Molecular Ecology Notes (2004) **4**, 547–550 doi: 10.1111/j.1471-8286.2004.00728.x

PRIMER NOTE

Development of polymorphic markers for the root pathogen *Thielaviopsis basicola* using ISSR-PCR

MARIA M. GELDENHUIS,* JOLANDA ROUX,* MICHAEL J. WINGFIELD* and BRENDA D. WINGFIELD†*

**Department of Microbiology and Plant Pathology*, †*Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa*

Abstract

***Thielaviopsis basicola* is a soil-borne fungal pathogen affecting many important agricultural crops. Little is known regarding the population biology or origin of this pathogen. Polymorphic markers developed for *Ceratocystis fimbriata*, a species complex phylogenetically closely related to *T. basicola*, were tested and found not to be useful for *T. basicola*. In this study 14 primer pairs, seven of which resulted in the amplification of single polymorphic fragments in *T. basicola* were developed. These primers will enable further studies on this economically important pathogen, and will result in an enhanced understanding of its population structure in different parts of the world.**

Keywords: *Ceratocystis*, codominant markers, polymorphic loci, population diversity

Received 27 February 2004; revision received 22 April 2004; accepted 28 May 2004

Thielaviopsis basicola is a soil-borne plant pathogenic fungus that is found in many parts of the world (Nag Raj & Kendrick 1975). It causes serious root diseases on a wide range of economically important crop plants including cotton, beans, carrots and tobacco. In some cases, it is one of the most important constraints to production (Yarwood 1981).

Very little is known regarding the origin or genetic diversity of *T. basicola*. This is partially due to a lack of appropriate tools to assess these characteristics. In this regard, codominant molecular markers have proved to produce highly reliable markers, providing information regarding the origin, spread and probable success of pathogen management practices. The aim of this study was to develop appropriate markers that can be used to gain an enhanced understanding of the population biology of *T. basicola*.

Thielaviopsis basicola is phylogenetically closely related to the important canker and wilt pathogen *Ceratocystis fimbriata* (Paulin & Harrington 2000). Microsatellite primers recently developed for *C. fimbriata* (Barnes *et al*. 2001a; Table 2) were tested using DNA extracted from two *T. basicola* isolates (CMW 5463, CMW 4098). These primers were AG 1/2, AG 7/8, AG 15/16, AG 17/18, CF 11/12, CF 15/16, CF 21/22 and CF 23/24. PCR mixtures and reaction conditions were the same as those described by Barnes *et al*. (2001a). In addition to the specific annealing temperature for each primer pair described by Barnes *et al*. (2001a; Table 2), temperatures two degrees below and above the specific annealing temperature were also tested. None of these primers successfully amplified DNA for either of the *T. basicola* isolates.

In order to develop codominant polymorphic markers for *T. basicola*, the internal-short sequence repeat (ISSR)-polymerase chain reaction (PCR) technique (Van der Nest *et al*. 2000; Burgess *et al*. 2001) was used. Two isolates of *T. basicola* from South Africa (CMW 5482, CMW 5528) and two from Ecuador (CMW 4098, CMW 4457) were used for marker development. These isolates and the developed markers were subsequently compared with *T. basicola* isolates from different hosts and parts of the world (Table 1). DNA was extracted from all fungal isolates using the protocol described by Barnes *et al*. (2001b). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Correspondence: B. D. Wingfield. Fax: +27 12 420 3947;
E-mail: wingfield@fabi.up.ac.za
*Correspondence: B. D. Wingfield, Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, 0001 Pretoria, South Africa.
E-mail: brenda.wingfield@fabi.up.ac.za

Table 1 Alleles (base pairs) observed at each of the seven loci for 10 *T. basicola* isolates from eight different countries

Isolate number*	Country	NG 3/4	NG 5/6	NG 13/14	NG 15/16	NG 17/18	NG 19/20	NG 21/22
CMW 5482, CMW 5528	South Africa	435	451	304	385	341	324	378
CMW 4098, CMW 4100, CMW 4381, CMW 4685, CMW 4689	Ecuador	405	448	303	378	341	341	382
CMW 4684		405	449	304	378	341	341	382
CMW4686		395	433	300	378	346	332	385
CMW 4457		395	434	300	378	347	331	385
CMW 5451	USA	427	452	304	386	341	316	379
CMW 6714	Australia	396	445	301	378	342	341	385
CMW 5896	Uganda	408	445	301	378	341	331	385
CMW 7065	Netherlands	408	446	301	378	341	331	385
CMW 7067	Belgium	405	454	303	377	341	351	385
CMW 7070	Switzerland	435	451	304	385	341	316	378
Number of alleles		6	9	4	4	4	5	4

*CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

ISSR-PCR was performed on a South African *T. basicola* isolate (CMW 5482) using seven primers, namely 5′ DDB$(CCA)_5$, 5′ DHB$(CGA)_5$, 5′-NDB$(CA)_7$C, 5′ YHY$(GT)_5$G, 5′ DBD$(CAC)_5$, 5′ $(CAT)_5$, and 5′-NDV$(CT)_5$ following the approach of Barnes *et al*. (2001a) except that an annealing temperature of 49 °C was used. The resulting amplicons were cloned, colonies screened for inserts of suitable size and these were then sequenced. Inserts were sequenced with T7 and SP6 using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq®DNA Polymerase, FS (Perkin-Elmer, Warrington, UK) following the manufacturers protocols, on an ABI Prism 377 DNA sequencer. Sequences were screened for tandem repeats ($n > 2$) and primers designed to flank these regions. No perfect tandem repeats of longer than eight repeats were found.

Fourteen primer pairs were designed to flank microsatellite-like regions. These were tested on the two South African and two Ecuadorian isolates (Table 1). PCR reactions were carried out in a total volume of 50 μL on a HYBAID thermocycler (Teddington, UK). The PCR mix included 2 ng DNA template, Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme), 0.2 μM of each primer, 200 μM of each dNTP and *Taq* Expand™ High Fidelity polymerase mixture (1.75 U) (Roche). Reaction conditions were the same as those described by Burgess *et al*. (2001). Specific annealing temperatures were used for each primer pair (Table 2). The PCR products were separated using PAGE (6% polyacrylamide in 50 mM TBE buffer for 7 h at 140 V) and visualized by silver staining (Blum *et al*. 1987). Five of the primer pairs produced multiple bands, two primer pairs were monomorphic and the remaining seven primer pairs produced one band that was polymorphic for isolates from South Africa and Ecuador (Table 2).

One primer from each polymorphic primer pair was labelled with the phosphoramidite fluorescent dyes FAM or TET (MWG) (Table 2). The same PCR reactions and conditions described above were used with the labelled primers to amplify all isolates (Table 1). Differences in product size were determined, relative to the internal size standard (TAMARA) by separating the labelled PCR products using PAGE on an ABI Prism 377 DNA sequencer. Analyses were carried out using GENESCAN 2.1 (Perkin-Elmer Corp.) and GENOTYPER (Perkin-Elmer Corp.).

For 16 *T. basicola* isolates from eight different countries, 11 genotypes and 36 alleles could be detected across the seven loci (Table 1). The number of alleles per locus ranged from three to nine. Each isolate had a different genotype except for the two South African isolates that had the same genotype. Five of the Ecuador isolates also had the same genotype, resulting in four genotypes out of the eight cultures isolated from carrots. The different genotypes, observed from only a few isolates from a single host, suggest that there is some degree of diversity in Ecuador.

In this study the ISSR-PCR technique was used to successfully develop seven codominant polymorphic markers for the important root pathogen *T. basicola*. The results suggest a different genetic composition for different geographical regions. This indicates that the makers will be valuable in assessing diversity and spread of the pathogen within countries and between continents. Knowledge gained from the application of these markers should contribute to the development of improved management strategies to reduce the impact of *T. basicola*.

Table 2 Primer pairs designed for amplification of *T. basicola* sequence characterized amplified regions

Primer pair	Primer sequence	Core sequence	T_m† (°C)	T_a‡ (°C)	GC percentage	Banding pattern	GenBank Accession no.
NG1	5′-GCT GGT GGG CGG AGA ATG-3′	*A_2CTA_5*$A_4GA_2GA_8$*$(GA_2GA)_2CA_2GA$*	60.5	62	66.7	Monomorphic	AY55940
NG2	5′-GGA TGG CCA GGG CCC CTC-3′		65.1		77.8		
NG3[c]	5′-GGC CCA GGC CAA AGG CAG-3′	*$(C_2AT)C_2AC(C_2AT)_4(C_2AC_2A_2T)_3$	62.8	62	72.2	Polymorphic	AY559433
NG4	5′-GCT ATC AAA GGG CAT GGC-3′	C_2AC*$(C_2T_2)_3$*A_3CA_5*	58.8		57.9		
NG5[c]	5′-CCT TTG ATG TCT CCT CCT GTC-3′	*$CATC(CATA)_4$*$T_3CT_3C_3T_7GT_2(GCT)_3$*	59.8	64	52.4	Polymorphic	AY559434
NG6	5′-CCT GAG TCG TCT GCT TGT GG-3′		61.4		60		
NG7	5′-CCA GTC CTG ATT GAT CGC C-3′	Sequence rich in C and T repeats	58.8	60	57.9	Monomorphic	AY559440
NG8	5′-GAG ATG GTC TAT GGC CGC-3′		58.2		61.1		
NG9	5′-CCC ACC TGC CGA ACA ACG-3′	Sequence rich in A repeats	60.5	60	66.7	Multiple bands	AY559441
NG10	5′-CTG ACT CTG AAG CCC GTC-3′		58.2		61.1		
NG11	5′-CTG TGA CGT CTG TAC GTC TC-3′	*$CT_2GT_2GCT(GT_2CT_2)_2GT_2$*	59.4	61	55	Multiple bands	AY559439
NG12	5′-GAC GCC CAT GCC GGT GTC-3′		62.8		72.2		
NG13[d]	5′-GGG GAC GCG ACT TAG TGC C-3′	*$A_2(GA)_4A_2(GA)_2$*	63.1	64	68.4	Polymorphic	AY559435
NG14	5′-GTC CAG AAT CTG CCC TGA CG-3′		61.4		60		
NG15[d]	5′-GCG AGT TTG CGG GAG TTT G-3′	*A_5*$A_5CGA_2GA_8$*$(GA)_4$*	58.8	62	57.9	Polymorphic	AY559437
NG16	5′-CGC TAC GCT GAG GGT CCC-3′	$(C_2AG_2)_2GAC(C_2AG_2)C_2A_2G_2A_2$*	62.8		72.2		
NG17[c]	5′-GGA GAA GCC TCG ATG TGT AG-3′	*$(T_2C)_2C(T_2C_2)T_4G_2(T_2C_2)_2T_2(CAT)_2$*	59.4	62	55	Polymorphic	AY559436
NG18	5′-CCG CCA GGA TCA GCC GGG-3′		65.1		77.8		
NG19[d]	5′-GGC CAG CAG AGC CCC AAG-3′	*$T_4A(T_2C)_2T_3CT_2C_2T_4$*$(CT)_2CACT(CA)_2(CT)_2$*$(CT)_4$	62.8	62	72.2	Polymorphic	AY559432
NG20	5′-CAA GAC TAC CAC GGC ACC G-3′	$CA(CT)_3CACTCA(CT)_2CA$*$(TCTG)_2$*$TC_3)_2T_2CA_2C_3$*	61.0		63.2		
NG21[c]	5′-GAA GAG CAA TCT ACA GTG CGC-3′	*$T_8CA_3CA_2GA_6$*$C_2T_8(CT)_2C_2(CT)(CCT)_2(CT_4)_2T_3$*	59.8	62	52.4	Polymorphic	AY559438
NG22	5′-GCA GTC GAG GGA GCC TAA G-3′		61.0		63.2		
NG23	5′-GAC TGC CCC GCC AAA CTC-3′	*$(CA)_4GA(CA)_3$*	60.5	60	66.7	Multiple bands	AY559442
NG24	5′-GGT AGT CTG GGA TCT GGG-3′		58.2		61.1		
NG25	5′-GGT GGA CAC GAG TGG CTC-3′	*$T(CT_5)_3T_8$*$(GA)_4$*$GT(CT)_4$*	60.5	62	66.7	Multiple bands	AY559443
NG26	5′-GCC TGG CCT GTG CTG GTC-3′		62.8		72.2		
NG27	5′-CGT CTA TTT GCT GCG GTA GC-3′	*$(GT)_7CT$*	59.4	62	55	Multiple bands	AY559431
NG28	5′-GCT GCG CCA GCT GTG TGA G-3′		63.1		68.4		

[a]T_m = melting temperature.
[b]T_a = annealing temperature.
*Variable length of sequence.
[c]primer labelled with FAM.
[d]primer labelled with TET.

Acknowledgements

We thank the members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry and the National Research Foundation (NRF), South Africa, for financial support. We are also grateful to Dr Treena Burgess and Mrs Irene Barnes for valuable advice regarding the development of the markers and data analysis.

References

Barnes I, Gaur A, Burgess T, Roux J, Wingfield BD, Wingfield MJ (2001a) Microsatellite markers reflect intra-specific relationships between isolates of the vascular wilt pathogen, *Ceratocystis fimbriata*. *Molecular and Plant Pathology*, **2**, 319–325.

Barnes I, Roux J, Wingfield MJ, Coetzee MP, Wingfield BD (2001b) Characterisation of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. *Plant Disease*, **85**, 317–321.

Blum H, Beier H, Gross HJ (1987) Improved silverstaining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8**, 93–99.

Burgess T, Wingfield MJ, Wingfield BD (2001) Simple sequence repeat (SSR) markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology*, **67**, 354–362.

Nag Raj TR, Kendrick B (1975) *A monograph of Chalara and allied genera*. Wilfred Laurier University Press, Waterloo, Ontario, Canada.

Paulin AE, Harrington TC (2000) Phylogenetic placement of anamorphic species of *Chalara* among *Ceratocystis* species and other ascomycetes. *Studies in Mycology*, **45**, 209–222.

Van der Nest MA, Steenkamp ET, Wingfield BD, Wingfield MJ (2000) Development of simple sequence repeat (SSR) markers in *Eucalyptus* from amplified inter-simple sequence repeats (ISSR). *Plant Breeding*, **119**, 433–436.

Yarwood CE (1981) The occurrence of *Chalara elegans*. *Mycologia*, **73**, 524–530.