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Monitoring changing virulence patterns of *Uromyces appendiculatus* in the resistant pinto bean cultivar Olathe by rep-PCR

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Abstract Corresponding molecular markers associated with avirulence or virulence genes in the bean rust pathogen are currently unknown, although host resistance genes have been linked to molecular markers in bean. The changing virulence patterns in pathotypes of *Uromyces appendiculatus* collected over a 14-year period after the release of the *Ur*-6 resistance gene on the USA high plains were therefore analyzed using rep-PCR molecular markers. Isolates from two neighbouring pathotype groups from Colorado and Nebraska were screened using the rep-PCR primer Box-AIR. The PCR fragment Box_{475} was cloned and sequenced and a specific primer set ATA-2 was designed. This primer yielded 10 polymorphic products which allowed separation of these isolates into two distinct groups and will be useful for future analysis of the population genetics of this organism.

Keywords Bean rust · Virulence · Epidemiology

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Uromyces appendiculatus, the cause of rust on common bean (*Phaseolus vulgaris*) produces periodic epidemics throughout Africa and the Americas and is characterized by highly diverse virulence phenotypes (Stavely 1984; McCain et al. 1990; Mmbaga et al. 1996). Current bean rust disease management strategies include fungicide applications, modification of cultural practices, periodic pathogen virulence surveys and deployment of new resistant cultivars developed by marker-assisted selection (Pastor-Corrales and Stavely 2002; Miklas et al. 2006). The pinto bean cv. Olathe, first described in 1978 by Ballantyne (McCain et al. 1990), contains a single dominant rust resistance gene *Ur*-6, of Andean origin (Stavely et al. 1989; Park et al. 2004). Olathe was first released in Colorado in 1979 (McCain et al. 1990), and in the USA central plains states of Minnesota, North Dakota and Nebraska in the 1980s (Steadman et al. 1996; Gross and Venette 2002), where it was initially resistant to all local rust races pathogenic on bean. However, annual pathogen surveys since its release indicate increasing virulence of rust isolates to Olathe in the central plains (Steadman et al. 1996; Stavely 2000).

In this study, a set of *U. appendiculatus* isolates collected from Nebraska, Colorado and North Dakota after the release of the *Ur*-6 resistance gene in the bean cv. Olathe was monitored by rep-PCR markers. The objective was to identify potential molecular markers for monitoring the epidemiological changes observed in *U. appendiculatus* isolates in the USA central plains in relation to the cv. Olathe, which

contains the single dominant resistance gene *Ur*-6 (Stavely et al. 1989).

A total of 145 pathotypes comprising isolates of *U. appendiculatus* on cv. Olathe, including a group of 17 published rust races and two reference races (Table 1), was used in this study (Stavely et al. 1989). Each isolate was derived from a single rust uredinium. Field collections of rust were identified as raised rust-coloured lesions or leaf spots on bean plants; these were carefully removed, placed in a clean, dry paper bag, and stored in a cool place or refrigerated. Rust isolates were then increased on the 12 bean differentials based at the UNL rust laboratory (Linde et al. 1990; Araya et al. 2004). Primary leaves of cv. Olathe were inoculated in triplicate with a spore suspension of each isolate as described by Mmbaga et al. (1996). Reactions 1 to 3.2 were considered resistant (R) reactions, and reactions 3.3 to 6 were susceptible (S) reactions (Mmbaga et al. 1996). Inoculation and evaluation of all isolates on Olathe were repeated once as described above.

DNA extraction was based on the method described by Linde et al. (1990) and previously reported by Araya et al. (2004). Rep-PCR reactions were comprised of 10.6 μl of ddH_2O, 1X Gitschiers buffer (200 mM $(NH4)_2SO_4$, 200 mM Tris–HCl pH 8.0, 200 mM $MgCl_2$, 100 mM EDTA pH 8.8, 2.8 mM β-mercapto-ethanol), 10% DMSO, 1 μM BOX primer, 60 μM DNTPs, 2 μM BSA, 2 u μl^{-1} TAQ DNA polymerase and 40 pg of genomic DNA. PCR amplification consisted of one cycle at 95°C for 2 min, 30 cycles of 94°C for 3 s, 92°C for 30 s, 50°C

Table 1 Virulence of *Uromyces appendiculatus* isolates on pinto bean cv. Olathe

Isolate	Origin[a]	Year of Collection[b]	Reaction on Olathe (R/S)[c]
NE57, NE65	Nebraska	1981	R
NE1, NE13	Nebraska	1982	S
NE69, NE70	Nebraska	1984	R
NE16, NE31, NE50, NE52, NE67	Nebraska	1985	R
NE54	Nebraska	1985	S
NE20, NE35	Nebraska	1986	S
NE8,NE11, NE21	Nebraska	1991	
NE27, NE79	Nebraska	1991	R
NE2,NE3-4, NE6-7, NE9-10, NE12, NE15, NE17-19, NE22-26, NE28,NE30, NE34, NE37-49, NE53, NE55-56, NE58-62, NE53, NE64	Nebraska	1992	S
NE5, NE29, NE51		1992	R
CO1-70	Colorado	1994	S
FA80[d]	North Dakota	1980	R
FA85[d]	North Dakota	1985	S
R46	Florida	1979	R
R47	Florida	1979	S
R44	North Dakota	1979	R
R53, R67	Florida	1980	S
R49	Nebraska	1982	R
R84	Colorado	1984	S
R38	USA	–[e]	S
R39	USA	–	R
R40-43	USA	–	S
R78	Tennessee	–	R
R86	Brazil	–	S
R93, R108	Honduras	–	R
R94	Honduras	–	S
R110	Zimbabwe	–	S

[a] Represents the US state, or country of origin of the isolate

[b] Original year of collection of each isolate

[c] Reaction on Olathe based on Rust disease scale

[d] Provided by Pat Gross, North Dakota State University, Fargo

[e] Year collected unknown and were provided by M. Pastor-Corrales, USDA, Beltsville Maryland.

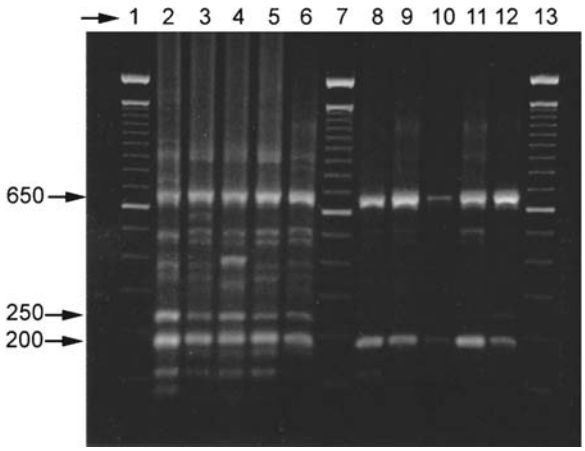

Fig. 1 Primer ATA-2 screened against five isolates of *Uromyces appendiculatus* from Nebraska in lanes *2* to *6*, and five from Colorado in lanes *8* to *12*. A 100-bp marker is shown in lanes *1*, *7* and *13*. PCR products at 650 and 200 bp are found in all isolates. A band at 250 bp is found only in rust isolates from Nebraska and not in those from Colorado

for 1 min, 65°C for 8 min, and a final extension at 65°C for 8 min conducted in a Techne Genius thermal cycler (Techne Inc, Princeton NJ). Amplified DNA fragments were separated by electrophoresis in 0.5× TBE on 1.5% agarose gels at 40 mA for 3 to 4 h. ERIC, REP and BOX primers (Louws et al. 1996) were initially screened in separate experiments and the BOX primer was selected for further use. All PCR reactions were confirmed by amplification of duplicate DNA extractions and repeated at least twice.

Thirty-two polymorphic fragments were scored using the *BOX-A1R* primer (Fig. 1). Chi-square analysis of two common fragments (Box 1800 and Box 475) with the virulence data showed that they were significantly ($P<0.05$) associated with these isolates but could not distinguish between virulence and avirulence to cv. Olathe. Amplified Rep-PCR fragments 475 absent in *E. coli* positive controls but present in pathotypes from Colorado and Nebraska and the rust race R38 (Table 2), were cloned into the TA cloning vector PCR4-TOPO (Invitrogen, Inc., San Diego, CA) according to the manufacturer's instructions. Two individual clones each, from independent PCR reactions of isolates R86, NE37 and CO58, were sequenced (Table 2) at the UNL Genomics Core Research Facility by capillary electrophoresis with a Beckman/Coulter CEQ2000XL 8-capillary DNA sequencer. BLASTN 2.2.5 (Altschul et al. 1997; http://

Table 2 Presence of rep-PCR amplified fragments in isolates of *Uromyces appendiculatus*

Isolate	Origin	Presence (+)/ Absence of M475	Presence (+)/ Absence of ATA-2	Reaction on Olathe (R/S)[a]
NE52	Nebraska	−	+	R
NE16	Nebraska	−	+	R
NE31	Nebraska	−	+	R
NE65	Nebraska	−	+	R
NE70	Nebraska	−	+	R
NE20, NE35	Nebraska	−	+	R
NE27	Nebraska	−	+	R
NE 57	Nebraska	+	+	R
FA80[e]	North Dakota –	−	+	R
FA 85[e]	North Dakota	+	+	R
R53	Nebraska	−	−	S
R 49	Nebraska	+	+	R
R 44	North Dakota	+	+	R
R 38	USA	+	+	S
R 39	USA	+	−	R
R 84	Colorado	−	−	S
CO17	Colorado	+	−	S
CO29	Colorado	+	−	S
CO30	Colorado	+	−	S
CO31	Colorado	+	−	S
CO35	Colorado	+	−	S
CO37	Colorado	+	−	S
CO43	Colorado	+	−	S

[a] Reaction on cv. Olathe based on the rust disease scale

www.ncbi.nlm.nih.gov/blast/bl2seq/cgi) was used to determine sequence similarity between bands from isolates CO58-3, CO58-6, and NE37-3.

Specific PCR primers were designed and aligned with the original Box rep-PCR primer. DNA derived from *E. coli* clones, excised original DNA fragments, and genomic DNA derived from the respective individual isolates were amplified using the specific primer ATA-2 (5′-cgcagcgcggaaggcgtcatgtg-3′). Rep-PCR reactions comprised 12.2 μl of ddH_2O, 1× Gitschiers buffer, 5% DMSO, 1 μM ATA-2, 60 μM DNTPs, 3 μM BSA, 1 u μl^{-1} TAQ DNA and 40 pg of genomic DNA and were amplified and separated by gel electrophoresis as described above.

Primer ATA-2 produced 10 polymorphic fragments (Fig. 1) characterized by a single fragment at 250 bp present in all Nebraska isolates but absent in those from Colorado and will be very useful for further analysis of the population genetics of this organism. In this study, we showed that general rep-PCR primers could amplify rust DNA but could not distinguish between virulent and avirulent phenotypes on the bean cv. Olathe. However, the specific rep primer, ATA-2, was able to separate rust isolates from Nebraska and Colorado and also placed the two reference rust races R38 and R39 into two separate groups. These two snap bean rust races produce distinctly different reactions on Olathe, although they were isolated from the same host (Pastor-Corrales and Stavely 2002). The distinction between Nebraska and Colorado rust isolates may be based on different local responses to an *Avr*/*Vir* gene complex in both pathogen and host, over time. Therefore, while genetic similarities may exist within each of these groups, genetic variation within and between both geographic populations is also evident both by cultural reaction to Olathe as well as by rep-molecular markers. The two pathogen populations may therefore mirror the epidemiological changes in *Ur*-6 resistance occurring in the region. The primers designed to target these products may need to be screened in a larger population of virulent isolates from neighbouring states to determine if a similar change in virulence in Olathe can be monitored in this way. Although endemic virulence to Olathe may also be found in other regions, genetic differences between Nebraska and Colorado rust isolates in this study indicate local events may be critical for assessments of gene deployment and rust disease management in the central plains. With the presence of such a complex resistance gene system and the ability of rust pathotypes associated with the *Ur*-6 gene in a small population to change, it is important to monitor and understand genetic changes so that virulence changes can be managed along with host resistance gene deployment.

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