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Development of strain-specific primers for a strain of *Gliocladium catenulatum* used in biological control

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Abstract

The randomly amplified polymorphic DNA (RAPD) technique was used to develop strain-specific primers for *Gliocladium catenulatum* strain J1446, which is promising in biological control. One of the primer pairs developed proved to be strain-specific; strain J1446 was differentiated from 16 *G. catenulatum* strains and six other strains of two *Gliocladium* species, as well as from *Trichoderma virens*, and isolates of *Nectria* spp. and *Fusarium* spp. Specific primers were also tested with DNA isolated from cucumber leaves, treated or untreated with a solution made from *Gliocladium* powder. The expected amplification product was produced only from treated leaves. DNA isolated from *Gliocladium*-treated potato tubers and fungi grown in peat was also used in amplification reactions. Strain-specific primers detected strain J1446 when the amount of DNA was 5 pg or more. Some variation between the *Gliocladium* strains was found by the random amplified microsatellites method (RAMS) and the universally primed polymerase chain reaction method (UP-PCR), but no clear fragments specific to strain J1446 were produced. Cross-blot hybridisation of UP-PCR products differentiated strain J1446 from *T. virens*, but not from the *Gliocladium* isolates. The 28S rDNA sequences and β-tubulin sequences were identical or very similar in all *Gliocladium* strains. Thus, it is possible that the *Gliocladium* strains of the present study are conspecific, which means that a revision in the taxonomy of *Gliocladium* species may be necessary.

Introduction

The efficacy of biological control agents in controlling soil-borne fungal pathogens has been intensively studied (Knudsen et al., 1997). There is social and economic pressure, arising out of environmental concerns, to reduce the use of fungicides. In Finland only a few fungicides are registered for soil fumigation and their use is limited to greenhouse production. In open-field crop production, many soil-borne diseases cause severe yield losses. The most commonly used biocontrol fungi belong to the genus *Trichoderma*. In addition, several *Gliocladium* species are known to be destructive mycoparasites of pathogenic fungi (Papavizas, 1985).

During the years 1990–1993 an Inter-Nordic research programme, initiated by the Nordic Joint Committee for Agricultural Research and entitled 'Biological Control of Seed-borne Diseases in Cereals', was carried out in Denmark, Finland, Sweden and Norway. The aim was to obtain antagonists well adapted to different Nordic environments and to develop them as effective biological control agents (BCAs) (Knudsen et al., 1997). In the Finnish experiments, 1700 isolates obtained from the soil in different Finnish climatic regions were screened in sand against *Fusarium culmorum* and after that in sphagnum peat and field soil (Teperi et al., 1998). Two hundred and ten isolates were included in a comparative screening experiment carried out in the field using spring wheat

cv. Luja, naturally infected with *F. culmorum*. The results of the field experiment and the initial screenings showed that isolates of *Gliocladium* spp. were highly antagonistic. The most promising *Gliocladium* isolates were also tested as biocontrol agents in peat substrate against *Pythium ultimum* on sugar beet and *Rhizoctonia solani* on cauliflower. The best isolate of *G. catenulatum* has been formulated and patented by Kemira Ltd (patent no. 101631).

The purpose of the present study was to develop strain-specific primers for the *G. catenulatum* strain J1446, which is promising for biological control, in order to be able to identify the particular strain from other *Gliocladium* strains and directly from plant material. The DNA sequence data was also used for phylogenetic comparison of the strains, which had before been investigated by RAPD-PCR and internal transcribed spacer (ITS) sequence analyses (Yli-Mattila et al., 1997). An additional purpose was to determine how long this strain would survive in peat, on potato tubers and on cucumber leaves after mixing into the soil or spraying the fungus onto leaves.

Materials and methods

Fungal isolates and DNA extraction

Mycelia of 23 *Gliocladium* isolates from Finland including *G. catenulatum*, *G. roseum* (= *Clonostachys rosea*, teleomorph *Bionectria ochroleuca*, Schroers et al., 1999) and *G. nigrovirens* isolates and one *T. virens* (= *G. virens*, Rehner and Samuels, 1994) isolate were obtained from the Agricultural Research Centre of Finland (Table 1). The identification of the most interesting *Gliocladium* isolates at the species level was confirmed by DSM in Germany and by Dr. W. Gams in The Netherlands. On the basis of preliminary experiments, we concentrated on ten isolates which in RAPD-PCR experiments (Yli-Mattila et al., 1997) proved to be most closely related to J1446. Mycelia were grown on cellophane membrane on potato dextrose agar (PDA) for 4–5 days at 25 °C at constant light. To test strain-specific primers, five Danish isolates of *G. roseum* (Bulat et al., 1998) and four *Nectria* and eight *Fusarium* isolates were used in addition to *Gliocladium* isolates (Table 1). In order to simplify the interpretation of the results, our own naming of the strains, as shown in Table 1, will be used.

DNA was extracted and purified with the chloroform/octanol method (Paavanen-Huhtala et al., 1999). Also the DNeasy plant mini kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions in some DNA extractions of cucumber leaves. The DNA concentration was estimated by comparison with DNA standards after gel electrophoresis.

RAPD amplification and purification of products

Amplifications for RAPD analyses were carried out as described in Yli-Mattila et al. (1996) except that 10× Dynazyme reaction buffer (Finnzymes, Espoo, Finland) [1× is 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100] was used and the amount of DNA was approximately 10 ng per reaction. Twenty-three RAPD-PCR oligonucleotide primers were used in 48 different primer pair combinations as in the Sequencing With Arbitrary Primer Pairs (SWAPP) method (Burt et al., 1994). In addition, a few RAPD primers were also used alone. The sequences of the primers used, except for the sequences of OPA1-20 primers (Operon Technologies, Alameda, CA) are shown in Table 2. Most amplifications were performed with a PTC-200 DNA Engine thermal cycler (MJ Research Inc., Watertown, MA, USA) using calculated control and heated lid. The programme for RAPD consisted of one cycle of 90 s at 94 °C followed by 39 cycles of 35 s at 93 °C, 25 s at 35 °C and 95 s at 72 °C. In some RAPD amplifications the HB-TR3-CM-220/110 OmniGene cycler (Hybaid, Middlesex, United Kingdom) was used (Paavanen-Huhtala et al., 1999). RAPD amplification products were electrophoresed and fragments specific for the GC1 strain which were not present when either primer was used alone were searched for (Burt et al., 1994).

The desired amplification product was cut from the gel and the DNA content was extracted using the β-agarase enzyme and chloroform/isoamylalcohol or simply by melting the agarose. In some cases, the fragment was reamplified using 0.5–1 µl of purified amplification product as a template under the same conditions as in the initial amplification. The purified products were sequenced from both ends with an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Dept. of Biology, Univ. of Turku) with the same primers as used in the amplification.

Cloning and sequencing of amplification products

The DNA fragments specific to GC1 were ligated into pCR2.1 from the TOPO TA cloning

Table 1. *Gliocladium*, *Trichoderma*, *Nectria* and *Fusarium* isolates

Species	Strain no.	Original no.	Geographic origin	Identification
G. catenulatum	GC1	J1446	Ylistaro	W. Gams/DSM
	GC2	M67-6	Mikkeli	W. Gams/DSM
	GC3	J2734	Pernaja	W. Gams/DSM
	GC4	M2423	Laukaa	W. Gams/DSM
	GC5	M3081	Pälkäne	W. Gams/DSM
	GC6	M66-25	Mikkeli	MTT
	GC7	J138	Kiukainen	MTT
	GC8	J1052	Toholampi	MTT
	GC9	J1414	Laukaa	MTT
	GC10	J1471	Sotkamo	MTT
	GC11	M88-1	Vampula	MTT
	GC12	J2087	Mouhijärvi	MTT
	GC13	J2081	Anjala	MTT
	GC14	M2248	Kokemäki	MTT
	GC15	M2530	Joroinen	MTT
	GC16	J2735	Pernaja	MTT
	GC17	M2554	Somero	MTT
G. roseum	GR1	M67-7	Mikkeli	MTT
	GR2	J380	Laukaa	W. Gams
	GR3	IBT 9790	Denmark	KVL
	GR4	IBT 9354	Denmark	KVL
	GR5	IBT 9366	Denmark	KVL
	GR6	IBT 9367	Denmark	KVL
	GR7	IBT 9368	Denmark	KVL
G. nigrovirens	GN1	M66-19	Mikkeli	W. Gams
	GN2	J1419	Laukaa	W. Gams
	GN3	M81-3	Orisuo	W. Gams
	GN4	J1248	Sotkamo	MTT
T. virens	TV1	J96	Riste	W. Gams
N. pityrodes	NP18	J76	Jokioinen	W. Gams/CS
	NP19	J1431	Övermark	W. Gams/CS
	NP20	J1432	Övermark	W. Gams/CS
	NP21	M2247	Peipohja	W. Gams
F. redolens	FR10	93139	Siuntio	MTT
F. oxysporum	FO1	93144	Lieto	H. Nirenberg
	FO6	93138	Espoo	MTT
F. avenaceum	FA38	92015	Nummi	MTT
	FA20	92024	Harjavalta	MTT
F. arthrosporiella	FArt2	92012	Myllyniemi	H. Nirenberg
F. tricinctum	FT43	93101	Kokemäki	H. Nirenberg
	FT92	92008	Mynämäki	H. Nirenberg

MTT = Agricultural Research Centre of Finland, DSM = Deutche Sammlung von Mikroorganismen und Zellkulturen GmbH, CS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, KVL = The Royal Veterinary and Agricultural University.

kit (K4500-01; Invitrogen, Leek, The Netherlands) following the manufacturer's instructions. Competent *Escherichia coli* 'One Shot' cells (Invitrogen BV, Leek, The Netherlands) were transformed and plated onto LB plates with 50 μg/ml ampicillin and 40 μl of X-gal (20 mg/ml). After overnight incubation at 37 °C, colonies containing recombinant plasmids were selected and cultured in LB medium. The plasmid DNA was isolated using the alkali lysis method according to Sambrook et al. (1989) or with the Quantum Prep kit (Bio-Rad Laboratories, Richmond, CA, USA). DNA fragments ligated into plasmids were sequenced using M13 Forward and Reverse primers. Sequences from the GenBank most

Table 2. List of primers used in the study

	Name	Sequence (5′to 3′)
RAPD primers	91,300	CGAGGTTCGC
	91,299	CGATTCGGCG
	X	GATAACGCAC
	Y	CGAGACACAC
UP primers	AA_2M_2	GAGCGACCCAGAGCGG
	0.3-2	TGAGGACAACGGTTCC
RAMS primer	CGA	(G, A or T)(A, T or C)(G, T or C)$(CGA)_5$
Specific primers	Gc1-1	CCGTCTCTTATCGAGCCAAGAT
	Gc3-2a	GCCCATTCAAAGCGAGGCATTA
	Gc3-2b	GGAATAATCAACGCACCGCCCA
	Gc3-3	AGTCAGCCACGTTCAATGCTTCG
	Gc10-1	ATGACTGCGCGCAGCCGATTTG
	Gc18-1	TCGCGAGATCACATGGGATGCC
	Gc10-2	TGATCGCAGAAGTGGGCTGGAT
	Gc18-2	GGTGACCGTATTCAGAAAAGGG
	ITS1	TCCGTAGGTGAACCTGCGG
	ITS4	TCCTCCGCTTATTGATATGC

similar to the DNA sequence obtained were also searched by the FASTA (Pearson and Lipman, 1988) program of the server of the DNA Data Bank of Japan.

Primer design and PCR assay

Oligonucleotides were designed according to the sequences obtained. Three of the primers were designed to be used as sequence-characterised amplified region (SCAR) primers (Paran and Michelmore, 1993). These primers contained the original RAPD primer plus the next 12 internal bases. Strain-specific primers were tested in 50 or 25 µl standard PCR reactions to amplify 0.1–1µl (ca. 1–10 ng) of the fungal DNA extracts. The reaction mixture of 50 µl contained 150 µM each of dNTP, 60 ng forward and reverse primers, 5 µl 10× Dynazyme reaction buffer and 1.6 U Dynazyme polymerase (Finnzymes, Espoo, Finland). Amplifications were performed in a PTC-200 DNA Engine thermal cycler. During the first cycle DNA was denatured at 94 °C for 3 min. For the subsequent 30 cycles the tubes were kept at 94 °C for 50 s, at 58 °C for 60 s and at 72 °C for 90 s, followed by an extension at 72 °C for 3 min. In some of the amplifications annealing temperatures of 55, 61 or 63 °C were tested. All DNA samples were tested with primers ITS1 and ITS4 (White et al., 1990) to check the quality of the DNA, in order to avoid false negative results in the specific PCR. The amplified products were separated by 1.0% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV illumination. The use of reamplification with nested primers was also tested in order to increase the sensitivity of the amplification.

PCR assay with DNA isolated from cucumber leaves, peat and potato tubers treated with GC1

Strain-specific primers were tested with DNA isolated from cucumber leaves. Five cucumber plants (cv. Boloria EZ) were sprayed (five leaves per plant, 20 ml of suspension per leaf) with a 0.5% *Gliocladium* suspension made of fermented and lyophilised mycelia and spores of GC1 strain. Control plants were not treated with the *Gliocladium* suspension. One leaf of each plant was taken on every sampling date: the first ones one day after the treatment and the next at intervals of one week, the last five weeks after the treatment. The survival of *G. catenulatum* on cucumber leaves was also tested for four weeks after treatment by counting colony-forming units. About 2 g of *Gliocladium*-treated leaves were put in 100 ml of sterile water and shaken for 2 h. Dilutions of 10^{-2} and 10^{-3} were made, of which 750 µl were spread on to PDA plates containing streptomycin (0.2 g/l). Colony-forming units were counted after seven days incubation. From leaf discs taken from treated and untreated plants and placed on

PDA plates for seven days the presence of *Gliocladium* and other fungi was assessed with a stereomicroscope.

The primers were tested with DNA isolated from pure cultures of fungi grown in peat treated or untreated with *G. catenulatum* strain GC1. The experiment with potato started with the treatment of tubers with a 0.5% *Gliocladium* suspension. Part of the tubers were treated with an 0.15% imazalil solution; control tubers were untreated. After treatment, the tubers were stored at 5 °C for about five months before planting. The first tuber and root samples were collected five weeks after planting; the last samples were collected 15 weeks after planting. The roots were rinsed with water and stored at −80 °C until the extraction of the DNA used for the PCR assay. Pieces of roots and soil were also placed on PDA plates and the presence of *G. catenulatum* strain GC1 was assessed according to morphology. Strain GC1 was isolated from roots and grown as a pure culture; the identity of some of the pure cultures was confirmed with strain-specific primers.

Dilution experiments

The amount of fungal DNA needed to amplify a PCR product was tested. The DNA was diluted with sterile water so that the amount of DNA in the amplification reaction was from 1 ng to 5 pg. Dilution was also performed with cucumber DNA: the amount of fungal DNA was from 1 ng to 30 pg, and combined with the cucumber DNA the total amount of DNA was between 2 and 10 ng. Amplification was also performed using a constant amount of fungal DNA (1 ng) and changing the amount of cucumber or pumpkin DNA (20–100 ng). The possible inhibiting effect of potato root DNA extract in the PCR reactions was tested by adding different amounts of potato root DNA extract into amplification reactions in which strain GC1 was used as template.

RAMS, UP-PCR and dot-blot hybridisation analysis

Amplification with one random amplified microsatellite (RAMS) primer was done according to Hantula et al. (1996) with 14 *Gliocladium* isolates. The amplification products were separated by electrophoresis in agarose gel with 0.6% Synergel (Diversified Biotech, Boston, MA, USA) and 1.2% agarose. Two universally primed (UP) primers were used to amplify random segments from the same 14 *Gliocladium* isolates as in the RAMS assay. The program for UP-PCR consisted of one cycle of 3 min at 94 °C followed by 30 cycles of 50 s at 92 °C, 60 s at 55 °C and 70 s at 72 °C. The amplification products of UP-PCR were electrophoresed in MetaPhor agarose gel (FMC BioProducts, Rockland, ME,USA).

The UP-PCR amplification products of AA_2M_2 primer from 14 *Gliocladium* isolates were used for cross dot-blot analysis according to Yli-Mattila et al. (1998) and Bulat et al. (1998) using whole PCR products from strain GC1 as a radioactive probe. In making the probe only three UP-PCR cycles were used and the annealing (90 s) and synthesis time (7 min) were longer than in the nonradioactive UP-PCR reaction. After three cycles an extra synthesis of 7 min was used. In addition, oil was added to the PCR tubes to prevent evaporation of the radioactive dCTP (Amersham, AA0005).

Sequencing of 28S rDNA and β-tubulin

The 28S rDNA sequence was amplified from six strains and the β-tubulin sequence from four strains to see if there were any differences between strains and for phylogenetic comparison. Primers and programs for the amplification of 28S rDNA and the purification of PCR products were as described in Yli-Mattila et al. (1997), while the amplification of β-tubulin was done as described in O'Donnell and Cigelnik (1997). The sequences obtained were aligned with the Clustal W program (Thompson et al., 1994).

Results

RAPD-PCR with arbitrary primer pairs

At the beginning of the study, ten *Gliocladium* isolates were analysed for RAPD profiles using different primer pair combinations. Of the 28 arbitrary primer pair combinations used, almost all amplified from all isolates one to five fragments which were not present when either primer was used alone. Primer pair OPA1/OPA3 amplified a fragment of ca. 400 bp (Figure 1) which was unique to *G. catenulatum* strain GC1 when compared to all other *G. catenulatum* strains. *Trichoderma virens* strain TV1 also produced a fragment of ca. 400 bp

(Figure 1). This fragment was sequenced with primers OPA1 and OPA3 (acces. no. AF139829) and was found to be nonhomologous with that of GC1.

Design and testing of strain-specific primers

The strain-specific DNA fragment amplified with the OPA1/OPA3 primer pair was sequenced with the OPA1 and OPA3 primers. Only ca. 375 bp of the fragment was obtained due to unsuccessful sequencing with OPA3 primer. According to the sequence obtained, one forward primer was designed, Gc1-1, and two reverse primers, Gc3-2a and Gc3-2b (Table 2). When the DNA fragment was cloned into plasmids and sequenced with M13 primers, the whole 397 bp sequence (acces. no. AF133569) of the fragment was obtained, including the primer sequences. According to this longer sequence, one more reverse primer was designed, Gc3-3, to obtain a longer specific amplification. Primer Gc3-3 includes the sequence of OPA3 and can be determined as a SCAR primer. Primer Gc1-1 was not replaced with a new SCAR primer because an insufficiently high GC content was found at that end of the DNA fragment.

When the specific primers Gc1-1 and Gc3-2a were tested with all isolates, a fragment of 237 bp was produced from isolates: GC11, GC13, GC14, GC15, GN2 and GN4. The situation was the same with primer pair Gc1-1/Gc3-3 (Figure 2). When the fragment of

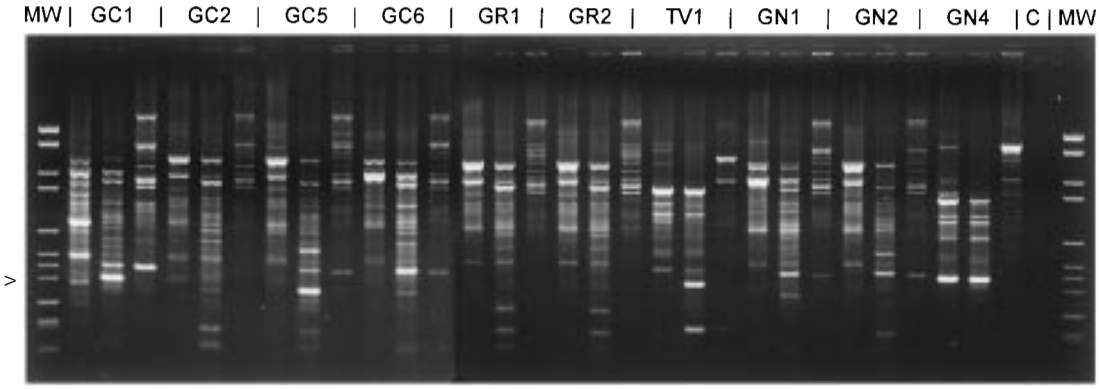

Figure 1. PCR amplification with single and paired RAPD primers OPA1 and OPA3. The lane on the right of each *Gliocladium* isolate is amplified with primer OPA3, the lane in the middle is amplified with both primers and the lane on the left is amplified with primer OPA1. Lane C is the negative control and MW the molecular weight marker VI (Boehringer/Mannheim) with fragment sizes 2176, 1766, 1230, 1033, 653, 517, 473, 394, 298, 234, 220 and 154 bp. The arrow indicates the approximate position of the strain-specific product isolated from strain GC1 (lane 2).

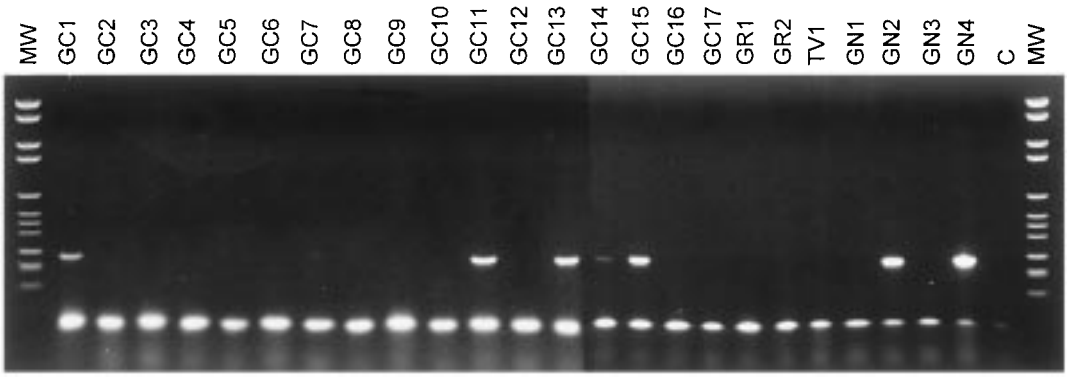

Figure 2. Gel electrophoresis of DNA products of *Gliocladium* isolates and one *Trichoderma* isolate after PCR with specific primers Gc1-1 and Gc3-3. The amplification product of 298 bp is obtained from seven isolates.

298 bp produced from isolates GC1, GC11, GC13, GC15 and GN2 was sequenced it proved to be identical in all isolates except that the fragment of isolate GN2 (acces. no. AF133570) differed in about 18 nucleotides and isolates GC13 and GC15 (acces. no. AF133574) differed in one nucleotide. The specific primers Gc1-1 and Gc3-2b were tested with all isolates except GC2, GC3, GC5 and GC7, using an annealing temperature of 55 °C. A 212 bp fragment was amplified from *G. catenulatum* strains GC1, GC11, GC13, GC14, GC15 and *G. nigrovirens* strains GN2 and GN4. Some weak larger fragments were also amplified from all isolates tested.

Since the first primers developed were not strain-specific, new RAPD amplification products specific for GC1 were searched for, using 20 more primer pairs. From the primer pairs tested, OPA10/OPA18 amplified a fragment of 1593 bp which was selected for further study. When all *Gliocladium* isolates were amplified with primer pair OPA10/OPA18, this 1593 bp fragment was found only in *G. catenulatum* strain GC1 (Figure 3).

The fragment was sequenced with OPA-10 and OPA-18 primers and after cloning with M13 primers. According to the 1593 bp sequence (acces. no. AF133568) obtained, two SCAR primers were designed, Gc10-2 and Gc18-2. In addition, two inner primers, Gc10-1 and Gc18-1, were produced. When primers Gc10-1 and Gc18-1 were tested with all

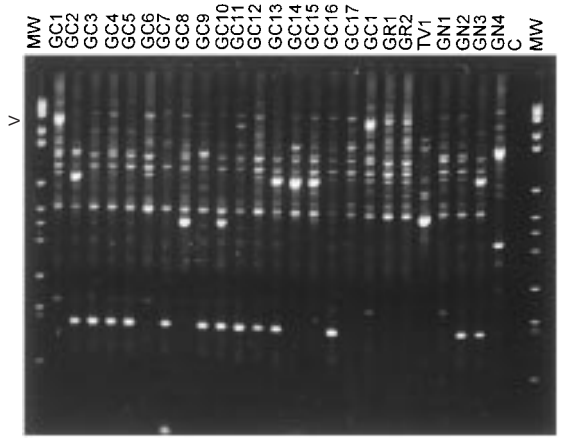

Figure 3. RAPD patterns for 23 *Gliocladium* isolates and one *Trichoderma* isolate obtained with primers OPA10 and OPA18. The arrow indicates the approximate position of the strain-specific product isolated from strain GC1 (lanes 1 and 18). C is the negative control and MW the molecular weight marker VI (Boehringer/Mannheim).

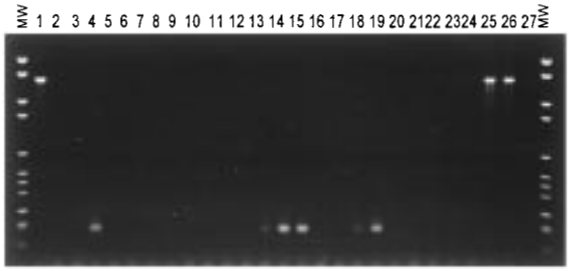

Figure 4. PCR products obtained with strain-specific primers Gc10-2 and Gc18-2 only from samples containing *G. catenulatum* strain GC1. Lanes 1–17: *G. catenulatum* strains GC1–17; lanes 18–19: *G. roseum* strains GR1–2; lane 20: *T. virens* strain TV1; lanes 21–24: *G. nigrovirens* strains GN1–4; lanes 25–26: *G. catenulatum* strain GC1 isolated from potato roots; lane 27: negative control. MW is the molecular weight marker VI (Boehringer/Mannheim).

24 Finnish *Gliocladium* and *Trichoderma* isolates a fragment of 878 bp was amplified strongly from 14 *Gliocladium* isolates (results not shown). Primers Gc10-2 and Gc18-2, on the other hand, amplified a 1593 bp fragment only from *G. catenulatum* strain GC1 out of all *Gliocladium* and *Trichoderma* isolates (Figure 4) as also *Nectria* and *Fusarium* isolates (results not shown). Using an annealing temperature of 55 °C, some weak extra fragments were amplified, but in an annealing temperature of 63 °C they disappeared, except for a fragment of about 230 bp which was amplified from six strains but not from GC1.

The GenBank databases were investigated in order to find sequences with the greatest degree of homology with the region amplified with the primer pairs Gc1-1/Gc3-3 and Gc10-2/Gc18-2. Very close sequences were not found. The closest matches (60–70%) were with mouse ornithine decarboxylase mRNA, *Caenorhabditis elegans* cosmid and some human and *Campylobacter* genes.

Survival of strain GC1 on cucumber leaves, in peat and on potato tubers

When specific primers Gc3-3 and Gc1-1 were tested with DNA isolated from cucumber leaves, the expected amplification product was produced only from leaves treated with the *Gliocladium* suspension (Figure 5). Fragments amplified from DNA of leaf samples, which were collected one day or one to two weeks after treatment, were strong. DNA from samples collected three or four weeks after *Gliocladium* treatment produced

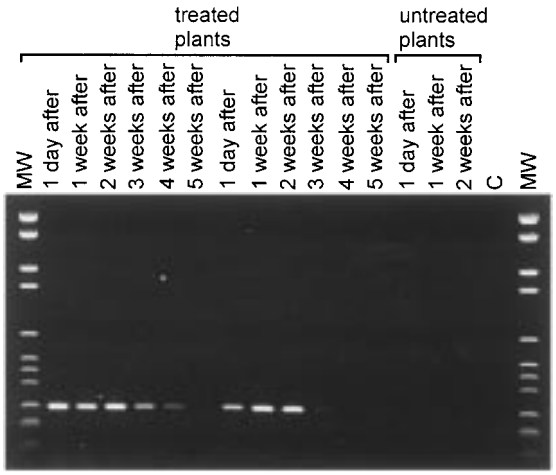

Figure 5. Detection of *G. catenulatum* strain GC1 with specific primers Gc1-1 and Gc3-3 on *Gliocladium*-treated and untreated cucumber leaves. The first sample was taken one day after treatment and the last one five weeks after treatment. C is the negative control, MW the molecular weight marker VI (Boehringer/Mannheim).

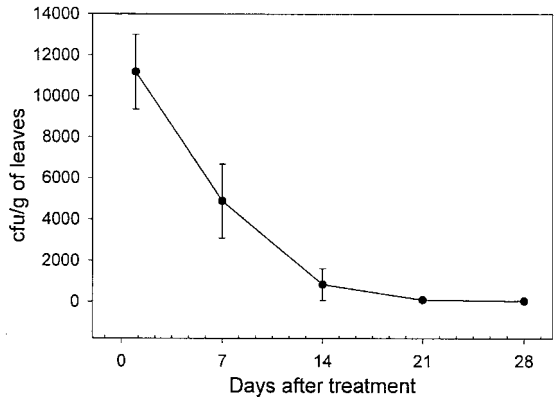

Figure 6. Survival of *G. catenulatum* on cucumber leaves according to colony-forming units (cfu) per gram of cucumber leaves. Dots represent the mean of cfu/g of five treated cucumber plants: the highest value is 11 186 and the lowest value 32. Bars = SE. Where no bars are shown the SEs are smaller than the dots.

weaker bands, while DNA from samples collected five weeks after treatment usually did not produce any fragment. Amplification products were not obtained from other samples. With specific primers Gc10-2 and Gc18-2, no or weak amplification products were obtained from cucumber samples. When amplified samples were used as templates and amplified again with nested primers Gc10-1 and Gc18-1, strong amplification products were obtained from leaf samples which had been collected one day or one to three weeks after treatment (results not shown). The survival of *G. catenulatum* on cucumber leaves is presented in Figure 6. One day after treatment, the mean cfu/g of cucumber leaves was over 11 000. The amount of *Gliocladium* decreased constantly and after four weeks the amount was about 30 cfu/g of leaves. *G. catenulatum* was found on every leaf disc taken from a treated cucumber plant. *Ostracoderma*, *Penicillium*, *Trichoderma*, *Botrytis* and *Cephalosporium* were found on the leaves of untreated plants and on treated leaves two weeks after the treatment.

Specific primers Gc1-1 and Gc3-3 amplified a fragment from all 21 DNA samples isolated from fungi grown in peat, even when *G. catenulatum* strain GC1 was not added to the peat. The same result was obtained previously; when peat samples were tested with three RAPD primers, all banding patterns proved to be identical. Strain GC1 was not detected with specific primers from DNA samples isolated from potato roots which were grown from tubers treated with strain GC1. Instead pure cultures of GC1 were obtained from potato roots, tubers and soil and they were identified with specific primers.

Sensitivity of strain-specific primers

In the dilution series of fungal DNA with primer pairs Gc1-1/Gc3-3 and Gc10-2/Gc18-2, a clear fragment was produced when the amount of DNA was over 20 pg and faint band was detected when the amount was 10 or 5 pg (Figure 7). The result was the same regardless of whether the DNA was diluted with water or with cucumber DNA. When the amount of fungal DNA was 1 ng, the expected band was produced regardless of the amount of cucumber or pumpkin DNA (20–100 ng). Only with cucumber or pumpkin DNA no fragments were detected. When different amounts of potato root DNA (1–60 ng) were added to fungal DNA (1 ng), there was no difference in the amplification compared to amplification with fungal DNA alone.

RAMS and UP-PCR

Isolates TV1 and GN4 had different UP-PCR and RAMS patterns compared to the others (Figure 8A,B). In addition, isolates GC1, GC4, GN1, GN2 and GN3 had slightly different UP-PCR patterns compared to other *Gliocladium* isolates, which had very similar patterns. No clear fragments specific to strain GC1 were

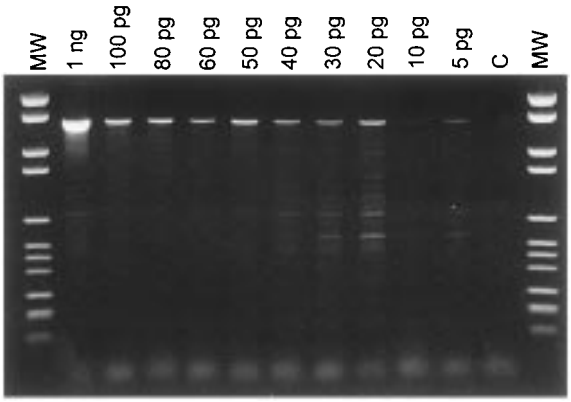

Figure 7. Sensitivity of the strain-specific PCR assay with primer pair Gc10-2/Gc18-2. An amplification product is obtained when the amount of DNA of *G. catenulatum* strain GC1 is 5 pg or over. C is the negative control, MW the molecular weight marker VI (Boehringer/Mannheim).

produced by UP-PCR and RAMS primers. The two *G. roseum* strains were identical with each other and had RAMS patterns slightly different from those of the *G. catenulatum* and *G. nigrovirens* strains. No bands present only in *G. nigrovirens* strains GN1, GN2 and GN3 and separating them from *G. catenulatum* and *G. roseum* strains could be found. The UP-PCR products of GC1 hybridised with all other isolates except for TV1 of *T. virens* (Figure 8C).

Sequence comparisons of the strains

According to the successful amplification of the ITS1-5.8S-ITS2 region with primers ITS1 and ITS4, the quality of DNA in all samples was suitable for PCR amplification. This rDNA region proved to be about 550 bp in all isolates except for *T. virens* isolate TV1, in which this region was about 600 bp, as shown in a previous study (acces. no. AF008923 and AF019199, Yli-Mattila et al., 1997).

The 28S rDNA sequences of ca. 400 bp obtained by primer F24 were identical in all *G. catenulatum*, *G. roseum* and *G. nigrovirens* strains studied (GC1, GC3, GR1, GR2 and GN4, acces. no. AF019200), while in *T. virens* strain TV1 the same DNA sequence was clearly different (acces. no. AF019201). Beta-tubulin products of ca. 1450 bp were obtained in strains GC6, GR1, GN4 and TV1 as well as in *N. pityrodes* isolates with primers T1 and T22. About 500–600 bp of the PCR product were sequenced with primer T1. The β-tubulin sequences of the *G. catenulatum* and *G. roseum* strains were identical (acces. no. AF133571) and very similar to that of the *G. nigrovirens* strain GN4 (ca. 14 nucleotides were different, acces. no. AF133572), while the β-tubulin sequence of *T. virens* strain TV1 (acces. no. AF133573) was clearly different from those of *Gliocladium* strains. The best scores (identity value $>90\%$) for the *T. virens* isolate TV1 (acces. no. AF019201) and the *Gliocladium* isolates (acces. no. AF019200) were obtained at the 28S rDNA region of *Cordyceps bifusispora* (acces. no. Z49794). In the β-tubulin region the best score for *T. virens* and *Gliocladium* isolates was *T. viride*, with identity values higher than 70% in 496 (*T. virens*) to 318 (*Gliocladium*) bp overlap.

Discussion

In the present study, we have developed strain-specific primers for one *G. catenulatum* strain GC1, promising in biological control, in order to facilitate the monitoring of this strain. With the primer pair Gc10-2/Gc18-2 strain GC1 can be distinguished from 16 *G. catenulatum* strains, 11 other strains of two *Gliocladium* species, *T. virens* strain as also from *Nectria* and *Fusarium* species. The specificity of the primers was also verified using DNA isolated from cucumber leaves treated with strain GC1 as a template.

Specific primers have been developed for fungal species (e.g. Schilling et al., 1996; Doohan et al., 1998; Ward and Adams, 1998) especially to identify pathogenic species in infected plant material. The development of strain-specific primers, on the other hand, has proved to be difficult, because for the development of strain-specific primers a higher mutation rate and thus more variation is needed than that for instance of the ITS of nuclear rDNA.

RAPD primer pair OPA-10/OPA-18 and strain-specific primer pair Gc10-2/Gc18-2 amplified a 1593 bp fragment from GC1. Primers Gc10-1 and Gc18-1 amplified about 840 bp of that fragment, which is 730 bp less than the whole fragment. This shorter fragment was amplified from 14 *Gliocladium* isolates. This means that bases which differentiate GC1 from other isolates are included in those missing 730 base pairs. The base pair differences in GC1, which allow the amplification of the fragment can be due to point-mutation, deletions or insertions. The RAPD primers OPA-1 and OPA-3 amplified a 397 bp fragment in GC1. Primers Gc1-1 and Gc3-3 designed according to this

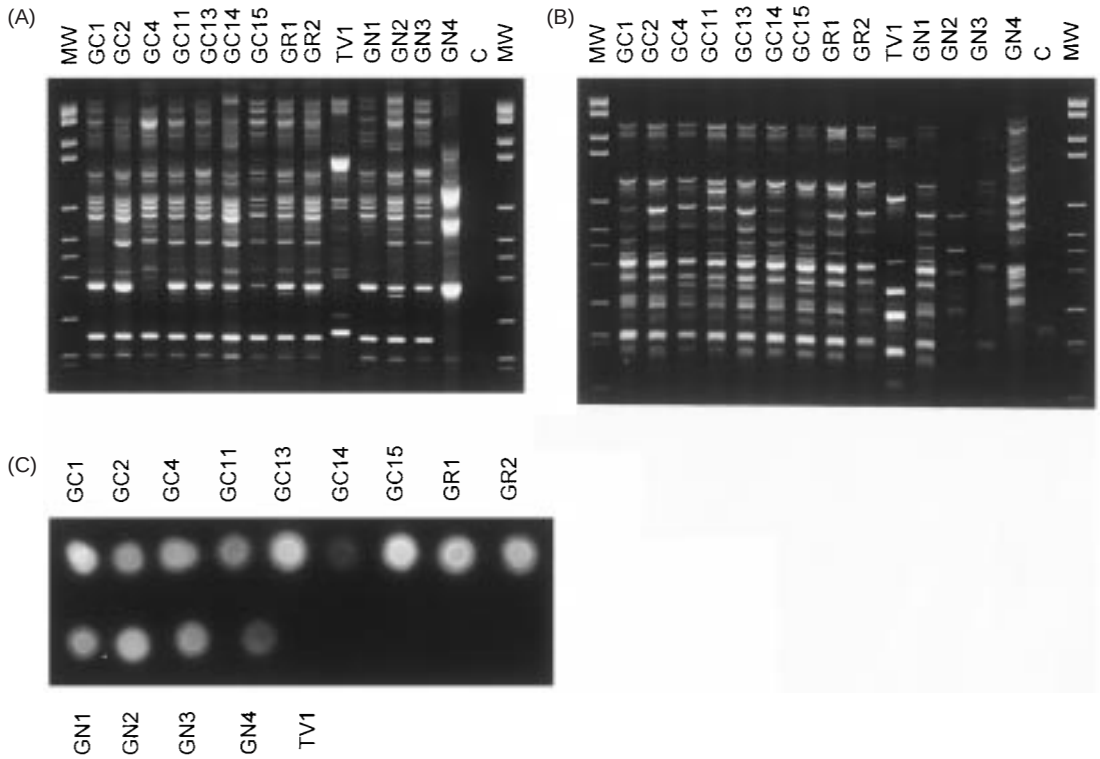

Figure 8. (A) UP-PCR patterns with primer AA_2M_2 and (B) RAMS reaction with CGA primer on 13 *Gliocladium* isolates and one *Trichoderma* isolate. C is the negative control, MW the molecular weight marker VI (Boehringer/Mannheim). (C) Dot-blot hybridisation of UP-PCR products with primer AA_2M_2 of *Gliocladium* isolates.

fragment amplify only 298 bp of this fragment. Primer Gc1-1 could be 100 bp further from Gc3-3, but since the G/C content was considered to be insufficiently enough at the end of the fragment the primer was not designed to the end of the fragment. If the primers amplified the total 397 bp fragment, strain GC1 could probably be differentiated from all other strains, because when all *Gliocladium* isolates were tested with OPA-1 and OPA-3 the 397 bp fragment was found only in GC1. From these results mentioned above and from results in the paper of Lübeck (1997), it can be concluded that in designing specific SCAR primers it is better to include the original RAPD or UP-PCR primers among the specific primers, and not to make the PCR product much shorter than the original RAPD or UP-PCR product. The cloning of the fragment and sequencing with M13 primers make this task easier to obtain the complete sequence. In direct sequencing with the original RAPD primers a shorter sequence, lacking both ends of the fragment, is obtained in cases where the fragment is too long to be totally sequenced with one primer.

In this study, *G. catenulatum* strain GC1 could be identified on cucumber leaves treated with the *Gliocladium* suspension not more than five weeks prior to sample collection. One reason why GC1 was no longer detectable four weeks after the treatment is that the leaf samples were taken from leaves which had developed after the treatment. The amplification with primers Gc10-2 and Gc18-2 was much weaker than with primers Gc1-1 and Gc3-3. With nested primers Gc10-1 and Gc18-1 a strong fragment was obtained from samples already amplified with primers Gc10-2 and Gc18-2 but which were not visible on the gel. This means that there has been some amplification with primers Gc10-2 and Gc18-2. This is probably due to the fact that the template DNA must be of better quality

for a longer fragment than for a shorter one. Also the synthesis time in the PCR must be enough long so that the whole fragment can be amplified. Fungi were also isolated from peat samples, some of which had been treated with *G. catenulatum* strain GC1 while others had not. Unexpectedly, all samples produced a band with primers Gc1-1 and Gc3-3. This was probably due to the spreading of spores of strain GC1 into all the peat samples; thus no conclusions can be drawn from these results. Primer pairs Gc1-1/Gc3-3 and Gc10-2/Gc18-2 did not amplify the DNA of strain GC1 in potato roots. Instead, pure cultures were obtained from these roots. Since the roots were washed with water, the amount of fungal DNA from spores and hyphae was probably too small to be detected with specific primers. To obtain a pure culture, even one spore is enough if the growing conditions are good. The possible inhibitory effect of potato roots in amplification was tested, but no detectable inhibiting effect was found. There were also problems in detecting strain GC1 in strawberries with specific primers (unpublished results). In the future, the treatment of roots and also leaves together with the DNA isolation methods should be developed so that *G. catenulatum* strain GC1 can be identified directly, without the need for pure cultures.

According to the results of the dilution assay, the amount of DNA needed for amplification with specific primers proved to be 5 pg. The amount of DNA was evaluated after electrophoresis by comparison to standards. Such evaluation is always only approximate; since the amounts of DNA are small and pipetting errors may occur, the exact concentrations of DNA are difficult to determine. These results, however, are in accordance with the results of the study of Schilling et al. (1996), in which 50 pg of *F. culmorum* and 5 pg of *F. graminearum* DNA were sufficient to yield PCR products with species-specific primers.

The best scores for the isolates of *T. virens* and *Gliocladium* were at the 28S rDNA region of *Cordyceps bifusispora* and in the β-tubulin region of *T. viride*. The 28S rDNA and the β-tubulin sequence data, together with the previous and ITS sequence data (Yli-Mattila et al., 1997) are in accordance with the RAPD-PCR (Yli-Mattila et al., 1997), UP-PCR and RAMS results, according to which all the *Gliocladium* strains of the present work are very closely related to each other, except strain GN4, which may belong to a different species. RAPD-PCR, UP-PCR and RAMS analyses are more sensitive than morphological identification in separating *Gliocladium* strains, but the phylogenetic groups obtained in this and previous work (Yli-Mattila et al., 1997) do not support dividing the strains into three species. Thus, the strains used in the present work may be conspecific, which means that the taxonomy of this genus still needs revision. This idea is also supported by the hybridisation results of the present work, according to which all *G. catenulatum*, *G. nigrovirens* and *G. roseum* strains belong to the same 'genospecies' (Bulat et al., 1998). The results are also in agreement with those of Rehner and Samuels (1994), who suggested that *Gliocladium virens* should be referred to as *Trichoderma virens*, in the sense that strain TV1 was clearly different from other strains and cannot be closely related to *Gliocladium* species.

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