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Short communication

Improved polymerase chain reaction (PCR) detection of *Gaeumannomyces graminis* including a safeguard against false negatives

Elaine Ward
Plant Pathology Dept, IACR, Rothamsted, Harpenden, Herts., AL5 2JQ, UK

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Abstract

A previously reported method for polymerase chain reaction (PCR) detection of *Gaeumannomyces graminis* was modified to simplify it, improve its specificity and decrease the possibility of contamination of the assay. The modified method also allowed discrimination between the *G. graminis* varieties (*tritici* and *avenae*) that are pathogenic to wheat and oats respectively and the variety weakly pathogenic to wheat (var. *graminis*). An internal positive control for the PCR was also added to the test by including a second pair of primers in the reaction. This positive control has wider application in PCR tests for detection of other fungi.

Gaeumannomyces graminis var. *tritici* (Ggt) and var. *avenae* (Gga) cause take-all and take-all patch, important diseases of cereals and turf grasses respectively [Asher and Shipton, 1981; Walker, 1981; Hornby and Bateman, 1991]. Another variety, var. *graminis* (Ggg) is weakly or non-pathogenic on cereals but is pathogenic on rice [Ou, 1972; Datnoff, 1993] and bermudagrass [McCarty and Lucas, 1989; Elliott, 1991]. Some isolates of *Phialophora* sp. (lobed hyphopodia), which is frequently found on cereals in the U.K. have been shown to be anamorphs of Ggg. *Phialophora graminicola* (Deacon) Walker (teleomorph *G. cylindrosporus* D. Hornby *et al.*) (Pg) is a non-pathogenic fungus, found on cereal and grass roots, and which resembles in some respects *G. graminis* on roots and in culture.

Identification of isolates by conventional methods such as pathogenicity testing on different hosts and determining morphological characteristics [Bateman, Ward and Antoniw, 1992; Mathre, 1992], is slow and sometimes inconclusive. Because of these problems alternative methods of identification using molecular biological techniques have been tried in recent years [summarized in Ward and Akrofi, 1994; Tan *et al.*, 1994].

Currently, the best molecular detection methods are those based on the polymerase chain reaction (PCR) since these are quick, simple and very sensitive. A specific PCR assay for *G. graminis* was one of the first PCR detection tests to be developed for any plant pathogenic fungus [Schesser *et al.*, 1991]. Although this assay has now been used extensively to detect these organisms in pure culture and infected wheat plants [Schesser *et al.*, 1991; Henson, 1992; Henson *et al.*, 1993; Elliott *et al.*, 1993], it uses nested PCR which has the disadvantages of being more costly and more labour intensive than single-stage PCR tests. The extra operations involved also allow more opportunity for contamination to occur. Nested PCR was used because initial experiments suggested that a single pair of primers (KS1F/KS2R) did not give the required specificity [Schesser *et al.*, 1991]. In the initial study DNA was also amplified from the unrelated fungi *Fusarium culmorum* and *Cochliobolus sativus*. However, the conditions used for the amplification using the single primer pair were not highly specific. It should be possible to use a much higher annealing temperature for these primers than the 42 °C used; using the approximation of 2 °C for each A or T and 4 °C for each G or C to calculate the Tm [Thein and Wallace, 1986; Innis *et al.*, 1990] an applicable annealing tem-

perature, (Tm −5) °C, for both KS1F and KS2R is 69 °C. In this new work the KS1F/KS2R protocol has been modified to achieve the required specificity in a single-stage PCR. The principal difference is the use of a higher annealing temperature although there are other differences in the cycling programme and the components of the reaction mixture that may also have an effect.

One of the main problems with PCR detection is the possibility of false negatives, samples which should test positively but do not because there is some problem with a particular PCR test. However, most workers seem not to have addressed this problem, or at best they have only checked that the DNA is of suitable quality for PCR by testing it with other universal/consensus primers in a separate experiment [Ward, 1994]. A much better way to check that the DNA is of suitable quality and quantity for PCR and that there are no other barriers to amplification is to include an internal control in the PCR test. The duplex PCR test described here should ensure that the result is unequivocal.

There were various considerations in choosing the primer pair for this control function. The primers needed to have (Tm−5) °C values comparable to those of KS1F and KS2R, so that they could operate at the same annealing temperature as this pair. The primers should also amplify DNA from all fungal species (as far as this can be determined), and ribosomal DNA (rDNA) primers were an obvious choice. The second amplified band should also be a different size from the KS1F/KS2R band so that the two can easily be resolved by agarose gel electrophoresis. Primers Pn3/ Pn8 [Sherriff *et al.*, 1994] fulfilled all three criteria and were therefore chosen for this study. The calculated (Tm−5) °C values for Pn3 and Pn8 are 77 °C and 75 °C respectively. These primers amplify a region of nuclear rDNA from the 3′ end of the small ribosomal subunit through the two ITS regions and the 5.8S rDNA to just beyond domain 1 of the 5′ end of the large subunit gene.

Details and references on the sources of the isolates used are given in Table 1. Methods for extracting purified DNA from fungal cultures, infected roots and for releasing DNA from boiled mycelial material are fully described in Ward and Akrofi [1994], Ward and Gray [1992], Bateman *et al.* [1992] and Ward & Bateman [1994].

Each 25 μl reaction mixture contained 25pmol of each primer, 0.5 units of *Taq* polymerase, 0.2mM deoxyribonucleoside triphosphates, reaction buffer (10mM Tris pH8.8 at 25 °C, 1.5mM $MgCl_2$, 50mM KCl and 0.1% Triton X-100) and 20ng fungal DNA. Reaction conditions were 25 cycles of 94 °C for 30 sec, 60 °C for 2 min and 72 °C for 2 min. The primers used were KS1F/KS2R [Schesser *et al.*, 1991] and Pn3/ Pn8 [Sherriff *et al.*, 1994]. The sequences of these primers are as follows: KS1F, TACGGCTGTACCGCATGATCTACTA; KS2R, ATGAGGCCAGAGGTCCCGTCAAAA; Pn3, CCGTTGGTGAACCAGCGGAGGGATC; Pn8, GCTGCATTCCCAAGCAACCCGACTC. A negative control (no DNA) was included in each PCR experiment. After amplification the PCR products were checked by electrophoresis of 10 μl of the sample in 1% agarose gels containing ethidium bromide to stain the DNA.

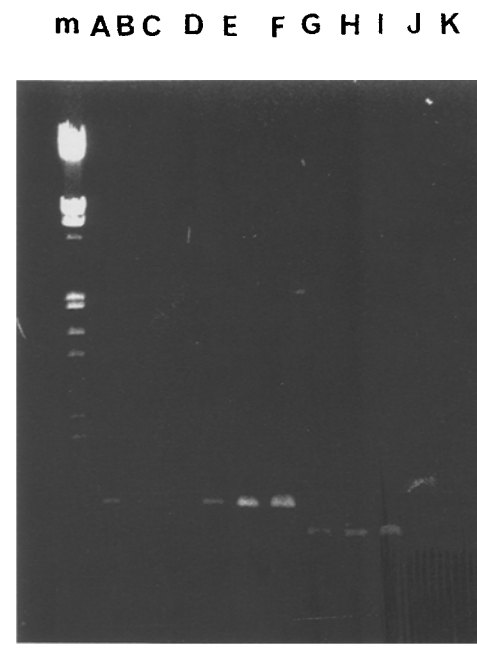

Fig. 1. An example of a 1% agarose gel showing testing of isolates using PCR with primers KS1F and KS2R. Key: m, size marker lambda DNA cut with *Eco*RI and *Hin*dIII; A, Ggt 184; B, Ggt 90/2-4; C, Ggt 90.GL0(ADAS); D, Ggt 87/7-4; E, Gga 174; F, Gga PO86/439; G, Ggg 247; H, *Phialophora* sp. (lobed hyphopodia) 85/23-1; I, *Phialophora* sp. (lobed hyphopodia) 89/5-1; J, *Phialophora graminicola* 74/1736-2; K, *P. graminicola* 89/2-1B.

The results obtained using primers KS1F/ KS2R alone are shown in Table 1 and an example of a gel showing testing of samples is shown in Fig. 1. All the isolates were tested at least twice. All of the *G. graminis* isolates tested gave a product. For Ggt and Gga isolates the band was 600 bp and for Ggg the band was at least 50bp smaller (size range 435–550 bp). No

Table 1. List of fungal isolates used and results obtained

Isolates	Reference/source	KS1F/2R result & size
G. graminis var. *tritici*		
184, 88/10–1, 90/2–4, 90/GL0.ADAS, Og12N,90.GLR.17, 87/7–4, 180	1	+ 600bp
92/3–2, 92/15–4A, 92/24–2	2	+ 600bp
92/5–2	3a	+ 600bp
92/18–1, 92/ 19–2	3b	+ 600bp
92/21–2, 92/24–3	3c	+ 600bp
93/17–2, 93/18–1	4a	+ 600bp
G. graminis var. *avenae*		
174, 175, 177, 179, PO86/441, ABL2, YZ1, 61 'felty', PO86/439	1	+ 600bp
178	4g	+ 600bp
G. graminis var. *graminis*/ *Phialophora* sp. (lobed hyphopodia)		
247, 85/23–1, 89/3–1, 89/5–1, 89/5–3, 122, 148	1	+ 490bp
176	1	+ 550bp
93/G1.1	6b	+ 490bp
92/38–1A, 92/49–2A, 92/ 52–1A	3d	+ 490bp
111, 119, 123, 124	4h	+ 490bp
120, 125, 126	4h	+ 435bp
Gaeumannomyces cylindrosporus/ *Phialophora graminicola*		
85/17–3B, 74/1736–2, 89/2–1B, 76/73–2	1	-
92/1–1, 92/2–1A, 92/2–3	2	-
92/37–2B, 92/42–3, 92/45–3	3d	-
93/1–2, 93/3–3, 93/4–4	3e	-
93/5–1, 93/10–1	3f	-
2502, 1850, 1826	5b	-
Gaeumannomyces incrustans		
1823, 1829, 2518, 2676, 2679, 2680	5b	-
Gaeumannomyces spp. from Cyperaceae		
G. caricis	4c	-
153	4i	-
Other fungi		
Cochliobolus sativus DM214	5a	-
Cochliobolus sativus GP0380	4b	-
Epicoccum purpurascens GP0479	4d	-
Fusarium avenaceum Fa2	6a	-
Fusarium culmorum DM409	5a	-
Fusarium culmorum Fc31	6a	-
Fusarium solani var. *coeruleum* 158	7a	-
Fusarium sulphureum 21	7b	-
Gibellina cerealis GP0436	4e	-
Helminthosporum solani GAH\B\S	7c	-
Leptosphaeria korrae 1828	5b	-
Magnaporthe grisea 2690, 2692, 2694	5b	-
Magnaporthe poae 1832, 2562, 2669	5b	-

Table 1. Continued

Isolates	Reference/source	KS1F/2R result & size
Microdochium bolleyi GP0381	4f	-
Phialophora malorum 1847	5b	-
Phoma foveata 30	7d	-
Polymyxa betae F41	8a	-
Polymyxa graminis F32	8b	-
Polymyxa graminis F36	8c	-
Polymyxa graminis F40	8d	-
Pseudocercosporella herpotrichoides 38.31	6d	-
Pseudocercosporella anguioides 86.46.40C	6c	-
Rhizoctonia solani	7	-

Source/ References
1. Ward & Gray (1992)
2. Ward & Akrofi (1994)
3. From R.J. Gutteridge, Rothamsted Experimental Station (RES). (a) RES, triticale, 1992 (b) RES, wheat, 1992 (c) RES, barley, 1992 (d) Abbotts Ripton, Hunts, UK, wheat bioassay, 1992 (e) Boxworth, Cambs, UK, wheat, 1993 (f) Woburn, Beds, UK, wheat, 1993.
4. From D.Hornby, RES (a) Wigton, Cumbria, barley (b) 1978, RES, Barley (c) tentative identification, from *Carex acutiformis*, Suffolk, UK (d) maize, Cambridge, 1975 (e) RES, 1969 (f) Papplewick, UK, barley, 1978 (g) bentgrass, NSW Australia (h) Australia (from P.T.W. Wong) (i) Wales, *Carex*, 1979. Originally this isolate was tentatively identified as Ggg, but it has now been reclassified.
5. From J. Henson, Montana State University. (a) Schesser *et al.*, 1991 (b) Henson, 1992.
6. From G.L. Bateman, RES, isolates from wheat at RES (a) 1987 (b) 1993 (c) 1986 (d) 1988 7. From P.J. Read, RES, isolates from potato (a) RES, 1984 (b) Edinburgh, 1976 (c) RES, 1991 (d) RES, 1974.
8. From M.J. Adams, RES (a) sugar beet, Norfolk, UK (b) barley, Sunstedt, Germany (c) Prince Edward Island, Canada, wheat (d) Xiaoshan, China, barley.

bands were amplified from isolates of species other than *G. graminis* using these primers.

When *G. graminis* isolates (all those listed in Table 1) were tested with primers KS1F/KS2R and Pn3/Pn8 together, one band was seen corresponding to the KS1F/KS2R-amplified DNA and one band corresponding to the Pn3/Pn8-amplified DNA (usually approximately 800bp). Again all the non-*G. graminis* isolates tested (all those listed in Table 1) failed to amplify using KS1F/KS2R but they did amplify using Pn3/Pn8. The two PCR products were of different size and easily distinguished (see Fig. 2). Some Ggt isolates (184, 88/10–1, 90/2–4, 87/7–4, Og12N, 180, 90/GL0(ADAS), 92/3–2) were also tested using DNA from mycelium boiled in Tris buffer and both the Pn3/Pn8 and KS1F/2R-derived bands were seen in each case.

In this paper, an existing PCR detection method for *G. graminis* has been modified to make it more specific, simpler, quicker, cheaper, and less prone to contamination. In the experiments reported here only DNA from *G. graminis* isolates was amplified. None of the isolates from other species, including *G. incrustans*, *G. cylindrosporus*/ *Phialophora graminicola* and two isolates of *Gaeumannomyces* spp. isolated from Cyperaceae, showed any amplification using KS1F/KS2R primers. This test is therefore more specific than that used previously, since in earlier work [Henson, 1992] several of these *G. incrustans* isolates (1823, 1829, 2518, 2676 and 2680) and *G. cylindrosporus*/ *P. graminicola* isolates (2502, 1850) tested positively under some conditions. Other non-*G. graminis* isolates that tested positively in previous tests [Henson, 1992] also tested negatively here. These isolates were *Magnaporthe poae* 1832, 2562 and 2669, *Magnaporthe grisea* 2690, 2694, 2692 and *Phialophora malorum* 1847. Also there was no amplification of DNA from the *Fusarium culmorum* (DM409) or *Cochliobolus sativus* (DM214) isolates which in the earlier experiments with these primers [Schesser *et al.*, 1991] had tested postitively and prompted the development of the nested primer PCR test. Another fungus that tested negatively was *Leptosphaeria korrae* which, with *Magnaporthe poae* and *M. grisea* causes turfgrass diseases that can be confused with those caused by *G. graminis* [Landschoot, 1993; Smiley, 1993]. As with the version of

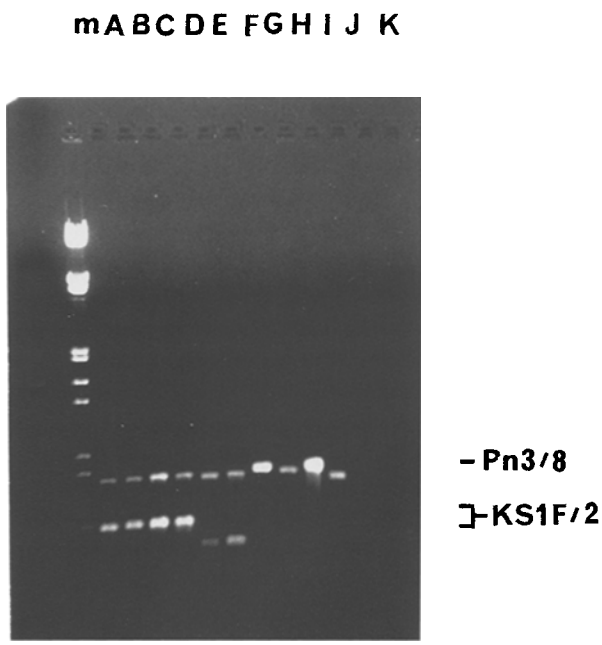

Fig. 2. An example of a 1% agarose gel showing testing of isolates using PCR primers KS1F/KS2R + Pn3/Pn8. Key: m, size marker lambda DNA cut with *Eco*Rl and *Hin*dlll; A, Ggt 90/2-4; B, Ggt 87/7-4; C, Gga 177; D, Gga 174; E, *Phialophora* sp. (lobed hyphopodia) 92/52-1A; F, *Phialophora* sp. (lobed hyphopodia) 89/5-1; G, *Phialophora graminicola* 92/2-1A; H, *Gaeumannomyces incrustans* 1829; I, *Cochliobolus sativus* DM124; J, *Fusarium culmorum* DM409; K, water control.

the test used by Henson and co-workers [Henson, 1992; Henson *et al.*, 1993; Elliott *et al.*, 1993] the test described here would also be useful for distinguishing turfgrass diseases caused by *G. graminis* from those caused by other fungi.

The most likely reason for the increased specificity over the initial experiments done by Schesser *et al.* [1991] using these primers is the use of a much higher annealing temperature. This is supported by experiments in which several non-*G. graminis* isolates were checked for amplification using the same protocol as described in Methods but using a 42 °C annealing temperature (results not shown). Some of these gave faint bands using this temperature, including *G. incrustans* 1829, *Fusarium culmorum* DM409, *Cochliobolus sativus* DM124 and two *Phialophora graminicola* isolates (89/2–1B and 74/1736–2).

The size of the KS1F/KS2R PCR products from Ggt and Gga isolates was 600bp and that from Ggg was discernibly smaller (550bp or less), but of more variable size. This size difference is useful as it allows discrimination between the varieties pathogenic on wheat and oats (Ggt and Gga respectively) and the variety weakly pathogenic on these cereals (Ggg). Almost all Ggg isolates gave a band of approximately 490 bp, the only exceptions being a few Australian isolates (120, 125 and 126) that gave a smaller product (435 bp) and an isolate from soybean (176) that gave a larger product (550 bp).

All the DNA samples used here had been tested previously with universal/ consensus fungal primers (ITS4/ITS5, for the method see Ward and Akrofi, 1994) to check that the DNA used was of appropriate quality and quantity for PCR. However, this was time consuming and did not guarantee against an individual PCR test failing for some other reason. Therefore, a duplex PCR assay using primers KS1F, KS2R, Pn3 and Pn8 was developed. Using this method *G. graminis* isolates gave 2 bands (one for KS1F/KS2R products and one for the Pn3/Pn8 products), whereas the non-*G. graminis* isolates gave a single band (Pn3/Pn8 product). The presence of this Pn3/Pn8 band in the non-*G. graminis* samples allowed a much greater degree of confidence in the assay since it confirms that DNA must be present and PCR is possible. Any failure to produce the KS1F/KS2R product must therefore be due to the absence of complementary sequences in the DNA. If no bands are produced in a particular PCR assay, the result is invalid and the sample's test should be repeated. Similar duplex PCR tests could be devised for other fungi using Pn3 and Pn8 or other suitable primers, in conjunction with primers specific for the fungus.

The method was also tested on DNA extracted from wheat roots infected with Ggt or Pg, but the interpretation of results was more complex as several bands were usually present; primers Pn3 and Pn8 also amplify bands from uninfected plant DNA. However, a band of 600bp (presumably corresponding to the Ggt KS1F/KS2R amplicon) was seen only when the roots were infected with Ggt, not with Pg or in uninfected roots. This PCR method therefore also shows promise for use with infected roots, but probably only in conjunction with a subsequent hybridization to confirm the presence of the KS1F/KS2R-amplified band. The method is probably best suited to testing DNA from mycelial pieces from agar plates and several Ggt isolates were successfully tested in this way. Therefore, once an agar culture is available, a test could be done within one day. When testing infected roots from the field, placing them on agar and culturing for a few days could be used to provide suitable material for this test.

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