Genetics

Isozyme Variation of Physiologic Races of *Ustilago hordei*

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ABSTRACT

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Sixty-three haploid isolates of the barley covered smut fungus, *Ustilago hordei*, were examined for isozyme variation by starch gel electrophoresis. Fifty-five isolates from North Dakota, representing different virulence genotypes, and eight isolates from Ethiopia of unknown genotype were tested. Activity was detected for nine enzymes. A single allele common to all isolates was detected for aconitase, adenylate kinase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase, and peptidase. Two alleles were detected for each of the enzymes isocitrate dehydrogenase and malate dehydrogenase, and three alleles, including a null, for phosphoglucomutase (PGM) were detected. In these latter three enzyme systems, greater than 70% of all isolates had the most common allele. Isolates from Ethiopia could not be differentiated from isolates from North Dakota by isozymes. Five electrophoretic types and two clusters based on the isozyme data were identified using genetic diversity and cluster analysis. Clusters from isozyme data in no way coincided with those based on virulence data. Mean allelic diversity was 0.189 based on isozyme data and 0.401 based on virulence data. Haploid sporidia from F_1 progeny also were obtained from a cross of race 7 × race 11 isolates, which carried two of the three alleles for PGM, and were analyzed. Isozyme patterns revealed that the enzyme PGM is coded for by at least two different alleles at a single locus.

Ustilago hordei (Pers.) Lagerh. is the causal agent of covered smut of barley (*Hordeum vulgare* L.). It occurs throughout the world wherever barley is grown. The life cycle of the fungus begins when a seed- or soilborne, diploid teliospore germinates to form a promycelium. The nucleus migrates into the promycelium, meiosis occurs, and four haploid sporidia form (23). The fungus has a bipolar mating system, so that two sporidia of opposite mating type are required for fusion and formation of a dikaryotic mycelium. Young barley seedlings can be infected by this mycelium. The mycelium infects through the coleoptile and grows along with the growing point of the plant. The mycelium invades the floral tissues; karyogarny occurs followed by the formation of teliospores from the mycelium. In this manner, the floral tissues are replaced by masses of teliospores contained within membranous sacs or sori. Spores are liberated by threshing and thus can contaminate seed and soil.

In the laboratory, teliospores germinate after 15 h on water agar, and the four haploid sporidia can be separated by micromanipulation. These sporidia can be transferred separately to potato-dextrose agar, where they divide in a yeastlike manner to produce haploid cell cultures. Sporidial cultures can then be maintained on agar plates or slants and can be preserved on silica gel for long-term storage. Such ease of manipulation and storage make *U. hordei* an ideal fungal pathogen for laboratory studies.

Physiologic specialization in *U. hordei* was alluded to as early as 1924 (10). Tapke (27,28) identified the existence of 13 different races based upon the differential host response of eight barley cultivars. The actual genes governing virulence in the pathogen and resistance in the host have been elucidated in more recent work (8,9,26,29).

The literature contains numerous reports on the usefulness and successfulness of isozyme analysis in studies of taxonomy, population genetics, and evolution. Isozyme analysis has allowed researchers to differentiate different pathogenic species that infect the same host or have similar host ranges (22,31). The differentiation of species within a given genus has been most successful (2,3,6,19,20). Such a method for the determination of species is useful for identification purposes in quarantine situations, disease resistance programs, and for additional verification of distinct species previously based on often variable morphological characteristics. Intraspecies differences have also been detected and have identified and/or confirmed classification of formae speciales (4,6,19,20,22). Actual race differences have not been as successful (11). Evolutionary pathways of species (7) as well as hybridization

and population genetics have been clarified by the use of isozyme analysis (12,25,30).

The purpose of this study was to evaluate isolates of *U. hordei* for isozyme activity, determine the amount of isozyme variability present among races of *U. hordei*, and to examine the inheritance of specific enzymes between race 7 and race 11.

MATERIALS AND METHODS

Haploid sporidia of *U. hordei* stored on silica gel were obtained from the laboratory of the late Dr. Person, Department of Botany, University of British Columbia, Vancouver, British Columbia. Of the 63 sporidial isolates analyzed in this study, 55 were derived from teliospores collected in North Dakota (Table 1) and represented 11 of the 13 races as originally described by Tapke (27,28). The remaining eight isolates were obtained from teliospores collected in Ethiopia. In addition, 32 sporidia from F_1 teliospores, from 1987 experiments (21), were analyzed. These teliospores previously were obtained from crosses between compatible sporidia from two single teliospores representing race 7 and race 11. From each of the eight crosses made, a single teliospore was chosen, and its four sporidia were analyzed.

Sporidia were grown on potato-dextrose agar (PDA) and then transferred to fresh PDA plates and slants. Cultures were maintained at room temperature for 2–3 wk and then stored at 10 C in the dark. Transfers to fresh media were made every 6–8 mo.

Two to three small square agar blocks (0.5 cm) of sporidia were removed from the PDA plates and transferred to approximately 50 ml of potato-dextrose broth in 125-ml Erlenmeyer flasks. Shake cultures (Lab-Line Orbit Environ-Shaker, Lab-Line Instruments, Melrose Park, IL) were grown for 1 wk; sporidia were collected by centrifugation (Beckman Model J2-21M, JA-17 rotor, Beckman Instruments, Fullerton, CA) for 10 min at 550 g, and homogenized to a fine powder with liquid nitrogen by using a mortar and pestle.

To obtain the protein extract, 1 ml of homogenization buffer (1.21 g of Tris, 0.292 g of EDTA, 38 mg of NADP, 1 L of water, pH 6.8) was added to the powdered sporidia. The samples then were spun down with a Sero-Fuge centrifuge (Clay Adams, Parsippany, N.J.) at 3,400 rpm for 7 min. The supernatant was collected into disposable culture tubes and frozen at −80 C until used.

Horizontal starch gel electrophoresis was used and the procedures given by Selander et al (24) were followed. Buffer and stain recipes (Table 2) followed those given by Selander et al (24) and May et al (18). Homogenization, electrophoresis, and analysis of all isolates were done three times.

Band migration was measured, and relative mobility was calculated using the main band of isolate 65, which was arbitrarily chosen as the standard. Bands were attributed to alleles at single loci; alleles were numbered sequentially from the anodal end of the gel. Only the predominantly stained band was scored when multiple bands appeared. Capital-lettered abbreviations refer to enzymes, whereas abbreviations with only the first letter capitalized refer to the specific loci coding for the enzyme (18).

A statistical computer program "ETDIV and ETCLUS" (T. S. Whittam, Department of Biology, The Pennsylvania State University, University Park, PA) was used to calculate genetic diversity at each enzyme locus, to generate a genetic distance

TABLE 1. Isolate number, origin, race designation, electrophoretic type, and alleles at nine enzyme loci for 63 isolates of *Ustilago hordei*[a]

Isolate number and origin	Race[c]	ET[d]	Enzyme Locus[b]								
			Aco	*Ak*	*G-6-p*	*Idh*	*Mdh*	*Pep*	*Pgm*	*6-Pg*	*Pgi*
North Dakota											
12–17	1	1	1	1	1	1	1	1	1	1	1
20–23	3	2	1	1	1	2	1	1	0	1	1
28, 31	4	1	1	1	1	1	1	1	1	1	1
36, 37, 39, 40	6	3	1	1	1	1	2	1	1	1	1
42–44, 47, 48	7	1	1	1	1	1	1	1	1	1	1
50–52	8	4	1	1	1	2	1	1	1	1	1
53–56	9	1	1	1	1	1	1	1	1	1	1
57–60, 62	10	4	1	1	1	2	1	1	1	1	1
65–72	11	5	1	1	1	1	1	1	2	1	1
73, 74, 77–80	12	1	1	1	1	1	1	1	1	1	1
81–88	13	1	1	1	1	1	1	1	1	1	1
Ethiopia											
127–129, 131–135	ND	1	1	1	1	1	1	1	1	1	1

[a]Alleles were numbered sequentially from the anodal end of the gel.
[b]Enzymes as given in Table 2.
[c]Race as determined by Tapke (27, 28); ND = not determined.
[d]Electrophoretic type as determined by diversity analysis.

TABLE 2. List of enzymes analyzed, enzyme commission numbers, buffer systems, number of alleles per locus, and genetic diversity determined for haploid isolates of *Ustilago hordei*[a]

Enzyme[b]	E.C. number	Buffer[c]	Number of alleles	Genetic diversity
Aconitase (ACO)	4.2.1.3	B	1	0.00
Adenylate kinase (AK)	2.7.4.3	H	1	0.00
Glucose-6-phosphate dehydrogenase (G-6-P)	1.1.1.49	A	1	0.00
Isocitrate dehydrogenase (IDH)	1.1.1.42	H	2	0.60
Malate dehydrogenase (MDH)	1.1.1.37	A	2	0.40
Peptidase (phenylalanyl-L-leucine) (PEP)	3.4.x.x	B	2	0.00
Phosphoglucomutase (PGM)	2.7.5.1	A	3	0.70
6-Phosphogluconate dehydrogenase (6-PG)	1.1.1.43	A	1	0.00
Phosphoglucose isomerase (PGI)	5.3.1.9	H	1	0.00

[a]See Materials and Methods for calculation.
[b]Stain recipes from Selander et al (24).
[c]Buffer recipes A and B from Selander et al (24). Buffer A: tray buffer = tris-citrate, pH 8; gel buffer = tray buffer diluted 1:30, run at 130 volts. Buffer B: tray buffer = tris-citrate, pH 6.3; gel buffer = tris-citrate, pH 6.7, run at 150 volts. Buffer recipe H from May et al (18). Buffer H: tray buffer = histidine; pH 6.5; gel buffer = tray buffer diluted 1:4, run at 350 volts.

matrix, and to perform a cluster analysis. Genetic diversity was calculated as follows: $h = 1 - \Sigma\, x_i^2\, [n/(n-1)]$, in which x_i = frequency of the ith allele at the locus and n is the number of isolates in the sample. Races were assigned to electrophoretic types (ET) based on unique combinations of alleles at each of the nine loci (multilocus genotype). The genetic distance matrix was generated by calculating the number of different alleles between all possible pairwise combinations of ETs. The distance matrix was subjected to the cluster analysis that grouped the ETs according to relatedness. Based on the calculated distance matrix and cluster analysis, a dendrogram was drawn representing a hypothetical phylogenetic tree indicating the average genetic divergence among races.

Virulence data from Tapke (27,28) were also analyzed with ETDIV and ETCLUS to calculate genetic diversity and genetic distance. The avirulent phenotype was assigned to races that produced <10% smutted heads and the virulent phenotype was assigned to races that produced ≥10% smutted heads.

RESULTS

A total of 21 enzymes in five different buffer systems were stained; however, only nine enzyme-buffer combinations had sufficient activity to be scored reliably (Table 2). A single allele common to all isolates was detected in the enzyme systems aconitase (ACO), adenylate kinase (AK), glucose-6-phosphate dehydrogenase (G-6-P), peptidase (PEP), phosphoglucose isomerase (PGI), and 6-phosphogluconate dehydrogenase (6-PG) (Table 1).

For the enzymes isocitrate dehydrogenase (IDH) and malate dehydrogenase (MDH), two alleles were observed for each enzyme. For IDH, isolates had either allele 1 corresponding to the electromorph having a relative mobility of 100 (isolates of races 1, 4, 6, 7, 9, 11, 12, 13, and the Ethiopian isolates) or allele 2, which corresponded to an electromorph having a relative mobility of 70 (isolates of races 3, 8, 10). For MDH, allele 1 (corresponding to an electromorph with a relative mobility of 100) was common to all isolates except those of race 6. Allele 2 was present in race 6 isolates and corresponded to an electromorph with a relative mobility of 66.

For the enzyme phosphoglucomutase (PGM), three alleles were detected. Allele 1, which corresponded to a band with a relative mobility of 111, was present in isolates of races 1, 4, 6, 7–10, 12, 13, and the Ethiopian isolates. Race 11 isolates had allele 2, corresponding to a band having a relative mobility of 100. Race 3 isolates were assigned a null allele, as no bands were detected.

F_1 progeny from the cross of race 7 × race 11 were analyzed for the enzyme PGM, as this enzyme differentiated race 7 isolates having allele 1 from race 11 isolates having allele 2. A clear 1:1 segregation pattern was noted; for each cross, two sporidia carried allele 1 (relative mobility of 113) while the other two sporidia carried allele 2 (relative mobility of 100) (Fig. 1). This indicates that PGM is coded for by at least two different alleles at a single locus.

Genetic diversity at the nine enzyme loci ranged from 0.0 to 0.70 with an average diversity of 0.189 (Table 2). Isolates were grouped by race as no isozyme polymorphisms were detected within races; races were assigned to one of five electrophoretic types (Table 1) based on their unique combinations of alleles at each of the nine enzyme loci. Isolates of races 1, 4, 7, 9, 12, 13, and the Ethiopian isolates all belonged to a single electrophoretic type (ET 1). Likewise, isolates of races 8 and 10 had identical alleles and were assigned to ET 4. As shown in Figure 2A, isolates of race 3 (ET 2), and ET 4 were closely related having a genetic distance of 0.111 as were ET 1 and isolates of race 11 (ET 5). Isolates of race 6 (ET 3) were related to ETs 1 and 5 at a distance of 0.125. ETs 1, 5, and 3 formed one cluster, which was related to the second cluster composed of ETs 2 and 4 at a distance of 0.169.

Based on the virulence data, races appeared to fall into four clusters (Fig. 2B). Cluster 1 was composed of races 4, 10, and 11; cluster 2 of races 5 and 6; cluster 3 of races 1, 7, 8, and 12; cluster 4 of races 2, 3, 9, and 13. Mean allelic diversity (0.401) and genetic distances between races were much greater based on virulence data than based on isozyme data.

DISCUSSION

The original differentiation of races in *U. hordei* was made by Tapke (27,28). Thirteen races were established based on the differential host response of eight barley cultivars. In the present study, a single allele common to all isolates representing 11 of the 13 races was detected for six enzyme systems. In the remaining three enzyme systems, there were two different alleles observed. In these cases, more than 70% of all the isolates had the most common allele. Allele differences were detected among some races but never within a race; no race was unique at all loci that were polymorphic. Thus, from these results, it becomes apparent that races of *U. hordei* cannot be identified solely by the isozymes and buffer systems used in this study.

Genetic diversity analysis, however, revealed five ETs. The ETs were closely related but could be grouped into two clusters. The mean diversity calculated (0.189) over all nine enzyme loci was greater than that obtained for other smut fungi (e.g., 0.03 for *Ustilago spinificis* [1], 0.074 for *Tilletia indica* [3], and 0.048 for *Ustilago bullata* [14]. The limited number of samples and loci analyzed could account for the differences in genetic diversity.

Because races are differentiated by distinct virulence genes, genetic distances among races were much greater when the virulence data was analyzed. Furthermore, no relationships could be determined between clusters obtained by isozyme analysis and those obtained by virulence analysis. In almost all cases, races that were genetically close based on isozyme data were genetically distant based on virulence data. Such results support the idea of greater homogeneity at enzyme loci than at virulence loci.

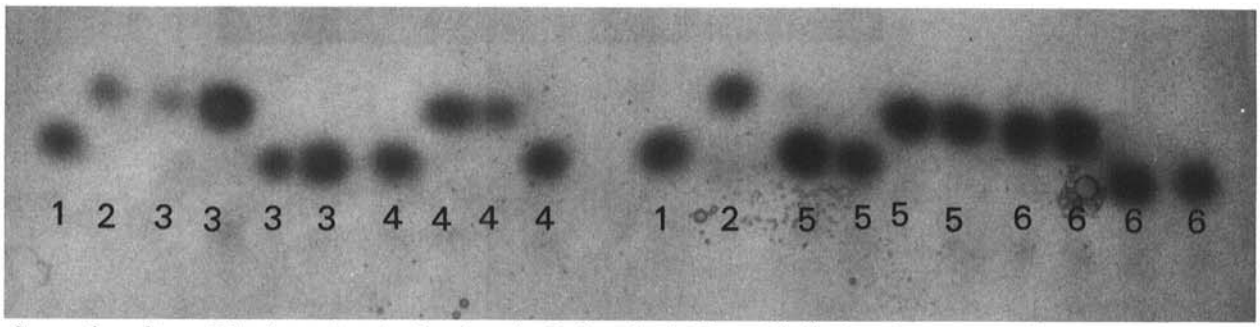

Fig. 1. Segregation of two alleles detected at the *Pgm* locus in *Ustilago hordei* F_1 sporidial isolates from a cross of race 7 × race 11 and parental isolates. Each F_1 teliospore produces two sporidia carrying each parental allele. Lane 1 is a single sporidial isolate from a teliospore of race 7, which was homozygous for allele 1. Lane 2 is a single sporidial isolate from a teliospore of race 11, which was homozygous for allele 2. The four sporidial isolates from single F_1 teliospore 3 (lanes numbered 3) carry the two *Pgm* alleles in a 1:1 ratio, as do F_1 teliospores 4 (lanes numbered 4), 5 (lanes numbered 5), and 6 (lanes numbered 6).

Gill and Powell (11) found isozymes of little use in differentiating physiologic races of *Phytophthora fragariae*. In other research (4–6,12,13,15–17,20), intraspecies differences have been detected, but have fallen into groups along the lines of pathotype or formae speciales. For instance, Bosland and Williams (4) found that isolates of *Fusarium oxysporum* could be divided into three groups that corresponded to natural host species. Thus, formae speciales could be distinguished from one another. Formae speciales of *Puccinia recondita* and *P. graminis* could also be differentiated by their isozyme phenotypes (6). No variation was detected within the formae speciales, namely *P. recondita* f. sp. *tritici* and *P. graminis* f. sp. *tritici* (5). Jeng and Hubbes (13) were able to differentiate between nonaggressive and aggressive strains of *Ceratocystis ulmi* using isozymes, and later Jeng et al (12) differentiated between Eurasian and North American strains within the aggressive strain group. These differences, however, also coincided with previously established morphological differences. Leung and Williams (15) found that 95% of the *Magnaporthe grisea* isolates from rice fell into only two groups, despite a high degree of variability in virulence among these isolates. Similarly, Linde et al (16) and Lu and Groth (17) identified only two to three groups within *Uromyces appendiculatus* despite the presence of a wide array of virulence phenotypes. Isolates of *Phytophthora megasperma* were divided into six groups that corresponded to host range (20). Based on these findings, relatively little if any variation has been found in isolates of a given species that infect a given host. Thus, the failure to separate races within *U. hordei* was not surprising.

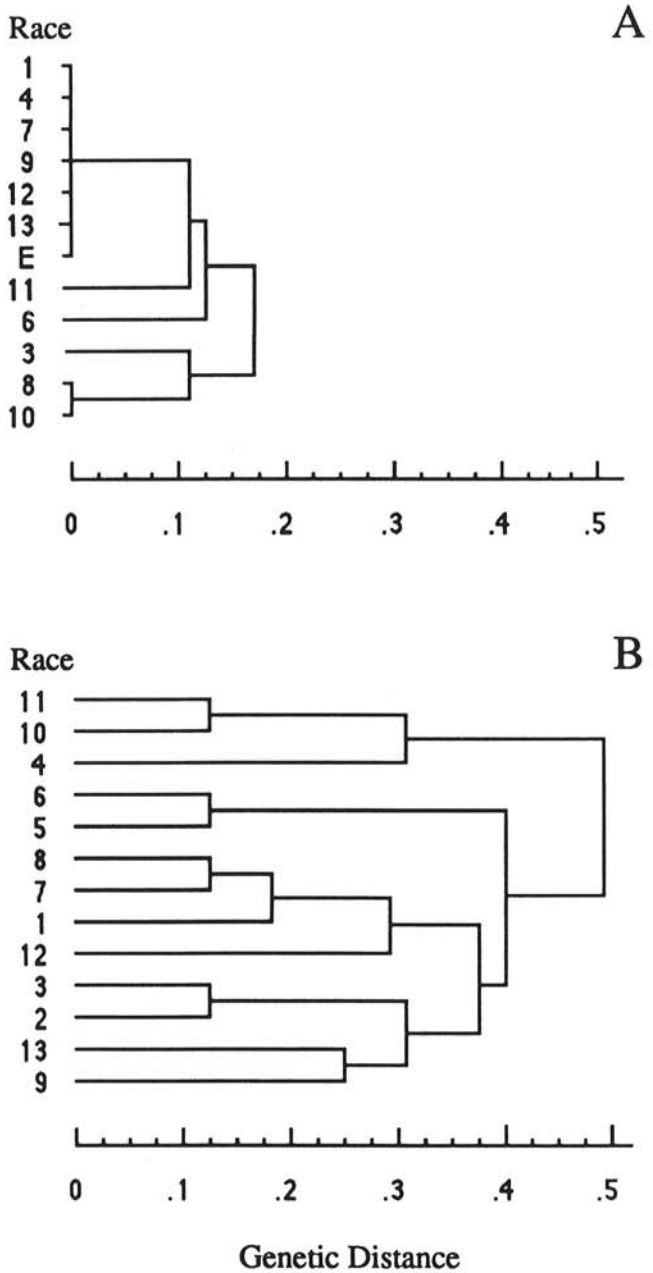

Fig. 2. Dendrogram illustrating genetic distances among races of *Ustilago hordei* based on **A,** isozyme data from Table 1 and on **B,** virulence data from Tapke (27,18). Isozyme data included 11 of 13 races and isolates from Ethiopia. Virulence data included only the 13 races.

The Ethiopian isolates produced identical banding patterns to those of the isolates from North Dakota. In the case where two different banding patterns were obtained, the Ethiopian isolates had the predominant banding pattern. Such uniformity between isolates from different geographic origins has been noted frequently. Bosland and Williams (4) found no differences in banding patterns based upon geographic origin in isolates of *F. oxysporum*. Likewise, Kirby (14) found that *U. bullata* from Australia and New Zealand were the same. Linde et al (16) and Lu and Groth (17) found no correlation between geographic origin and isozyme phenotype in *U. appendiculatus*. Leung and Williams (15) found relatively little variability between 335 isolates of *M. grisea* collected from rice hosts from 12 different geographic locations around the world. Newton et al (19) found intragroup uniformity among isolates of *Puccinia striiformis, P. recondita*, and *P. hordei*, despite diverse origins.

The lack of isozyme variation observed in the present study has significant meaning. The fact that the isozymes investigated are direct gene products distinguishes them from virulence markers, which involve not only the pathogen's genes, but the host's genes as well. Because of this, isozymes may be subject to different selection pressures. This feature, in itself, may in part explain the small variation detected in this study. The selection pressure on these enzymes may be so small that there is little advantage to be gained by enzyme variation. This would greatly lessen the value of isozyme analysis as a marker for pathogenic variability, an idea already alluded to by Leung and Williams (15).

Bonde (3) proposed that the high degree of isozyme variability in *T. indica* was due to the fact that basidiospores of the same basidium did not fuse, thus promoting greater genetic recombination. In contrast, almost no isozyme variability was found in either *T. foetida* or *T. caries*, in which basidiospores of the same basidium can fuse. This idea may also be true of *U. hordei*. The low isozyme variability may be due to the fact that compatible sporidia from the same promycelium fuse, thus promoting inbreeding and decreasing any variation which would result from fusion of sporidia from different promycelia.

The origin and evolution of species, or strains within species, has been elucidated in the work of several researchers. Isozyme analysis allowed for the detection of progeny resulting from either hybridization or selfing (25). The high degree of pathogenic diversity in *Phytophthora infestans* is believed to be due to interbreeding within the sexual population in Mexico, rather than the wide host range found there. Similarities in isozyme patterns between Australian and African rust isolates lead Burdon et al (7) to hypothesize an African origin of some of the Australian rusts. Lack of variation between other rust races indicated a possible common origin. Kirby (14) suggested a common origin for isolates of *U. bullata* from New Zealand and Australia based on the lack of isozyme polymorphisms. One might draw similar conclusions for *U. hordei* (i.e., that the isolates collected in North Dakota had a similar origin).

The lack of variation between isolates from North Dakota and those from Ethiopia also may point to a common ancestor. Adaptations for better fitness in the environment and to different host cultivars probably did not affect isozyme loci. However, a greater number of isolates from a broader geographic range and additional loci would have to be analyzed to support such a conclusion.

Inbreeding, common origin, and the lack of necessity for isozyme variation are therefore three explanations for the few iso-

zyme polymorphisms detected in *U. hordei*. Isozymes appear to be of greater use in interspecies differentiation, rather than intraspecies differentiation. The limited variation in isozymes as compared to virulence and the lack of variation based on geographic origin correspond well to the findings of other researchers.

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