

## *In situ* PCR for detection and identification of fungal species

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PCR and DNA sequence analysis have become standard tools for identification, detection and phylogenetic analysis of fungi. A large number of species are incapable of growth in the laboratory, making the preparation of pure DNA problematical. The amplification of DNA samples from impure material is subject to misinterpretation if more than one species is present. To overcome this problem, we designed an *in situ* PCR technique that links PCR amplification to the light microscopic image. The amplified tissue is stained, thus confirming which morphotype has been amplified. The PCR product can then be sequenced. We tested the technique on fixed *Blumeria graminis* spores and mycelia using primers derived from the sequence of the gene encoding the catalytic subunit of protein kinase A (*bka1*). This is the first report of *in situ* PCR on phytopathogenic fungal material. This technique allows positive confirmation of the origin of genes cloned from obligate pathogenic fungi and could be adapted for use on any samples containing mixed fungal species.

### INTRODUCTION

The use of PCR and DNA sequence analysis has become routine for the detection, identification and phylogenetic analysis of many fungal species (e.g. Henson & French 1994, Oliver 1993, Taylor *et al.* 2000). It has been particularly valuable in the analysis of fungal phylogenetics. Morphology-based fungal taxonomy was limited by the comparative lack of diagnostic microscopic features, a high degree of morphological variability, and the difficulty of incorporating such data into phylogenetic analyses. Molecular genetics has thus revolutionised fungal phylogenetics in recent years.

Many fungi, particularly soil fungi, are incapable of independent growth in culture. This applies to obligate pathogens (such as *Blumeria*), obligate symbionts (such as *Glomus*) as well as many free-living uncultivable species. The application of molecular phylogenetics to such species is complicated by the difficulty of obtaining pure tissue and, therefore, DNA samples. In addition, many applications of fungal molecular phylogenetics, such as detection and discrimination, involve samples

of fungi growing on other organisms. Examples would include infected seeds and diseased animals. A technique that could identify such species without the need to obtain a pure culture of the fungus would be valuable.

*In situ* PCR (ISPCR) combines two established technologies in molecular genetics, the polymerase chain reaction (PCR) and *in situ* hybridisation (ISH). ISPCR is based on the amplification of specific gene sequences within intact cells or tissues (Nouvo 1992, Long 1998). The technique was developed for animal tissues and used for the detection of viruses such as HIV (Patterson *et al.* 1993) and hepatitis C (Nuovo *et al.* 1993). It has also been applied to a variety of animal diseases and plant tissue (Johansen 1997).

Barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) is an ascomycete pathogen of barley (*Hordeum vulgare*). It is an obligate biotroph and thus it can only complete its lifecycle on a living host plant (Parry 1990). Nucleic acids isolated from infected material can therefore arise from either the plant or fungus. In the work presented here, we set out to adapt the *in situ* PCR technique used in plants (Johansen 1997) to *B. graminis*-infected plant material. The gene detected in the experiment is the catalytic subunit of Protein Kinase A (*Bka1*) cloned from resting conidia (Hall *et al.* 1999). The protein is part of the cAMP-dependent pathway and the gene is found as a single copy in the genome.

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Previously we isolated genes from *B. graminis* using conidia collected with a vacuum device from infected leaves. *In situ* PCR was therefore used to confirm that the genes isolated originated from *B. graminis*.

## MATERIALS AND METHODS

*Blumeria graminis* f. sp. *hordei* race 15 was used in all experiments. The barley cultivar Golden Promise was grown at  $18 \pm 2$  °C in a 18 h photoperiod. The plants were infected one week after germination and incubated for 24 h in a humid environment. Fungal spores were harvested 10 d after infection by gently shaking the leaves over glass slides. Epidermal strips of these infected leaves were peeled off and placed on glass slides for analysis of the mycelia.

Genomic DNA was extracted from resting conidia using a modified CTAB extraction Hall *et al.* 1999). PCR was done using 10 U Taq-polymerase, 1× buffer (both Promega), 2.5 mM MgCl<sub>2</sub>, 1 pmol of each of the primers pkaf (GTG GAA GGT GGC GAA CTC TT) and pkar (GGC GTA TAG CCA CAA AGC AT) (DNA technology) that amplify 445 bp of the *Bka1* gene (GenBank AJ243654), in a total volume of 20 µl. 25 cycles were run (30 s at 95 °, 2 min at 55 ° and 2 min at 72 °) using 25 ng of genomic DNA from *B. graminis*. To confirm that the fragment amplified was indeed *Bka1*, the band was purified from an agarose gel and sequenced by Dideoxy terminator sequencing using a PRISM™ BigDye Terminator kit (PE Biosystems). The reactions were analysed on an ABI Prism 377 DNA sequencer (Perkin Elmer). Gel fractionation was performed according to standard methods (Sambrook *et al.* 1989).

Conidia or infected epidermal strips from barley were placed on silane-coated *in situ* PCR slides (Perkin Elmer). The slides with conidia were incubated for 2 h ensuring that the spores had stuck to the glass. The glass slides with spores were then submerged in FAA (2% formaldehyde, 5% glacial acetic acid, 62% ethanol) for 20–30 min to fix the spores. The slides with mycelia/epidermis were fixed in 4% freshly prepared paraformaldehyde (in 0.05 M phosphate buffer, pH 7) for 30 min. The fixative was washed off with sterile water. Each specimen on the *in situ* PCR slide was encircled with wax (Pap Pen, Electron Microscopy Sciences). 250 µl Zymolyase (40 µg ml<sup>-1</sup> Zymolyase in 0.2 M PIPES, pH 6.9; Seikagaku America) was placed on the tissue (both spore and mycelia) and incubated at 37 ° for 20 min in a humid chamber to prevent evaporation. The pre-treatment with Zymolyase partially degrades the cell wall and ensures that the primers, nucleotides and polymerase have access to the target sequence. The Zymolyase incubation time depends on the tissue type and should be optimised for each fixation. We tested intervals of 3 min (from 10 to 30 min) to determine the optimal length of the Zymolyase incubation.

The PCR mix contained 10 mM Tris-HCl (pH 8.3),

50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 200 µM dNTP, 2 µM of DIG-dUTP and 1 mM of the primers pkaf and pkar. The PCR mix was heated to 70 ° for 10 min before adding 10 U AmpliTaq DNA Polymerase (Perkin-Elmer). The slides were preheated to 70 ° on the assembly tool before 40 µl PCR mix including polymerase was added and sealed with AmpliCover discs and clips (Perkin-Elmer). As negative controls, either primers or polymerase was omitted. The assembled slides were placed in the Perkin Elmer GeneAmp In Situ PCR system 1000 at 70 °. PCR amplification was performed running 30 cycles (95 ° for 1 min, 55 ° for 2 min, 72 ° for 2 min). After amplification, the discs and clips were removed. The slides were then washed in phosphate-buffered saline for 5 min followed by a 5 min wash in 100% ethanol before they were air-dried.

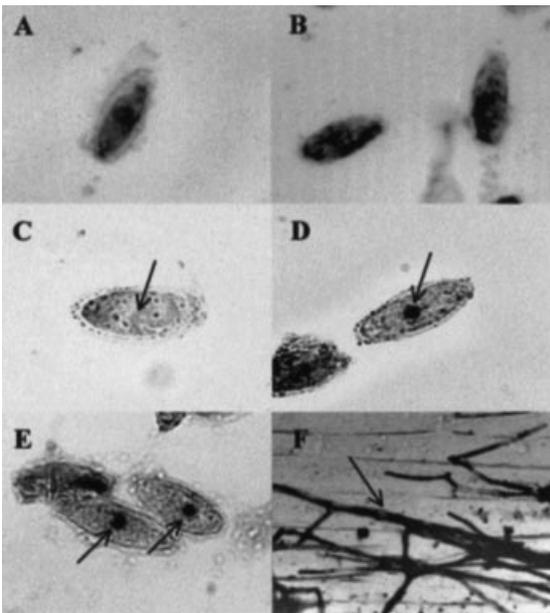
An indirect immuno-labelling method, using secondary antibodies conjugated to phosphatase, was used to detect the PCR product. First, blocking was carried out in 0.1% BSA (bovine serum albumin; Aurion) in 1×PBS (100 µl per sample) for 1 h. The liquid was removed gently with a pipette and monoclonal anti-digoxin (Sigma) diluted 1:100 in 1×PBS (50 µl per sample) was applied for 1 h. The slides were then drained and rinsed twice for 10 min in 1×PBS. The secondary antibody, anti-mouse conjugated to phosphatase (Sigma), was diluted 1:200 in 1×PBS (50 µl per sample) and applied for 1 hour. Detection of the alkaline phosphatase was carried out for 10–30 min, using NBT/BCIP ready-to-use tablets (1 tablet in 10 ml water; 50 µl per sample) (Boehringer Mannheim). After detection, the slides were rinsed in distilled water and analysed in the microscope (Zeiss, Axioplan).

## RESULTS AND DISCUSSION

A number of protocols were tested and modified during the course of the experiments, as described in Material and Methods and below. The fungal digestion conditions were found to be critical and may need to be modified for each application. Using the chosen protocol, amplification could be visualised in spores and mycelium of powdery mildew (Fig. 1A–B and F) where the PCR product is seen throughout the cells. To our knowledge, this is the first demonstration of ISPCR in fungal systems.

Gel electrophoresis showed that the primers only amplified one 445 bp band (not shown) and this band was sequenced and confirmed to be the expected fragment of *Bka1*.

In a control PCR in which the polymerase was left out, no PCR product was seen (Fig. 1C). Optimal fixing and enzyme treatment resulted in confinement of the PCR product to the nucleus (Fig. 1D–E). The enzyme (Zymolyase) chosen to degrade cell walls is a mixture of β-1,3-glucan laminaripentahydrolase and β-1,3-glucanase and is used for degrading yeast cell walls when making yeast spheroplast preparations. It is believed that both the cell wall and the intracellular 3-



**Fig. 1.** *In situ* PCR in mildew spores and mycelia. A–B, *In situ* PCR in mildew spores. The PCR product is detected throughout the spores. C, Negative control – no PCR product is detected. PCR was performed without polymerase. D–E, *In situ* PCR in mildew spores. A fragment of the PKA-c gene was amplified. Arrows indicate the nucleus in which the PCR fragment is trapped. F, *In situ* PCR on mildew mycelia growing on barley. The PCR product is detected throughout the mycelia as indicated by the arrow.

dimensional meshwork consisting of cross-linked proteins and carbohydrates has to be partly broken down for the primers and polymerases to access the target sequence (Gu 1995). When the enzyme treatment was too short, the PCR failed. In this work, an incubation of 20–30 min with Zymolyase was found to be optimal. Incubation of 20–30 min on mycelia was successful but as the PCR products were detected throughout the cytoplasm (Fig. 1F) and not confined to the nucleus as it is in the spores (Fig. 1D–E) a shorter time would presumably prove to be adequate.

The *in situ* PCR technique could be useful in confirming the origin of amplified genes. The technique could be applied not only to obligate fungi, as here, but also to any interaction involving a test fungus and other species. Examples would include the detection of pathogens in crop seed, planting material and animal tissues. The technique obviates the need to culture the fungus and extract DNA prior to amplification. It permits the correlation of morphology, determined

microscopically, with DNA sequence. It also has potential applications in the detection and identification of uncultivable fungi from soil, from infected plant material and from infected medical and veterinary samples.

In this study, we used primers that amplify a single copy gene (Hall *et al.* 1999) in order to test the sensitivity of the system. Multicopy genes such as the nuclear rDNA and the mitochondrial genome are commonly used in detection and identification protocols (White *et al.* 1990). These genes would add greatly to the sensitivity of the technique outlined here.

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