ISOZYME AND NUCLEIC ACID COMPOSITION OF PUCCINIA HELIANTHI - UNIFORMITY IN ALLOZYMES AND DS RNA MOLECULES

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SUMMARY

Soluble proteins and nucleic acids extracted from germinating urediniospores of 15 isolates of Puccinia helianthi collected in Australia were examined for isozyme and dsRNA banding patterns following electrophoresis. Sixteen enzymes were detected, but the isozyme phenotypes were invariant for all isolates. Double-stranded RNA molecules were found in P. helianthi, and the ten molecules detected ranged in size from 1090 to 5080 base pairs. The dsRNA patterns formed were uniform for all isolates. The virus-like particles in P. helianthi urediniospores were observed by electron microscopy to be spherical particles of estimated size 37.5 nm. The monomorphic nature of the isozymes and dsRNA molecules in P. helianthi in Australia contrasts with the variation in the virulence of the rust isolates.

INTRODUCTION

Characterization of Puccinia helianthi (sunflower rust) has been predominantly investigated by virulence variation in association with rust-resistance sunflower breeding programs (Putt and Sackston 1963; Sackston 1962). These studies have shown Puccinia helianthi to be polymorphic for genes determining virulence although the level of polymorphism varies with time and with location. In 1983, only two races of rust (races 1 and 3) were detected in eastern Australia (Kochman and Goulter 1984) based on patterns of reaction observed on differential sunflower lines developed in Canada (Sackston 1962). In contrast, all four races of rust that are able to be identified using these differential lines have been detected in Canada, the United States, and Argentina.

A study of the pathogenicity of P. helianthi throughout eastern Australia showed extensive virulence variation (Dry and Downes, unpublished data). An increased number of races of rust other than the two races previously identified by the Canadian differentials were detected. Some of these isolates were chosen for further analysis.

Potential alternative approaches to characterize the races of a fungal pathogen involve the use of distinctive soluble protein markers especially isozymes or dsRNA molecules occurring in the cytoplasm of the cell. Isozyme markers could be useful to determine genetic variation in soluble enzymes within Puccinia helianthi. Isozyme variation has been demonstrated within a wide range of fungal species including Neurospora (Spieth 1975), Pyricularia oryzae (Matsuyama & Kosaka, 1971) and Puccinia graminis f. sp. tritici (Burdon et al., 1982). Previously some of the isozymes present in Puccinia helianthi (Dry, 1985) were identified and here the genetic variability of these molecules among distinct isolates of this species is examined.

The nucleic acid composition of rust fungi has only recently been investigated. Pryor and Boelen (1987) demonstrated the presence of a number of double-stranded RNA molecules in P. sorghi using agarose gel electrophoresis, and Newton et al. (1985) detected ds RNA molecules in isolates of P. striiformis, P. recondita and P. hordei using polyacrylamide gel electrophoresis. Variability in ds RNA phenotypes were found within the latter two species. This suggests the presence of ds RNA molecules might provide markers to examine the genetic variation among isolates of P. helianthi.

MATERIALS AND METHODS

In April/May 1984, rust isolates were collected from commercial crops and wild populations of sunflowers in eastern Australia. Leaves with rust pustules were placed in paper bags, dried and stored at 4^{0}C during the field trip. Afterwards, rust isolates were stored at -10^{0}C.

Race 1, race 3, and collected isolates were grown up from single pustules on susceptible hosts in controlled, isolated conditions to maintain genetic purity. Urediospores were collected from infected leaves by tapping them over aluminium foil. Spores were used fresh or were dried under vacuum for three hours, sealed in ampoules, and stored at 4°C.

Electrophoretic analysis of isozymes Approximately 50 mg. of urediospores of each race were dispersed as a monolayer on distilled water and allowed to germinate overnight. The germinated spores were collected and ground with a mortar and pestle in .05 ml of 0.05 M phosphate buffer pH 7.0 containing 1 mg/ml dithiothreitol. The crude extract was absorbed on paper chromatography wicks (6 mm X 5 mm). Wicks were inserted in slots in a horizontal 12% starch gel, each sample in duplicate, and electrophoresis was carried out in one continuous (histidine buffer pH 8.0) and two discontinuous (lithium borate variant buffer pH 8.2 and tris citrate buffer pH 8.0) systems. Details of these systems are given in Broue et al. (1977), Moran and Marshall (1978), and Brown et al. (1978). In the continuous system, electrophoresis was conducted for 5 hours, and in the discontinuous systems, electrophoresis was carried out until the borate or citrate fronts had migrated 10 cm from the sample slot. Each gel was then cut horizontally into three slices, and the anodal portion of the gel was assayed for the range of enzymes described in Table 1. The staining procedures were similar to those described by Brewer and Sing (1970), Brown et al. (1978), and Burdon et al. (1980).

Double-stranded RNA The extraction of nucleic acids from the germinated spores followed the procedure developed by Pryor and Boelen (1987). Briefly, the monolayer of germinated spores (50 mg-1 g) was concentrated by removal of water and ground to a fine powder in liquid nitrogen. The total nucleic acids were extracted from the ground spores with an equal volume of Sarkosyl buffer (100 mM NaCl, 50 mM EDTA, and 2% Sarkosyl) and immediately shaken with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1). The interphase and phenol layers were reextracted with 0.5 volumes of the extraction buffer. The total nucleic acids in the combined aqueous phases were precipitated in 2 hours at -20°C or in dry ice for 30 minutes by the addition of 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate. After centrifugation at 10,000 g at 5°C for 10 minutes, the precipitate was dissolved in a small volume of 10 mM Tris-HCl, 5 mM EDTA pH 8.0 (TE) and incubated with pronase at a final concentration of 0.5 mg/ml for 3 hours at 37°C. The nucleic acid extraction was repeated with the Sarkosyl buffer, the phenol-chloroform mixture, and the ethanol precipitation. The total nucleic acids were redissolved in TE buffer and stored at -20°C.

Electrophoresis of ds RNA Electrophoresis was carried out on 0.7% or 1.2% agarose gels in tris/borate/EDTA pH 8 buffer. Ethidium bromide was incorporated in the gel to a final concentration of 0.5 ug/ml. The electrode buffer was tris/borate/EDTA pH 8 with ethidium bromide present at 0.5 ug/ml. Before electrophoresis, sucrose containing 2.5 mg/ml bromophenol blue was added to each sample. In addition to the P. helianthi samples, P. sorghi, and lambda phage DNA digested with restriction endonuclease Pst 1 (37°C, 30 mins) were included on each gel. The sizes of the ds RNAs were estimated from electrophoretic migration rates relative to lambda DNA Pst 1 restriction fragments of known lengths. To determine susceptibility to RNase degradation, the nucleic acid sample was treated with Ribonuclease A (Sigma) at 0.1 ug/ml in 0.3 M NaCl, 0.3 M NaCitrate pH 7.0 or S1 nuclease at 30 units/ul in a buffer pH 4.2 of 0.25 M NaCl, 0.3 M Na acetate, 0.02 M zinc sulphate for 30 minutes at 37°C to degrade single-stranded nucleic acids. Gels were run at 70 mA for 2-3/4 hours or at 10 mA for 22 hours, and bands were examined on a U.V. transilluminator.

Isolation of Virus-Like Particles One gram of urediospores was collected from the field and germinated overnight on a monolayer of distilled water. Powdered germtubes were prepared as described above and were suspended in 0.1 M phosphate buffer pH 7.5 and centrifuged at 10,000 g for 10 minutes. The supernatant was resuspended in 9% PEG 6000 and 2.5% NaCl at 4°C. (The PEG binds the virus particles and other macromolecules, and the salt enhances precipitation of virus bound to PEG). The pellet was resuspended in 0.1 M phosphate buffer after centrifugation at 12,000 gm for 25 minutes and was centrifuged again at 12,000 g for 20 minutes. The supernatant was centrifuged for a further 2 hours at 40,000 g, and the pellet formed resuspended in phosphate buffer. The resuspended pellet was placed on a

Caesium chloride cushion, density 1.406, and centrifuged at 45,000 rpm for 90 minutes. The layer present in the CsCl cushion was diluted with water and was centrifuged again at 45,000 g for 1-1/2 hours at 5°C. A sample was stained in 2% uranylacetate and examined under the electron microscope (JEOL JEM 100S).

RESULTS

Enzyme Electrophoresis

Enzyme activity was detected in 16 of 22 enzyme systems in germinated urediospores of 15 isolates of P. helianthi. Table 1 shows the positive enzymes, the number of presumed loci scored for each enzyme system, the buffer system and position in the gel, and the migration distance of the bands of enzyme activity. Most enzymes gave a single band, but three enzymes showed two bands. Although different buffer systems (chemical composition and pH) were explored to reveal other possible enzyme variation, no differences were found. All P. helianthi isolates showed homozygosity at all loci. All isozyme phenotypes for the enzymes detected were invariant for the fifteen rust isolates of P. helianthi. The monomorphic nature of the enzymes in this rust species contrasts with the variation in the virulence of the isolates (Dry and Downes, unpublished data).

Nucleic acid composition

The total nucleic acids prepared from the nine isolates of P. helianthi had very similar patterns on agarose gel electrophoresis. The largest band was RNAase resistant and S1 nuclease resistant and is probably fungal nuclear DNA. A smear towards the electrophoretic front is probably composed of small cytoplasmic RNA molecules and degradation fragments. The larger RNA bands present in P. helianthi and distinct from the pattern of bands formed in P. sorghi migrate as distinct species and appear to be double-stranded RNA. These bands were insensitive to treatment with RNAase (0.1 ug/ml ribonuclease A) under conditions of high salt concentration (0.3 M NaCl, 0.3 M NaCitrate). Similarly, when the nucleic acids were treated with S1 nuclease under conditions of high salt concentration (0.25 M NaCl), the same pattern of bands was obtained as with RNAase treatment. S1 nuclease gave better resolution of the bands and was the preferred reagent thereafter.

The sizes of the ds RNA molecules relative to the migration rates of lambda phage restriction fragments included five large ds RNAs of estimated size 5080, 4750, 4505, 4250, and 4000 base pairs. Three medium-sized bands of about 2140, 1900 and 1800 base pairs and two small RNAs of 1250 and 1090 base pairs. In some samples, a number of smaller bands were also detected of about 500 base pairs in length.

Virus-like particles (VLP) in P. helianthi were observed using electron microscopy. The preliminary study involved a P. helianthi isolate collected locally in 1984 distinct from races 1 or 3 by virulence studies. Negatively stained spherical particles were readily detected at magnifications of x70, x560, x80,000 and x106,664. The virus-like particles were observed in the preparation before the caesium chloride cushion. Spherical particles of estimated size 37.5 nm were more clearly identified after their separation from other debris in the CsCl gradient. The nature of these VLPs was very similar to those described by McDonald and Heath (1979), identified in thin sections of urediospores.

VLPs were precipitated with PEG, redissolved in phosphate buffer, cleaned of debris and PEG, and were then reprecipitated by high centrifugation. The supernatant of this fraction might be expected to obtain material from damaged VLP. Indeed, ethanol extraction of nucleic acids from this fraction was followed by electrophoresis on a 1.2% agarose gel. No banding equivalent to DNA was observed, but clear banding of dsRNA was found equivalent to the concentration obtained in a sample test.

TABLE 1 Details of the electrophoretic systems used to detect isozymes in P.helianthi

Symbol	E.C. Code	Enzyme	Buffer system and position	Migration distance of bands (cm)	No. of Loci.
ACO	4.2.1.3.	Aconitate hydratase	His, T	1.0	1
ACP	3.1.3.2	Acid phosphatase	TCpH 8.0, B	0.2	1
ADH	1.1.1.1	Alcohol dehydrogenase		n.d.	
CAT	1.11.1.6	Catalase	TCpH 8.0, B		1
ENDO	3.4.22.9	Endopeptidase		n.d.	
EST	2.1.1.2	Arylesterase	TCpH 8.0, M	6.3	1
GAL	3.2.1.23	B Galactosidase	TCpH 6.5, M	0.4	1
GDH	1.4.1.2	Glutamate dehydrogenase		n.d.	
GOT	2.6.1.1	Aspartate aminotransferase	LiV, B	4.2	2
G6PD		Glucose-6-phosphate dehydrogenase		5.5	1
HEXO	2.7.1.1	Hexokinase	LiV, M		1
IPO		Indophenoloxidase	LiV, T	7.0	1
LAP	3.4.11.1	Cytosol aminopeptidase	TCpH 6.5, M	7.2	1
LDH	1.1.1.27	Lactic dehydrogenase		n.d.	
MAL	1.1.1.40	Malic enzyme	His, B	0.6	1
MDH	1.1.1.37	Malate dehydrogenase		n.d.	
NADHD	1.6.4.3	NADH diaphorase	TCpH 8.0, M	3.2	2
PAL	3.1.3.1	Alkaline phosphatase	TCpH 8.0, T	0.2	1
PEP		Peptidase	TCpH 8.0, B	5.1	1
PGI	5.3.1.9	Glucose-6-phosphate isomerase	LiV, T	4.0	2
PGM	2.7.5.1	Phosphoglucomutase	His, B	0.8	1
SDH	1.1.1.1.25	Shikimate dehydrogenase		n.d.	

n.d.	- not detected	TC	- tris citrate	T	- top
LiV	- lithium borate variant	His	- histidine	M	- middle
				B	- bottom

DISCUSSION

Characterization of Puccinia helianthi has been limited to pathogenicity and temperature of germination of urediospores (Sood and Sackston, 1972). Differences were determined between the temperature of germination of urediospores for races 1 and 3 compared with races 2 and 4. Two alternative approaches were selected to assess any intraspecific isozymic variation, and ds RNA variation in P. helianthi. P. helianthi isolates showed reproducible enzyme activity in sixteen systems. Despite the range of virulence and geographical origins, no consistent differences in isozyme phenotype were detected. This isozymic uniformity within P. helianthi would appear to be a property of sunflower rust populations in Australia.

The absence of isozymic variation within species of the genus Puccinia was also found in Australian P. graminis f. sp. tritici and P. recondita f. sp. tritici isolates (Burdon et al., 1983) as well as United Kingdom isolates of P. striiformis, P. recondita, and P. hordei (Newton et al., 1985). The current study demonstrates monomorphism for isozyme phenotypes in P. helianthi, but polymorphism for virulence phenotypes. This further supports the hypothesis that pathogenicity and enzymic activity are subject to different selective forces (Burdon et al., 1983).

Perhaps the simplest explanation for the lack of isozyme variation between the races of sunflower rust present in Australia is to postulate a single introduction of the pathogen followed by point mutation at virulence loci. Selectively neutral isozyme markers would remain uniform. Race 3 may be derived from race 1 by mutation in the virulence genes since only race 1 was detected in the early 1970s. Commercial hybrids resistant to race 1

were introduced in 1972, and this resistance has been overcome by the pathogen, and there is a wide distribution of race 3 throughout sunflower-growing areas in eastern Australia (Dry and Downes, unpublished data). Similarly, some of the other rust isolates studied may be derived from race 3 by mutation in the virulence genes.

The extent of the sexual cycle in Puccinia helianthi in Australia is not well documented. As the studies of the wheat stem rust populations suggest that sexual recombination does maintain variability within pathogen populations (Burdon and Roelfs, 1985), and if this applies to P. helianthi, then the apparent uniformity in isozyme phenotypes between the races of rust in Australia would imply that predominantly asexual sunflower rust populations occur in Australia.

Double-stranded RNA

The nucleic acid composition of P. helianthi included molecules of ds RNA. The detection of ds RNA in these fragments was confirmed by resistance to RNAase or S1 nuclease digestion under conditions of high salt concentration. The presence of ds RNA in a fungal isolate suggests the genomic contents of a virus (Bozarth 1979, Buck 1980) located in the fungal cytoplasm. Monomorphic phenotypes of the ds RNA fragments in all isolates were observed although pathogenicity varied considerably between these isolates. An absence of variation in ds RNA phenotypes was also obtained in a recent study of P. striiformis f. sp. tritici isolates (Newton et al., 1983). However, isolates of P. recondita and P. hordei possessed unique ds RNA phenotypes. Likewise, in M. lini ds RNA, variation between races of flax rust was demonstrated (Lawrence, Boelen and Pryor, 1987).

The detection of double-stranded RNA in all isolates of P. helianthi coupled with the demonstration of ds RNA present in P. sorghi (Pryor and Boelen, 1987), P. striiformis, P. recondita and P. hordei (Newton et al., 1985) supports the hypothesis of the widespread occurrence of ds RNA in Puccinia species.

The effects of the VLPs on the metabolism of P. helianthi and on the sunflower rust:sunflower plant relationship is unknown. In this preliminary electron microscopy study of VLPs in Puccinia helianthi, only spherical particles were identified. However, in P. helianthi race 2, both spherical particles and narrow flexuous rods were observed in negatively stained thin sections of urediospores (McDonald and Heath, 1979). Further examination of VLP by electron microscopy would be necessary to determine whether variation occurs in particle morphology between races.

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