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DNA restriction fragment length polymorphisms in the wheat stem rust fungus, *Puccinia graminis tritici*

P. A. Anderson and A. J. Pryor *

CSIRO Divison of Plant Industry, CSIRO, PO box 1600, Canberra, ACT 2601, Australia

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Summary. A cDNA library was synthesized from poly A^+ RNA extracted from germinated urediospores of the wheat stem rust fungus, *Puccinia graminis tritici* (race 343-1,2,3,5,6). The library was used as a source of probes to detect RFLPs in genomic DNA from three major races of *P. graminis tritici* in Australia, as well as two *formae speciales* of *P. graminis*. DNA extracted from another *Puccinia* species infecting wheat, *P. recondita tritici* (wheat leaf rust), was included in the analysis. Nine different cDNA probes were analysed, and all detected polymorphisms between the races and *formae speciales* of *P. graminis* that were tested. Seven detected polymorphisms between *P. graminis* and *P. recondita*; the remaining two probes showed no detectable homology to *P. recondita* genomic DNA. The potential applications of RFLP markers to study the origin of genetic variability in *P. graminis tritici* are discussed.

Key words: RFLP – *Puccinia* – cDNA – Leaf rust

Introduction

Puccinia graminis Pers. f. sp. *tritici* Eriks. & Henn., the causal agent of stem rust of wheat (*Triticum aestivum* L.), is one of the world's more destructive and economically significant pathogens. Surveys of *P. g. tritici* populations have shown a high level of variation in pathogen virulence against many host resistance genes (Roelfs and Groth 1988; Luig and Watson 1970). The source of this variation has been attributed to the combined effects of several mechanisms such as mutation and/or recombination by either sexual or asexual events (Watson and Luig 1958; Ellingboe 1961; review by Watson 1981). The relative importance of these mechanisms in generating variation is not known.

In a survey of North American populations of wheat stem rust in which variation in isozymes as well as virulence was measured, a higher level of genetic variability was observed in a sexually reproducing population than in a population dependent only on asexual reproduction (Burdon and Roelfs 1985a, b). In Australia, the absence of the alternate host and/or the conditions for teliospore germination has meant that sexual reproduction of *P. g. tritici* is rare or non-existent. Since 1920 Australian isolates of *P. g. tritici* have been typed for virulence against major genes for resistance in wheat. Watson (1981) analysed these data to outline the probable origin of the major races of *P. g. tritici* in Australia from 1920–1980. He suggested that the majority of Australian *P. g. tritici* rust races have arisen from a limited number of parental races. Each of these has generated a series of races that are related to each other by step-wise changes (mutation) in virulence. The parental races are thought to have originated outside Australia, possibly in Africa. In 1981–1982, on the basis of virulence specificities, 16 pathotypes of *P. g. tritici* were reported in Australia. Burdon et al. (1983) could find no variation in these isolates at 11 different isozyme loci. In agreement with Watson, they suggest that the variation in virulence is the result of the selective step-wise selection of new virulent forms. Such a situation might explain the variation in virulence and the lack of variation in isozyme patterns.

In combination with mutation, somatic hybridization events could contribute to the generation of new virulence gene combinations of *P. g. tritici*. There are a number of putative somatic hybridization events documented in the literature, one being between two *formae speciales* of *P. graminis, P. g. tritici* and *P. g. secalis* (Watson and

* To whom correspondence should be addressed.

Luig 1959; Luig and Watson 1970). Both *P. g. tritici* and *P. g. secalis* infect the rough wheat grass *Agropyron scabrum*, the common host plant on which the somatic hybridization event is believed to have taken place. The putative hybrid is pathogenic on *A. scabrum* and the barley grass *Hordeum leporinum*, but non-pathogenic on wheat or rye. Isozyme analysis of the hybrid supported the hypothesis of its origin, and suggested that exchange of whole nuclei between the presumptive parents was responsible for the formation of the new rust (Burdon et al. 1981).

Population studies of *P. g. tritici* have been limited by the availability of useful markers. Virulence markers are subject to strong selection and isozyme markers have revealed relatively little variation. Molecular probes developed to detect RFLPs in genomic DNA of eukaryotic organisms (Botstein et al. 1980) have been successfully used as genetic markers in a number of phytopathogenic fungi (Hulbert and Michelmore 1988; McDonald and Martinez 1990; Christiansen and Giese 1990; Braithwaite et al. 1990; for review see Michelmore and Hulbert, 1987) and more recently in the rust fungi of maize, *P. sorghi*, and flax, *Melampsora lini* (Anderson and Pryor submitted, Anderson et al. submitted). The studies on rust fungi showed that the cDNA probes from *P. sorghi* have greatly reduced cross hybridization to other species of *Puccinia* and that there was little or no interaction between genera.

This paper describes the synthesis of a cDNA library specific for *P. g. tritici* and demonstrates the use of a set of these cDNA clones as probes capable of detecting RFLPs between rust races and *formae speciales* of *P. graminis*.

Material and methods

Rust strains

The origin of the races and *formae speciales* of *P. graminis* and *P. recondita tritici* used in the RFLP analysis is listed in Table 1. Urediospores of race 343, from which mRNA was extracted for cDNA synthesis, were typed for virulence specificities at the Plant Breeding Institute (University of Sydney) and multiplied from a single pustule on the wheat 'Transfer', a cultivar that is susceptible to *P. graminis tritici* and resistant to *P. recondita tritici*.

DNA and RNA extraction and cDNA synthesis

Urediospores were germinated on an air/water interface to which several drops of 1-nonanol (Aldrich) were added to stimulate germination (Burdon and Roelfs 1985a). All nucleic acid extraction and purification steps were conducted according to methods described previously (Anderson and Pryor submitted). Complementary DNA synthesis was performed using a kit provided by Amersham, and the cDNA sequences cloned, with *Eco*RI linkers (Boehringer), into the *Eco*RI site of the pGEM 7Zf(+) bacterial plasmid vector (Promega). Approximately 8.0×10^4 recombinant colonies were obtained, and the library was stored as bacterial cells and as purified plasmid DNA at −80 °C.

Table 1. Origin and description of the rust races used in the RFLP study

Rust races	Host plant	Origin[a]
Puccinia graminis tritici		
34-2, 12, 13, (accession no. 84552=427)	Wheat	PBI
126-5, 6, 7, 11 (accession no. 334=427)	Wheat	PBI
343-1, 2, 3, 5, 6	Wheat	Field isolate
P. graminis secalis (accession no. 57241B=915)	Rye	PBI
Scabrum rust (accession no. 71406=107)	*Agropyron scabrum*	PBI
P. recondita tritici (race 76-0)	Wheat	PBI

[a] PBI stands for the Plant Breeding Institute, University of Sydney

Southern blot and hybridization

Genomic DNA (2 μg) from germinated urediospores was digested with the restriction endonuclease *Eco*RI, *Hin*dIII, or *Bam*HI (Pharmacia) and separated on a 1% agarose gel. Electrophoresis, Southern blots, hybridization and labelling of probes were all done according to standard methods.

Results

A cDNA library of 8.0×10^4 colonies was constructed from poly A^+ RNA extracted from germinated urediospores of the wheat stem rust *P. graminis tritici*. Fifty-eight colonies were selected at random from the library and 41 were found to contain recombinant DNA ranging in size from 200 bp to 2.2 kbp. Nine clones were hybridized to Southern blots containing digests of genomic DNA from *P. g. tritici*, *P. g. secalis*, *scabrum* rust and *P. recondita tritici*. The autoradiographs of three of these hybridizations using the cDNA clones pPGtc10, pPGtc26 and pPGtc36 are shown in Fig. 1a–c, and the data from all the hybridizations are summarized in Table 2.

The three races of *P. g. tritici* and the *formae speciales* of *P. g. tritici* could be distinguished from one another by any one of the nine probes with at least one of the restriction enzymes tested (Fig. 1). Seven of the nine clones hybridized to DNA from wheat leaf rust (*P. recondita tritici*) and revealed RFLPs between the two species. The remaining two clones did not show any cross species homology (for example, refer to Fig. 1b, lane 6).

Within the *P. g. tritici* races, races 343 and 34 were more similar to each other than either was to race 126. Of the 27 probe/enzyme comparisons made, 23 detected an RFLP between race 126 and either of races 343 or 34, while only three RFLPs were detected between races 343 and 34.

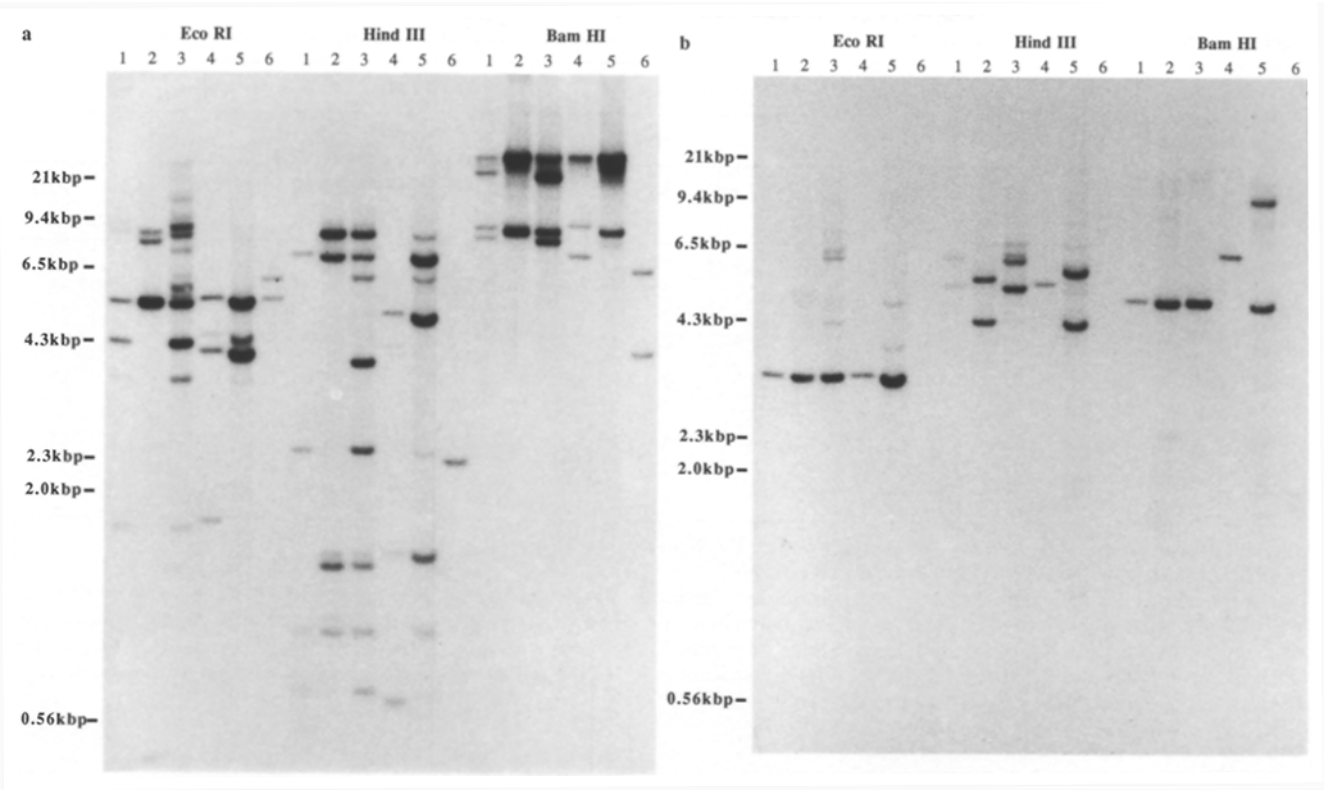

Fig. 1a–c. Autoradiographs of a Southern blot containing genomic DNA of *P. graminis tritici* digested with the restriction endonucleases *Eco*RI, *Hin*dIII and *Bam*HI, and hybridized to the cDNA probes pPGtc10 (**a**), pPGtc26 (**b**) and pPGtc36 (**c**). The order of the loadings are: *lane* 1 race 34, *lane* 2 race 126, *lane* 3 race 343, *lane* 4 *P.g. secalis*, *lane* 5 *scabrum* rust, *lane* 6 *P. recondita tritici*. The loading of genomic DNA was uneven with race 34 being less and the *scabrum* rust being more than in the other lanes. Genomic DNA from race 343 did not cut to completion in each of the digestion reactions, generating some faint partial bands hybridizing to the probes. These were disregarded in the RFLP analysis

Table 2. Summary of the RFLP patterns observed in races and *formae speciales* of *P. graminis* and *P. recondita tritici*

Enzyme		Restriction enzyme/DNA combination																	
		*Eco*RI						*Hin*dIII						*Bam*HI					
DNA Clone	Size	34	126	343	*P.g.s*	*sc*	*P.r.t*	34	126	343	*P.g.s*	*sc*	*P.r.t*	34	126	343	*P.g.s*	*sc*	*P.r.t*
pPGtc5	800	1	1	1	1	1	1[d]	2[a]	1[b]	2	2	2	1[d]	1	1[b]	1	2	1	1[d]
pPGtc7	800	2[a]	2[b]	2	2	2	1[d]	2[a]	2[b]	2[c]	2	2	2[d]	1[a]	2[b]	1	2	2	2[d]
pPGtc8	500	2[a]	1[b]	2	2	1	1[d]	2[a]	2	3	1	2	1[d]	2[a]	1[b]	2	2	2	1[d]
pPGtc10	900	2[a]	1[b]	2	2	2	2[d]	4[a]	4[b]	7[c]	4	4	1[d]	4[a]	2[b]	4	3	2	2[d]
pPGtc15	650	2[a]	2[b]	2	1	2	1	2	2	2	1	2	1[d]	2[a]	1[b]	2	1	2	1[d]
pPGtc26	1000	1	1	1	1	1	[e]	2[a]	2[b]	2	1	2	[e]	1	1	1	1	2	[e]
pPGtc29	1900	4[a]	3[b]	4	2	3	4[d]	3[a]	1[b]	3	1	2	3[d]	2[a]	2[b]	2	1	2	4[d]
pPGtc35	2100	2[a]	2[b]	3[c]	1	2	[e]	2[a]	1[b]	2	1	4	[e]	2[a]	2[b]	2	1	2	[e]
pPGtc36	800	1[a]	2[b]	1	1	2	3	1	1	1	1	1	1[d]	2[a]	2[b]	2	2	2	1[d]

P.g.s., Puccinia graminis secalis; sc, scabrum rust, *P.r.t., P.r. tritici*
Size: Size estimate of the insert DNA in base pairs (bp)
Numbers refer to the observed number of major bands hybridizing to a particular probe. The interpretation of the number of bands was difficult, and a number of faint bands was not counted in the present analysis. In some cases, particularly for smaller bands there is no simple explanation. Unequal loading particularly for race 34 DNA compared to DNA from races 126 and 343 caused some differences, and partial restriction such as for *Eco*RI digestion of race 343 DNA also caused some faint bands that were not counted. Results that are underlined are shown in Fig. 1 a–c.
[a] RFLP between race 34 and race 126 of *P. g. tritici*
[b] RFLP between race 126 and race 343 of *P. g. tritici*
[c] RFLP between race 34 and race 343 of *P. g. tritici*
[d] RFLP between *P. g. tritici* and *P. r. tritici*
[e] No detectable hybridization

The RFLP analysis of the *scabrum* rust was used to examine the hypothesis that this rust originated from a somatic hybridization event between *P. g. tritici* and *P. g. secalis*. In 22 out of 27 probe/enzyme combinations the observed banding pattern of the *scabrum* rust was in agreement with the inheritance of bands from either *P. g. tritici* and *P. g. secalis* (for example, refer to Fig. 1 a, *Hin*dIII, lanes 1–5). In the other 5 cases the *scabrum* rust contained bands that were not present in either of the putative parental rusts (for example, refer to Fig. 1 b, BamHI, lane 5). Out of the 22 cases where the *scabrum* bands could be identified in the parental races, the race of *P. g. tritici* from which the *scabrum* band may have originated could be distinguished on six occasions. In one instance the band could be traced to either race 34 or 343, and in the other five cases to race 126 (for example refer to Fig. 1 b, *Hin*dIII, lanes 2 and 5). Therefore, if the *scabrum* rust did originate from a somatic hybridization event, a race 126 or a derivative of it was the most likely *P. g. tritici* parental type.

Discussion

The results reported here demonstrate the successful application of RFLP analysis of genomic DNA extracted from the wheat stem rust fungus, *Puccinia graminis tritici*. The probes were derived from a cDNA library of 8×10^4 colonies of which 41 out of 58 or 70%, contain inserts of rust cDNA. This library is probably sufficient to cover most of the RNA species present in germinating urediospores of *P. g. tritici* since in comparison Johnson and Lovett (1984) have shown by hybridization kinetics that zoospores of the zygomycete *Blastocladiella emersonii* contain approximately 5,000 different RNA sequences.

The cDNA clones from one rust species have significantly reduced homology to DNA from other species. Of nine different cDNA *P. g. tritici* sequences used, two sequences did not hybridize to genomic DNA from wheat leaf rust (*P. recondita tritici*), and several others showed reduced homology. This supports previous results in which only 25% of the cDNA sequences from the maize rust *P. sorghi* showed interspecific homology to other *Puccinia* species, and none of them showed intergeneric homology to the flax rust (*Melampsora lini*) (Anderson and Pryor submitted). The phylogenetic significance of these results is difficult to assess. Baum and Savile (1985) suggested, primarily on the basis of spore morphology, that of the *Puccinia* species infecting cereal hosts, *P. graminis* and *P. recondita* were not closely related. At present there is insuffient data available from the DNA hybridization results to contribute to the understanding of evolutionary relationships between species of rust.

The origin of new virulent races of *P. g. tritici* in Australia has been attributed to the combined influences of the infrequent introduction of exotic races, step-wise selection for virulence and the possibility of other mechanisms for genetic reassortment uch as somatic recombination. Race 126 was a major race in Australia 70 years ago when virulence typing began, but died out in the 1960s. Races 34 and 343 represented 80% of all the isolates of *P. g. tritici* tested in the 1989/90 season (Park RF, personal communication). Race 343 is thought to be derived from race 326, which was first detected in Australia in 1969. Burdon et al. (1982) suggest on the basis of isozyme data and data provided by Luig (unpublished) and de Sousa (1975) that race 326 originated from Africa. Race 34-2, 12, 13 is believed to have originated from 34-2, which in turn originated from 21-0 (Watson 1981). Race 21-0 appeared in Australia for the first time in 1954, and Burdon et al. (1982) postulate that its origin was also from Africa. Step-wise mutation of 34-2 to virulence against Sr_{27} and Sr_{Satu} has resulted in the formation of 34-2, 12, 13 (Singh and McIntosh 1990).

The results of the RFLP analysis showed that variation between race 126 and either 34 or 343 is greater than between 34 and 343. This supports the results of an isozyme study in which Burdon et al. (1982) detected no variation between the two races. Taken together, the RFLP results and the isozyme data may indicate that race 34 and 343 share a common African ancestor.

The hybridization pattern of the cDNA probes to DNA from *P. g. tritici*, *P. g. secalis* and the *scabrum* rust support the existing hypothesis, based on virulence and isozyme studies, that the *scabrum* rust is a somatic hybrid between *P. g. tritici* and *P. g. secalis*. While the results reported here suggest that the *P. g. tritici* parent may have been race 126 or a derivative, they also indicate that a convincing demonstration of the occurrence of somatic hybridization in the generation of new virulent races will require a thorough documentation of the parental races involved.

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