DNA diagnostics for *Fusarium* wilt of cotton

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Abstract

Fusarium wilt of cotton has emerged as a major threat to cotton production, since it was first recorded in Australia in 1993. Control of fusarium wilt depends on understanding genetic diversity within the fungus that causes the disease, *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*). We have analysed genetic diversity within *Fov* by DNA amplification fingerprinting (DAF), and restriction fragment length polymorphism (RFLP) and sequence analysis of the intergenic spacer (IGS) region of the ribosomal (r) DNA. The Australian isolates of *Fov* were compared to overseas isolates that represented races 1, 2, 3, 4, 5, 7, 8 and A of the pathogen, and also to other formae speciales of *F. oxysporum* and species of *Fusarium*. We have identified two distinct genotypes amongst the Australian isolates of *Fov* that correspond with vegetative compatibility groups (VCGs) 01111 and 01112. We are using the information we have obtained on genetic diversity among Australian isolates of *Fov* to develop a polymerase chain reaction (PCR)-based DNA diagnostic system for the rapid detection of *Fov* directly from infected plants and seed, and infested soil. This diagnostic test will be an invaluable tool for the cotton industry, as early detection of fusarium wilt is critical to the containment and control of the disease.

Fusarium wilt

Fusarium wilt of cotton is responsible for serious losses of production in nearly all the major cotton growing areas of the world. The disease was first recorded in Australia in 1993, and has since been identified in several cotton growing regions of Queensland and New South Wales. Fusarium wilt is easily spread by the movement of contaminated soil attached to farm machinery and irrigation water. The disease is also transmitted through infected seed, so there is a high risk of establishment in new areas. Once a farm is infected with fusarium wilt, there is no commercially viable way to eliminate the disease from the soil.

The most effective way to control fusarium wilt is to select or breed cotton varieties with resistance to the disease. Other options for management rely on limiting the build-up of the disease through agronomic practices like stubble management and crop rotation regimes.

Genetic diversity within *F.oxysporum*

Control of fusarium wilt depends on an understanding of genetic variation within the fungus that causes the disease, *Fusarium oxysporum* f.sp. *vasinfectum* (*Fov*). The species *F.oxysporum* includes many plant pathogens that cause wilt diseases on a broad range of agricultural and ornamental plants, as well as saprophytic forms. The pathogenic strains of *F. oxysporum* are indistinguishable from saprophytic forms on a morphological basis. Among the pathogenic forms of *F. oxysporum*, strains with the same host range are grouped together into a 'forma specialis' (f.sp.). Within a forma specialis, strains that are specific for particular cultivars or varieties of the host plant are grouped together into a 'race'. More than 120 formae speciales and races have been described within *F. oxysporum* (Armstrong and Armstrong, 1981). These formae speciales and races can only be distinguished from each other by pathogenicity testing. The forma specialis that is specific to cotton is *F. oxysporum* f.sp. *vasinfectum* (*Fov*).

We have used several different techniques to estimate genetic variation within *Fov*, including vegetative compatibility group (VCG), DNA fingerprinting of genomic DNA, and RFLP and DNA sequence analysis of the ribosomal (r) DNA. We have analysed genetic variation among Australian isolates of *Fov*, and also compared them to isolates of *Fov* from overseas and other fusaria.

DNA fingerprinting

Arbitrary primer technology (APT) methods, such as DNA amplification fingerprinting (DAF), are popular techniques for generating DNA markers for genetic mapping, molecular taxonomy, and molecular diagnostics. These techniques generate genome specific DNA banding patterns in a PCR-based DNA amplification reaction directed by a single oligonucleotide primer of arbitrary sequence. APT methods are useful for quickly and conveniently estimating genomic variation.

We have used the DAF technique to analyse genetic variation among two hundred and thirty Australian isolates of *F. oxysporum* f.sp. *vasinfectum* (*Fov*). The Australian isolates were compared to 55 overseas isolates of *Fov* that represented races 1, 2, 3, 4, 5, 7, 8 and A and were from various countries, including Angola, Argentina, Benin, California, China, Egypt, Ivory Coast, Peru, Philippines, Sudan, Tanzania, and USA. The Australian isolates were also compared to 30 other fusaria, which represented other *F. oxysporum* formae speciales and other *Fusarium* species.

DNA fingerprinting analysis identified two different genotypes amongst the Australian isolates of *Fov* that corresponded to VCGs 01111 and 01112 (Davis *et al.*, 1996). The two Australian genotypes were distinct from all overseas strains of *Fov* and the other fusaria examined. The relationships amongst the overseas isolates of *Fov* that we determined by DNA fingerprinting analysis, supported previous molecular characterisation of VCGs and races of *Fov* based on RFLP and RAPD analysis (Bridge *et al.*, 1993; Assigbetse *et al.*, 1994; Fernandez *et al.*, 1994). We have set up a DNA fingerprint

reference database containing the DNA fingerprints of all of the Australian and overseas isolates of *Fov* that we have characterised, along with information on the host and geographical origin of each isolate. This database allows us to quickly identify isolates of *Fov* when new outbreaks occur, to determine which genotype of *Fov* is present. The DNA fingerprint database will also enable us to recognise new strains of fusarium wilt, if they arise or are introduced into Australia.

rDNA analysis

Useful phylogenetic sequence information is obtained from genes that have the same function in all taxa, evolve at approximately the same rate, and are present only once in the genome or behave like a single copy region. The rDNA genes from the nuclear and mitochondrial genomes fulfil these criteria. The systematic versatility of the rDNA arises from the numerous rates of evolution among the different regions of the rDNA (both within and among genes), the presence of many copies of most rDNA sequences, and the pattern of concerted evolution among repeated copies (Hillis and Dixon, 1991). The nuclear rDNA repeat includes both highly conserved genes and more variable spacer regions, allowing distinction at different taxonomic levels. The non-coding rDNA spacer regions, which include both the internal transcribed spacers (ITS) and the intergenic spacer (IGS), evolve more rapidly, have been used in evolutionary and taxonomic studies in fungi. The IGS region between rDNA repeat units, appears to be the most rapidly evolving region (Hillis and Dixon, 1991), and is therefore a good indicator of intraspecific variation that can be used to infer phylogeny among closely related taxa.

The ITS region of the rDNA has been used to differentiate between *Fusarium* species, and strains of *F. oxysporum* form a distinct group based on ITS sequence (Guadet *et al.*, 1989; Peterson, 1991; O'Donnell, 1992; Donaldson *et al.* 1995; Bateman *et al.*, 1996; Edel *et al.*, 1996; Waalwijk *et al.*, 1996). However, intraspecific variation has been found within the IGS region of the rDNA (Appel and Gordon, 1995, 1996; Edel *et al.*, 1995; Woudt, 1995; Kistler, 1997).

We have analysed genetic variation among Australian and overseas isolates of *Fov* by both RFLP and sequence analysis of the IGS region of the rDNA. The aims of our work were to (i) confirm the relationships amongst Australian and overseas isolates of *Fov* determined by DNA fingerprinting, and (ii) determine the relationship between *Fov* and other formae speciales of *F. oxysporum*. The IGS region (approximately 2.6 kbp) was amplified by the polymerase chain reaction, and digested with the restriction enzymes *Alu*I, *Bst*UI, *Dpn*II, *Rsa*I, and *Scr*FI. RFLP analysis of the IGS region revealed that each of the Australian genotypes of *Fov* (identified by DNA fingerprinting) also represented a unique IGS haplotype. The relationships between isolates of *Fov* and other formae speciales of *F.oxysporum* were also determined (S. Bentley, unpublished data).

By combining total genomic DNA fingerprinting methods such as DAF with other PCR-based methods such as RFLP and sequence analysis of the IGS region of the rDNA, we have thoroughly analysed genetic diversity within *Fov*, from the genus to the strain-specific taxon levels. Such a thorough analysis of genetic diversity provides a solid basis for the development of a detection and diagnostic system for fusarium wilt in cotton.

DNA diagnostics

We are currently developing a DNA diagnostic system for the detection and identification of the two Australian genotypes of *Fov*. This DNA diagnostic system uses the PCR to specifically detect DNA from each of the Australian genotypes of *Fov* in infected plants and seed, and infested soil. The advantages of using DNA-based methods are that DNA markers are generally more stable than phenotypic characteristics, they provide a more precise derivation of genetic relationships, and are they highly amenable to statistical and computer-based pattern analyses and data management protocols (Louws *et al.*, 1999). Furthermore, DNA-based methods that are also PCR-based are able to detect only small amounts of DNA and are generally more specific, sensitive and rapid.

Several other DNA diagnostic tests are available for different formae speciales of *F.oxysporum*. PCR assays have been developed for the specific detection of *F.oxysporum* f.sp. *ciceris* (Kelly *et al.*, 1998) and *F.oxysporum* f.sp. *gladioli* (de Haan *et al.*, 2000) based on sequence characterised amplified regions (SCARs); detection of *F.oxysporum* f.sp. *albedinis* (Fernandez *et al.*, 1998) and *F.oxysporum* f.sp. *dianthi* (Chiocchetti *et al.*, 1999) was based on transposable elements; and a genomic DNA probe has been identified for *F.oxysporum* f.sp. *canariensis* (Plyler *et al.*, 1999).

A PCR-based assay for detection of overseas isolates of *Fov* based on small nucleotide differences in the ITS region of the rDNA has been described by Moricca *et al.* (1998). We are doubtful of the suitability of this assay for the specific detection of Australian genotypes of *Fov* because there is little to no variation within the ITS region of different formae speciales of *F. oxysporum* (Guadet *et al.*, 1989; Peterson, 1991; O'Donnell, 1992; Donaldson *et al.* 1995; Bateman *et al.*, 1996; Edel *et al.*, 1996; Edel *et al.*, 2000; Waalwijk *et al.* 1996), and therefore we consider it unlikely that the test will specific for *Fov*. Moricca *et al.* (1998) evaluated the specificity of their PCR assay on a limited number of isolates, including only one isolate from one other forma specialis of *F.oxysporum*, three isolates from other species of *Fusarium* and three isolates from other genera. Furthermore, we have found that the primer sequences of Moricca *et al.* (1998) match DNA sequences in many other formae speciales of *F.oxysporum* (based on BLAST searches of sequence information published in Genbank). The lack of intraspecific variability within the ITS region of the rDNA, combined with the high degree of genetic diversity within *F.oxysporum*, requires a much more thorough evaluation of primer specificity to ensure the specificity of the assay.

We have targeted the IGS region of the rDNA as the basis for our diagnostic test because (i) it is a stable marker, (ii) it is present in multiple copies (allowing more sensitive detection), and (iii) the presence of both conserved and variable regions allows discrimination at different (taxonomic) levels of specificity. We have identified DNA sequence information that is unique to the two Australian genotypes of *Fov* and designed PCR primers that specifically amplify DNA only from Australian VCGs 01111 and 01112 of *Fov*. Database searches of DNA sequence information published in Genbank have indicated that there are no matches for these primers with any other organism, but we are currently completing the laboratory screening of the specificity of these primers. We will then adapt our laboratory PCR conditions for amplification of *Fov* DNA from infected plants and seed, and infested soil. There are several practical considerations for PCR amplification from such materials, including: (i) false positive reactions that can result from cross-amplification of non-target DNA, exogenous DNA from cultures or aerosols, or from contaminating DNA originating from carryover of previous experiments, (ii) false negative reactions that can arise for many reasons, including the presence of compounds derived from extracted substrates that inhibit *Taq* polymerase, degradation of the DNA target sequence, or reagent problems, (iii) PCR reaction inhibitors e.g. plant derived compounds that inhibit the PCR, (iv) sampling i.e. the large initial sample size needs to be consolidated to a 50 µl endpoint, and (v) amplification of non-viable cells (DNA from dead cells). After these issues are addressed the diagnostic test will require validation and field-testing, and then it will be made available to industry.

Conclusion

Thorough analysis of genetic diversity within a plant pathogen provides the groundwork for the development of DNA-based detection systems, which allow more accurate plant disease diagnosis and better disease management. We have thoroughly analysed genetic diversity among Australian and overseas isolates of *Fov*, from the genus to the strain-specific taxon levels. From this information, we are developing a PCR-based DNA diagnostic system specific for the Australian genotypes of *Fov*. This DNA diagnostic test is urgently required for the rapid identification of *Fov* from infected plants and seed or infested soil, as early detection of fusarium wilt is critical for containment and control of the disease. This test will also be a useful tool to monitor the distribution and spread of the disease throughout the cotton production areas of Australia, and to evaluate the effectiveness of different agronomic practices (e.g. crop rotation) on the survival of *Fov* in affected fields.

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