10.

MS-1 is avirulent on isolines containing the genes *Pm*2a, *Pm*3a, or *Pm*4a, and its genotype may be written *P*2 *P*3 *P*4. MO-10 is virulent on these same isolines and its genotype may be written *p*2 *p*3 *p*4. A cross between MS-1 and MO-10 produced progeny of eight types, as expected with unlinked genes at three loci. The inferred genotypes of MS-1, MO-10, and their progeny, as well as the position each of the five variant polypeptides takes in the parental and progeny isolates are given in Table 1. In each of the progeny, polypeptide spots 1, 3, and 4 appeared in either the position characteristic of one parent or the other, but never both. The positions of polypeptide 3 and 4 could not be confidently determined for progeny 3 and 6, respectively, even though the entire procedure was performed at least five times for each culture. None of the five polypeptide spot positions could be correlated with a particular *P* gene. Instead, genes for virulence and genes governing spot position assorted independently.

DISCUSSION

Two-dimensional electrophoresis can theoretically resolve over 7,000 polypeptides on a single gel (10). In practice, however, comparative analyses of protein spots on crowded gels is much more limited. In certain areas of the gels examined in this study, the

TABLE 1. The segregation of three genes determining pathogenicity in the progeny of *Erysiphe graminis* f.sp. *tritici* cultures MS-1 × MO-10 compared to the gel position phenotypes of five polypeptides that differentiate MS-1 and MO-10

Isolate	Genotype[a]	Gel position taken by[b]				
		Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5
MS-1	*P2 P3* P4	MS-1	Absent	MS-1	MS-1	MS-1
MO-10	*p2 p3 p4*	MO-10	MO-10	MO-10	MO-10	Absent
Prog. 1	*P2 P3 P4*	MO-10	MO-10	MS-1	MS-1	Absent
Prog. 2	*P2 p3 P4*	MO-10	MO-10	MO-10	MS-1	MS-1
Prog. 3	*P2 p3 p4*	MO-10	MO-10	?	MO-10	Absent
Prog. 4	*P2 P3 p4*	MO-10	MO-10	MO-10	MS-1	MS-1
Prog. 5	*p2 P3 P4*	MO-10	MO-10	MO-10	MS-1	MS-1
Prog. 6	*p2 p3 P4*	MO-10	MO-10	MO-10	?	Absent
Prog. 7	*p2 P3 p4*	MO-10	MO-10	MO-10	MS-1	MS-1
Prog. 8	*p2 p3 p4*	MS-1	MO-10	MO-10	MO-10	Absent

[a] Genotypes are inferred from the reactions of each isolate on three differential host isolines: *Pm*2a, *Pm*3a, and *Pm*4a. For example, an isolate avirulent on the *Pm*2a isoline is inferred to carry the corresponding *P*2 gene. An isolate virulent on *Pm*2a is inferred to carry the *p*2 allele.

[b] Peptides 1, 3, and 4 were always found in a position corresponding to that of one of the parents, MS-1 or MO-10, but never in both positions. Peptide 2 was either present, as in MO-10, or absent, as in MS-1. Peptide 5 was either present, as in MS-1 or absent, as in MO-10.

number of polypeptides exceeded the resolving power of the system. When silver was used to stain a gel that had already been stained with Coomassie blue, about twice as many spots were revealed than were originally observed (compare a to b and c to d in Fig. 1). The silver stain often revealed spot clusters that had the appearance of single spots when stained with Coomassie blue alone. The presence or absence of a spot within such a cluster was often difficult to determine. In areas of overcrowding, it is possible that peptides of the same or very similar isoelectric point and molecular weight masked polypeptides that were actually missing. Even for the five polypeptide differences detected between MS-1 and MO-10, replications were essential for interpretation.

The polypeptide maps obtained from a wild-type isolate of *C. lindemuthianum* and its mutant showed that a mutant induced with NTG can differ in many polypeptides. The great variation suggests that the mutation affected a basic part of the protein synthesis apparatus. For example, a mutation in a gene affecting the specificity of an amino-acyl *t*-RNA synthetase would be expected to affect many proteins. Because the sexual stage of this fungus is unknown, it was not possible to readily determine whether the mutation(s) induced with NTG affected one gene, or many.

The few differences observed in polypeptide maps for different cultures of *E. graminis* f. sp. *tritici* were surprising. Mutagenesis with NTG was expected to affect many genes. Yet when three mutants to increased virulence, obtained by NTG treatment, were compared to the wild type, no differences were detected among approximately 600 polypeptides. Furthermore, only five

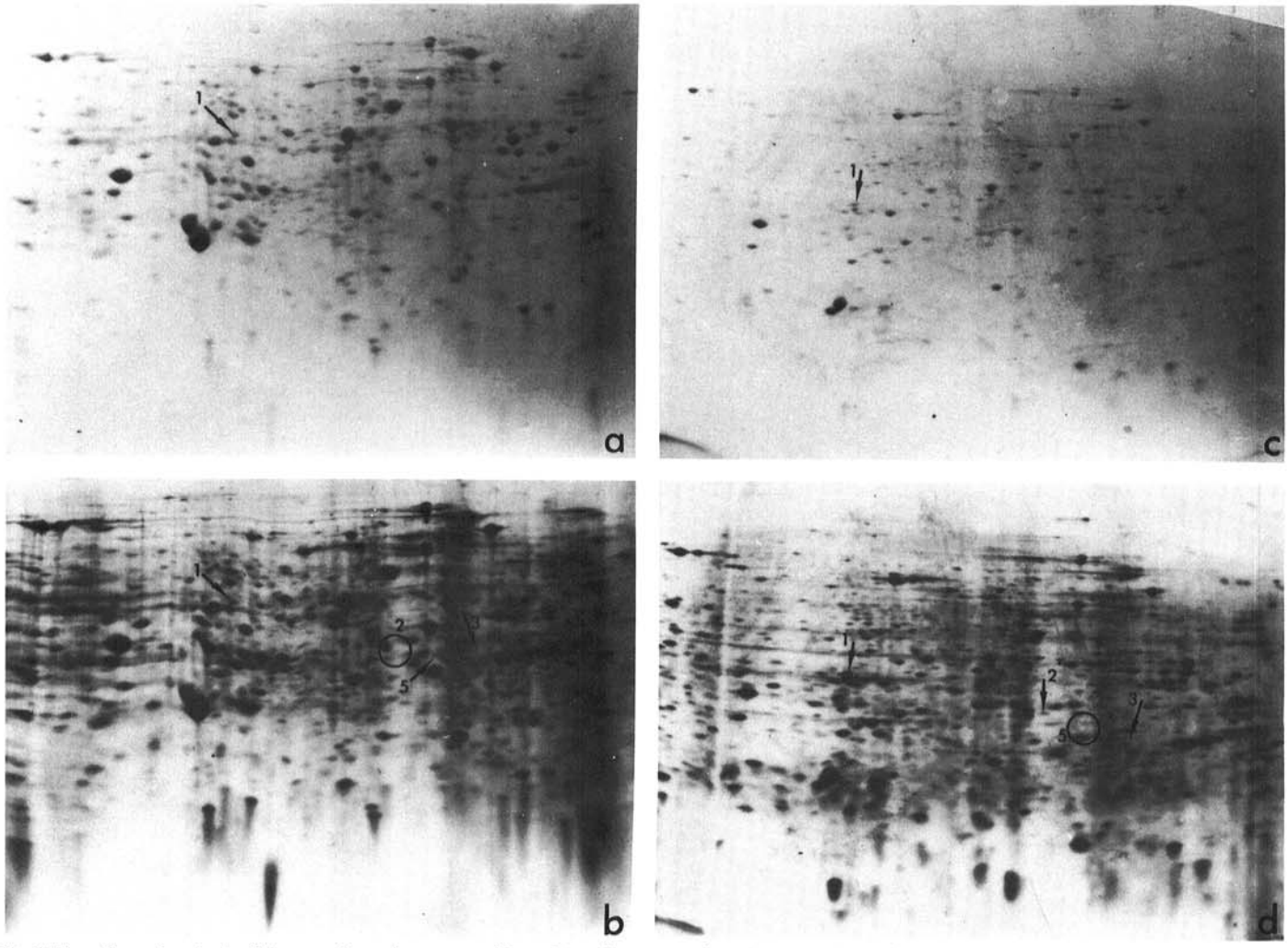

Fig. 1. Two-dimensional gels of denatured proteins extracted from *Erysiphe graminis* f. sp. *tritici*. **a,** MS-1, stained with Coomassie blue. **b,** The same gel as in "**a,**" but stained with silver after staining with Coomassie blue. **c,** MO-10, stained with Coomassie blue. **d,** The same gel as in "**c,**" but stained with silver after staining with Coomassie blue. The numbered arrows and circles indicate the relative positions of four variant polypeptides that distinguish MS-1 and MO-10 from each other.

polypeptide differences were observed between two different field isolates, MS-1 and MO-10. MS-1 was isolated from wheat in East Lansing, MI, in 1961 and has been maintained on Little Club wheat in a growth chamber for 19 yr. MO-10 was isolated from cleistothecia on wheat carrying the *Pm*4 gene in Monroe County, MI, in 1977. That cultures of such diverse history have so little electrophoretic variation is interesting. Conspecific plant populations have been found to exhibit electrophoretic variation in about 5% of the proteins observed (8). Similarly, natural populations of conspecific fruit flies (3) and rodents (1) exhibit variation in about 4–10% of the total proteins visualized by 2-d electrophoresis. Is it possible that an obligate parasite such as *E. graminis* is so precisely adapted to its host that almost any variation is lethal?

The low level of variation found in *E. graminis* f. sp. *tritici* was advantageous because it eliminated the need for following the segregation patterns of a large number of polypeptides. Progeny from a cross between MS-1 and MO-10 showed segregation of only five polypeptide variants. *E. graminis* f. sp. *tritici* is haploid. When a difference in position of a peptide is found between two isolates, progeny of a cross between the two should exhibit one parental type or the other, but never both if the two forms are the product of each of two alleles of one locus. This is what was found. No latent differences appeared as a result of segregation. The segregation pattern in the progeny of MS-1 × MO-10 showed that the peptide differences between the parents were not the products of the *P* genes examined (*P*2a, *P*3a, or *P*4a).

The three independently derived mutants to increased virulence were found to give polypeptide maps indistinguishable from that of the parent MS-1. The failure to find a polypeptide whose net charge was changed concommitantly with a mutation to increased virulence was not surprising because only about 600 polypeptides were observed; this probably is only a small portion of the total number of polypeptides present in the pathogen. The methods used revealed only the more abundant proteins. Furthermore, only approximately 30% of any mutational changes in nucleotides can be expected to lead to amino acid substitutions that give the peptide a net change in charge (12). Perhaps the three mutants examined did not contain a charge change in the peptides responsible for the changes in virulence.

The extraction procedures outlined here give highly reproducible polypeptide patterns on 2-d gels. We have other results suggesting that the patterns can be quite different if other extraction procedures are used. The number of peptides visualized can be increased by expanding the pH gradient and thus the resolution of the 2-d gels. Because at least 10 *P* loci are available for mutational analysis in *E. graminis* f. sp. *tritici,* the possibility of finding a product of a *P* gene with many mutants at each *P* locus is not unrealistic.

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