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Applications of PCR in Fungal–Plant Interactions

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13.1 Introduction

This chapter describes the use of PCR-based techniques for studying plant–fungal interactions. The first section describes the uses of PCR for detection of fungal pathogens and symbionts within their hosts and the other three sections cover approaches used to study the genes involved in plant–fungal interactions. Some genes involved in fungal infection and plant responses to infection are common to many host–pathogen combinations and some of these have been characterized at the nucleotide or amino acid level. Where this is the case, PCR allows similar genes from other interactions to be isolated and characterized relatively easily. A second approach, which does not rely on any previous sequence characterization, is to search for genes whose expression is altered during fungal interactions and PCR-based methods are available to do this. The final application discussed is the use of PCR to detect genomic differences between plants or fungi that differ in one characteristic only, e.g. fungal pathogenicity or plant resistance to a particular pathogen. Recent reviews on various aspects of plant–fungal interactions have described: plant resistance genes (Ellis *et al.*, 1988; Michelmore *et al.*, 1992; Keen, 1992); fungal pathogenicity and virulence genes (Schäfer, 1994; Van Etten *et al.*, 1994; Hensel and Holden, 1996); fungal avirulence genes (de Wit, 1992); genes involved in mycorrhizal interactions (Gianinazzi-Pearson, 1996; Gianinazzi-Pearson *et al.*, 1996); plant defence genes (Dixon *et al.*, 1994); and fungal colonization of plants (Takenaka, 1995).

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13.2 Applications

13.2.1 Detection, localization and quantification of fungi in plant tissues

PCR is now widely used to detect and identify microorganisms, and there are many examples of its use for fungi, including both plant pathogens and symbionts. This application has been reviewed by Foster *et al.* (1993), Ward (1994) and Bridge and Arora (Chapter 4) and will not be covered here. PCR assays developed for a fungus in pure culture can usually be adapted for detection of the fungus within the plant although procedures to overcome PCR inhibitors in some plant tissues may be necessary (Robb and Nazar, 1996). This then opens the way for using PCR to quantify the extent of fungal colonization of plant tissue or to localize the fungus within the plant.

One area in which PCR is beginning to be used is the quantification of fungal biomass in infected tissues. For reviews of quantitative PCR, see Ferre (1992), Diaco (1995) and Zimmerman and Mannhalter (1996). The use of PCR for quantification is not straightforward. This is because the PCR includes an exponential phase, so minute differences in efficiency of reaction between samples can give rise to dramatically different amounts of final product. To overcome this problem, internal control DNA, which is amplified in the same PCR reaction, should be included. The quantity of control product is then used to adjust the quantity of the test amplicon for variations in the efficiency of the PCR between tubes. The detection and quantification of the PCR products can be done by measuring the intensity of ethidium bromide-stained DNA on gels (e.g. using densitometry), by scintillation counting of labelled DNA, or by using colorimetric techniques.

Quantitative PCR has several advantages over other methods of determining fungal biomass, such as tissue maceration and dilution plating which is a lengthy and labour-intensive procedure. Also, the dilution plating assay may only indicate the amounts of fungus that can be isolated rather than the amount actually present, and it cannot be used for obligate pathogens or symbionts. PCR is a much more rapid technique and it does not require isolation of pure fungus from the tissue. Another method of biomass determination is to measure ergosterol or chitin levels, but this method does not allow discrimination between different fungi infecting the same plant, which is possible with PCR. Serological methods have also been used for fungal biomass determination but it is generally difficult to obtain antibodies with the required specificity for the fungus being studied. PCR has the potential to measure fungal biomass simply and with great sensitivity and specificity. Quantitative PCR has been used in the detection of mycorrhizal fungi (Simon *et al.*, 1992), *Verticillium* spp. (Hu *et al.*, 1993; Moukhamedov *et al.*, 1994; Robb and Nazar, 1996), *Leptosphaeria maculans* (Mahuku *et al.*, 1995) and *Microdochium nivale* (Nicholson *et al.*, 1996). The ability to monitor the amounts of different fungi infecting the same plant at the same time by quantitative PCR will be of great value in studying disease complexes

(Nicholson *et al.*, 1996) and interactions between fungi, e.g. synergism and competition.

Localizing fungi within plant tissues can be done simply by separating the different parts of the plant and testing each for the presence of the fungus; PCR was used to monitor the spread of *Verticillium* species through their hosts at different times after infection (Moukhamedov *et al.*, 1994; Hu *et al.*, 1993). A much more precise method, capable of localizing the fungus at the cellular level, is *in situ* PCR (Nuovo, 1995), in which thin sections of infected plant tissue are placed on a microscope slide and the PCR is performed directly on this. This is technically difficult and although there are now many applications in studying human infections, particularly those involving viruses, we are not aware of any applications to the study of fungi or plant–fungal interactions published yet. This could be an area for development in the future, not just for detection of the fungi themselves but also for the detection of mRNA from genes expressed in plant–fungal interactions.

13.2.2 Targeted approaches to cloning and analysing plant and fungal genes

These approaches can be used when physiological, biochemical and/or molecular data about a gene or gene product suggest that it is involved in a particular plant–fungal interaction. They are particularly suitable for genes and gene products that are common to many plants or fungi and/or are expressed in response to a range of pathogens, hosts or other stimuli. Many of the plant defence genes fall into this category. Nucleotide or amino acid sequence data from previously characterized genes or proteins are used to design primers that can amplify the same or homologous genes or identify cDNA clones for those genes. Whether designing primers from amino acid or nucleotide sequences, this approach usually involves the use of degenerate primers that allow for uncertainties in the nucleotide sequence (see Kwok *et al.*, 1995 for a review of their design and use). Examples of genes isolated using targeted approaches are given in Table 13.1.

Where a protein involved in a particular interaction has been characterized, amino acid sequence information can be used to design primers to amplify part of the corresponding gene from genomic DNA or cDNA. Because of the redundancy of the genetic code, a given amino acid may be encoded by different nucleotide triplets. Degenerate primers should be designed as a pool of all the possible combinations of nucleotides that could code for the amino acid sequence. Deoxyinosine can be used to reduce the complexity of the primer pool. In other cases, nucleotide sequence information can be used to design primers, e.g. where nucleotide sequence information is already known for related genes. This generally involves the use of degenerate primers because even within conserved regions of genes there is often some variation, e.g. between different plants or fungi or between different members of a gene family in the same plant.

Table 13.1. Genes isolated from various plant–fungal interactions using PCR-based targeted approaches.

	Gene product	Organism	Reference
Fungal genes for products involved in fungal attachment and penetration of the host plant	Pectin lyase	*Glomerella cingulata*	Templeton *et al.* (1994)
	Exo-β1,3-glucanase	*Cochliobolus carbonum*	Schaeffer *et al.* (1994)
	Endopolygalacturonase	*Fusarium moniliforme*	Caprari *et al.* (1993)
	Extracellular protease (*ALP1*)	*Cochliobolus carbonum*	Murphy and Walton (1996)
Genes for fungal toxins	Phytotoxins synthesized by cyclic peptide synthetases	*Cochliobolus victoriae*	Nikolskaya *et al.* (1995)
		Diheterospora chlamydosporia	Nikolskaya *et al.* (1995)
		Cylindrocladium macrospora	Nikolskaya *et al.* (1995)
Plant defence genes	Phenylalanine ammonia lyase	Rice	Zhu *et al.* (1995)
	Peroxidase	Wheat	Båga *et al.* (1995)
	Pathogenesis-related gene *RH2*	Pea root epidermis	Mylona *et al.* (1994)
	Chitinases	Sugar beet	Nielsen *et al.* (1994)
		Pea	Vad *et al.* (1993)
		Maize	Wu *et al.* (1994)
		Wheat	Liao *et al.* (1994)
	Polygalacturonase inhibitor (PGIP)	Soybean	Favaron *et al.* (1994)
	IWF (non-specific lipid transfer protein)	Sugar beet	Nielsen *et al.* (1996)
Fungal and plant genes involved in mycorrhizal interactions	Plant sugar transporter	*Medicago truncatula*	Harrison (1996)
	Fungal phosphate transporter	*Glomus versiforme*	Harrison and van Buuren (1995)

Once a small region of the gene of interest has been amplified using PCR, full-length clones may be obtained by screening genomic or cDNA libraries with labelled PCR-amplified DNA, or by using other PCR-based approaches. For cDNA clones the missing parts of the gene sequence can be cloned by an approach most commonly referred to as RACE (rapid amplification of cDNA ends; Frohman, 1995). RACE is also known as anchored PCR or one-sided PCR. RACE involves amplification using one primer derived from a short stretch of known sequence in the gene/mRNA and another primer that anneals either to the poly(A)-tail (to obtain the 3′-end) or to an appended homopolymer tail (for the 5′-end). There are several examples of its use in cloning genes involved in plant–fungal interactions (Vad *et al*., 1993; Nielsen *et al*., 1994, 1996; Murphy and Walton, 1996).

PCR-based approaches have allowed genes and their mRNA products to be cloned and analysed much more quickly than was previously possible. Once sequences are published it is possible for other workers to use the information to analyse similar genes immediately rather than waiting for the exchange of clones. There are various uses for the genes once isolated, in addition to characterizing the gene structure and organization. The spatial and temporal expression of the gene can be studied during infection by techniques such as Northern blotting (Vad *et al*., 1993; Wu *et al*., 1994; Zhu *et al*., 1995; Nielsen *et al*., 1996), reverse transcriptase (RT) PCR (Båga *et al*., 1995; Guo *et al*., 1995; Meyer *et al*., 1996) or *in situ* hybridization. The gene can be cloned into an expression vector allowing purification and further characterization of the protein and the production of antisera (Templeton *et al*., 1994; Guo *et al*., 1995). The promoter from the gene can be fused to a reporter such as β-glucuronidase (GUS), transformed into plants and the expression studied in various tissues at different times after infection or other treatments (Zhu *et al*., 1995).

In fungi, mutants lacking particular genes have been obtained by targeted gene disruption using cloned genes and this has allowed functional studies to be carried out. A gene encoding the exo-β-1,3-glucanase of *Cochliobolus carbonum* was cloned using degenerate primers based on amino acid sequence and this was then used to generate mutants that could not produce the enzyme. The mutant was still pathogenic to maize, indicating that the exo-β-1,3-glucanase gene was not essential for pathogenicity (Schaeffer *et al*., 1994). A similar approach was used to study an extracellular protease ALP1 from *Cochliobolus carbonum* (Murphy and Walton, 1996). The ALP1 mutants thus generated had the same growth and disease phenotypes as the wild-type strain, indicating that this gene by itself was not required for pathogenicity. Although the above studies could have been done without the PCR, its use enabled the work to be done much more easily and quickly than was previously possible.

PCR can also be used for *in vitro* mutagenesis to study the effects of particular amino acid residues on the biological activity of the gene product. Altered primers can be used to replace the nucleotides coding for single or

multiple amino acid residues in the protein. This approach was used to study the mode of action of the AVR9 elicitor peptide involved in the *Cladosporium fulvum* – tomato interaction (Honée *et al.*, 1994).

13.2.3 Studying plant and fungal genes involved in interactions by comparative genome analysis

The biochemical basis of the effects of most genes involved in plant–fungal interactions is not known. One approach devised for studying these genes is based on comparing the DNA of organisms with or without a trait of interest to find the regions that differ. In particular, this has been used to study plant resistance genes, but the methods described are applicable to other plant and fungal genes. The first stage in the analysis is to find a molecular marker that is closely linked genetically to the required gene. Two types of strategies are commonly used to 'home in' on the region of interest. The first is to use near isogenic lines, that differ primarily in the gene of interest (Horvath *et al.*, 1995). The second is a 'pooling approach' such as bulked segregant analysis (Michelmore *et al.*, 1991). Using this technique, markers linked to a trait of interest are identified using two pooled DNA samples, one from (homozygous) individuals that express the trait and the other from (homozygous) individuals lacking the trait. Any polymorphism between the two pools should be linked with the trait. Markers thus identified are then confirmed by mapping a segregating population.

Until the advent of PCR in the late 1980s most of the markers used were RFLP (restriction fragment length polymorphisms). Although these allowed much faster and easier screening than did the previously available phenotypic markers, the procedure required relatively large quantities of purified DNA and usually radioactive detection. PCR offered the opportunity of being able to screen for differences much more easily; the techniques do not involve radioactivity, are simpler and can be performed on small amounts of crudely prepared samples. Some of the previously developed RFLP methods were subsequently converted to PCR-based protocols by sequencing the clones and then designing primers based on these sequences (Balint-Kurti *et al.*, 1994; Kilian *et al.*, 1994). These markers are frequently referred to as SCARs (sequence-characterized amplified regions).

Additional PCR-based methods were also developed that allow the identification of useful markers much more quickly. The most widely used of these involves PCR at low stringency, with arbitrarily chosen primers, and is known by the acronyms RAPDs (random amplified polymorphic DNAs; Williams *et al.*, 1990), AP-PCR (arbitrarily primed PCR; Welsh and McClelland, 1990) and DAF (DNA amplification fingerprinting). The concept is essentially the same and hereafter RAPDs will be used to refer to all three techniques. In this technique, the primers are usually much shorter (10 bases) than those generally used for PCR (~20 bases) and one primer is

sufficient. Also the conditions of the PCR are made relatively non-specific, e.g. by reducing the annealing temperature and increasing the number of amplification cycles (e.g. from 25 to 45). Since the primers are so short and mismatches are allowed by low stringency conditions it is likely that many primer-binding sites will be present in the target DNA. There is also a high probability that pairs of sequences complementary to the primer will be arranged close enough together and in the correct orientation for PCR-amplification of the intervening sequences. Each RAPD is likely to result in the amplification of several (usually 3–10) bands that can be detected by agarose gel electrophoresis and ethidium bromide staining. Alternative detection methods, that reveal a larger number of bands, are silver staining of polyacrylamide gels (Caetano-Anollés *et al.*, 1991) radioactive labelling of products run on acrylamide gels detected by autoradiography (Welsh and McClelland, 1990) and denaturing gradient gel electrophoresis (DGGE) (Procunier *et al.*, 1995). Reactions with different RAPD primers can detect variation at different levels, including species, isolates and near-isogenic lines differing only in a single trait, e.g. plant resistance to disease or fungal pathogenicity.

Due to the ease of use and the speed at which markers can be developed using it, RAPDs have been used extensively in the few years since the method was first reported. Several reviews have covered various aspects of the technique and its applications in genetics and plant breeding (Waugh and Powell, 1992; Bowditch *et al.*, 1993; Rafalski and Tingey, 1993; Williams *et al.*, 1993; He *et al.*, 1994; McClelland and Welsh, 1995). Using RAPDs, markers have now been identified that are closely linked to the resistance genes for barley stem rust (Horvath *et al.*, 1995), barley powdery mildew (Xu and Kasha, 1992), wheat leaf rust (Schachermayr *et al.*, 1994), lettuce downy mildew (Michelmore *et al.*, 1991; Paran and Michelmore, 1993; Maisonneuve *et al.*, 1994), pea powdery mildew (Timmerman *et al.*, 1994), bean rust (Miklas *et al.*, 1993) and muskmelon Fusarium wilt (Wechter *et al.*, 1995). RAPDs have also been used as markers in fungi, but the work here has mainly been used for species and population analysis (McDermott *et al.*, 1994).

The major problem with the RAPD technique is the lack of reproducibility, although with appropriate precautions the effects of this can be minimized. Usually RAPD bands behave as dominant markers (only one of the alleles can be amplified) rather than co-dominant markers (each allele amplifies a different band) which would be generally more useful. Another feature of RAPDs is that multiple loci are amplified. Whilst this is useful initially, in that many loci can be analysed at once, it is a disadvantage in subsequent analyses because of the complexity of the patterns. Another potential source of problems is contamination and it is essential to include negative controls. Because of these problems RAPD markers are often converted to more reproducible markers (SCARs) after cloning and sequencing (Markussen *et al.*, 1995; Naqvi and Chattoo, 1996).

Another, more recently developed technique for isolating markers linked to traits of interest is AFLPs (amplified fragment length polymorphisms) (Vos *et al.*, 1995; Perkin Elmer, 1995). The technique is based on the selective amplification of restriction fragments generated from the genomic DNA. First the DNA is digested with restriction enzymes and oligonucleotide adaptors are ligated to it. Sets of restriction fragments are then amplified selectively and, finally, the amplified fragments are analysed after gel electrophoresis. The selectivity is achieved by using primers that contain the restriction site and variable 3′ extensions. The number of bands produced depends on the type of gel analysis used but typically 50–100 restriction fragments are resolved on denaturing polyacrylamide gels. The AFLP method is more robust and reliable than RAPDs because more stringent reaction conditions are used, but is more difficult and expensive to do. The technique has been used to select markers closely linked to the *R1* (resistance to *Phytophthora infestans*) gene of potato (Meksem *et al.*, 1995) and the tomato *Cf-9* gene (resistance to *Cladosporium fulvum*; Thomas *et al.*, 1995). Other PCR-based methods have also been developed that allow rapid identification of markers for genes of interest (for reviews, see Karp *et al.*, 1996; Staub *et al.*, 1996), including variable non-translated repeats (VNTRs) and microsatellite/simple sequence repeats (SSRs). Once developed, these markers are useful in screening breeding lines to track which progeny contains the desirable trait (marker-assisted selection). Molecular markers, and particularly those involving PCR, allow much faster and easier screening than do the previously available phenotypic markers, and this is important in these high throughput studies.

The markers can also be used to facilitate cloning of the gene of interest (for resistance, pathogenicity, etc.) by map-based cloning procedures (Young, 1990, 1995; Michelmore *et al.*, 1991). This is an approach whereby information about the gene's chromosomal location is used as the basis for cloning. After markers that are closely linked genetically to the gene of interest have been identified a physical map is constructed in which the distances between markers are calculated as numbers of nucleotides rather than recombination frequencies (e.g. using pulsed-field gel electrophoresis). Chromosome walking is then used to identify overlapping clones in the region of interest. The last stage is to pinpoint the target gene among the overlapping clones, usually by complementation of organisms with the recessive phenotype. This approach is a long-term, labour-intensive undertaking, but it has been used to isolate the genes conferring resistance to the bacterial pathogen *Pseudomonas syringae* in tomato (Martin *et al.*, 1993) and *Arabidopsis thaliana* (Mindrinos *et al.*, 1994).

It may also be possible to identify genes of interest which, for example, differ between wild-type and deletion mutants, by genomic subtraction methods. DNA that is found in one of the pair of organisms under test, but not the other, is selected and then amplified using PCR (Straus and Ausubel, 1990; Lisitsyn *et al.*, 1993). This approach has been used to clone the

Arabidopsis gibberellin synthesis (*GA1*) locus (Sun *et al*., 1992). Subtractive hybridization is also used on mRNA, to find mRNA species which are present in colonized plants but absent from the uninfected plant (see section 13.2.4).

13.2.4 Non-targeted approaches to characterizing altered gene expression during plant–fungal interactions

The use of PCR in combination with standard cloning techniques is now a widely adopted strategy to identify genes whose expression is altered during biological interactions and it has resulted in the saving of much time, effort and cost. Several methods have been developed and optimized in different laboratories to suit a specific need and a particular biological system. In this section we discuss three applications of PCR to investigate changes in gene expression during fungal–plant interaction, without prior knowledge of the genes or proteins that might be involved: (i) the use of PCR in combination with differential hybridization methods to screen for differentially expressed genes; (ii) RNA fingerprinting methods to analyse differential gene expression; (iii) the use of PCR in conjunction with subtractive hybridization to generate probes specific to differentially expressed genes.

Differential screening of cDNA libraries

Various papers discuss the use of PCR to construct cDNA libraries (Gurr and McPherson, 1992; Clackson *et al*., 1993; Lambert and Williamson, 1993). PCR can also be used during screening to estimate the average cDNA insert size (Güssow and Clackson, 1989). This allows the presence, size and orientation of inserts to be determined rapidly by amplification with flanking primers, and (for orientation) by including a single internal primer. PCR can be performed directly on single phage plaques, aliquots from phage libraries, or bacterial colonies, circumventing all DNA preparation. When the PCR-based assessment is used in combination with the IPTG and X-Gal colour selection method (Sambrook *et al*., 1989) to determine the relative numbers of recombinant (white) versus wild-type (blue) phage, it provides a simple and accurate assessment of the quality of cDNA library under investigation.

Differential screening allows the identification of cDNA clones corresponding to differentially expressed genes. Poly(A)$^+$ RNAs extracted from the two samples under comparison are used as templates to generate labelled cDNA probes that are hybridized separately to duplicate copies of the same cDNA library. Clones that hybridize to both probes correspond to genes that are constitutively expressed in both samples, whereas clones that hybridize to only one probe correspond to mRNAs that are expressed in only one sample (Sambrook *et al*., 1989). To ensure the purification of single clones, putative positive phage clones are screened through two or three rounds of differential hybridization. Due to the high number of phages

which are analysed during each round of screening, the method can be very time-consuming and costly. PCR can be used to simplify and speed up this process. An aliquot is taken from each phage clone as template for a PCR reaction using, for example, M13 forward and reverse sequencing primers to amplify the cDNA insert within the polylinker or any specific primers flanking the cloning site. There are several advantages of using this approach: (i) it shows whether the phage clone is single or contaminated by other surrounding phages; (ii) it gives an estimate of the size of the inserted cDNA; (iii) several phage clones can be screened at once; (iv) when combined with Southern blot analysis it allows analysis of the induction or repression of the clones under investigation. This approach has been used to clone symbiosis-related cDNAs from eucalypt ectomycorrhizae (Tagu *et al.*, 1993) and to study genes whose expression is altered during the colonization of tomato roots with the arbuscular mycorrhizal fungus *Glomus mosseae* (Tahiri-Alaoui and Antoniw, 1996).

RNA fingerprinting methods

Another approach to detecting and isolating differentially expressed genes is by comparing cDNA fingerprints generated from mRNA expressed under different conditions (see Chapter 3; McClelland and Welsh, 1995; McClelland *et al.*, 1995, for recent reviews and protocols). These methods are the RNA equivalents of the DNA fingerprinting methods described in Section 13.2.3. Liang and Pardee (1992) were the first to describe an RNA fingerprinting protocol, which they termed differential display reverse transcriptase PCR (DDRT–PCR). They used a primer for reverse transcription based on oligo-dT but with an anchor of two bases at the 3′-end. After reverse transcription and denaturation, PCR was performed on the first strand cDNA using the anchored oligo-dT primer together with an arbitrarily chosen 10-mer primer to generate a fingerprint of products analysed on a polyacrylamide sequencing gel. DDRT–PCT has been used in the investigation of gene regulation in both animal and plant systems. Although the principle of the technique is simple and elegant, there are several problems in practice, including the redundancy and under-representation of certain mRNA species and a rather high number of false positives that cannot be confirmed by Northern blot analysis. We have encountered such problems in our laboratory in investigating the interaction of barley roots with the take-all fungus, *Gaeumannomyces graminis* var. *tritici*, and potato infected with potato cyst nematode, *Globodera rostochiensis*. Despite extensive analysis, and although barley and potato showed dramatic physiological responses to the pathogen attack, no differences in gene expression were found between uninfected plants and plants responding to pathogen attack. This led to the design of an experimental system in which it was demonstrated that differential display showed a strong bias towards high copy number mRNA species (Bertioli *et al.*, 1995). There are few reports of the successful use of differential display in analysing

plant–microbe interactions (Martin-Laurent *et al.*, 1995; Martin-Laurent *et al.*, 1996). Various efforts have been made to optimize and streamline the method (Liang, *et al.*, 1993, 1994; Li *et al.*, 1994; Mou *et al.*, 1994).

In an alternative protocol, termed RNA arbitrarily primed PCR (RAP-PCR; Welsh *et al.*, 1992) arbitrary primers are used both for cDNA synthesis and PCR amplification. A method of RNA fingerprinting based on AFLPs has also been developed and used to analyse gene expression during potato tuber development (Bachem *et al.*, 1996).

Subtractive hybridization methods

This approach involves the subtraction (by hybridization) of the mRNAs expressed in uninfected cells from those expressed in infected cells to leave only those expressed during infection. PCR has been invaluable in amplifying the minute amounts of material that result from this process. The cDNA thus produced can be used to make subtractive cDNA libraries or used as a probe to screen existing cDNA libraries. There are several variations of the technique depending on the method used to separate the hybridized and non-hybridized molecules, including hydroxylapatite chromatography (Sambrook *et al.*, 1989), biotinylation (Wieland *et al.*, 1990) and magnetic beads (Sharma *et al.,* 1993). Examples of the use of the technique in studying plant–fungal interactions have been reported (Sharma *et al.,* 1993; Lönneborg *et al.,* 1995; Roberts and Pryor, 1995; Justesen *et al.*, 1996). Recent developments in subtractive hybridization techniques include representational difference analysis (RDA) and PCR-select cDNA subtraction.

RDA is a method originally developed by Lisitsyn *et al.*, (1993) to isolate differences between two complex genomes. Genomic RDA relies on the generation, by restriction enzyme digestion and PCR amplification, of simplified versions of the genomes under investigation known as 'representations'. If an amplifiable restriction fragment (the target) exists in one representation (the tester), and is absent from another (the driver), a kinetic enrichment of the target can be achieved by subtractive hybridization of the tester in the presence of excess driver. Sequences with homologues in the driver are rendered unamplifiable, while the target hybridizes only to itself, and retains the ability to be amplified by PCR. Successive iterations of the subtraction/PCR process produces bands on ethidium bromide-stained agarose gels corresponding to enriched target. The RDA method was adapted for cDNA and successfully used to identify a number of caffeine-induced cDNA fragments from pre-B cell lines (Hubank and Schatz, 1994). Although we are not aware of any published work using the cDNA-RDA method to investigate altered gene expression in plant–fungal interactions, the approach seems to be fast, sensitive, reproducible, with few false positives and capable of being applied to a wide range of biological problems.

PCR-select cDNA subtraction (Clontech, 1996) uses a new method called suppression PCR (Siebert *et al.*, 1995). The PCR-select cDNA primer

adaptors are engineered to prevent undesirable amplification, i.e. amplification of common sequences, during PCR. Suppression occurs when complementary sequences are present on each end of a single-strand cDNA. During each primer annealing step, the hybridization kinetics strongly favour the formation of a pan-handle secondary structure that prevents primer annealing. When occasionally a primer anneals and is extended, the newly synthesized strand will also have the inverted terminal repeats and form another pan-handle structure. Thus, during PCR, non-specific amplification is efficiently suppressed, and specific amplification of cDNA molecules with different adaptors at both ends can proceed normally. We have used this technique to generate two specific probes of mRNA that are specifically expressed in arbuscular mycorrhizal colonized tomato roots (tester) but not in control uninoculated roots (driver). The two probes were used to screen a cDNA library prepared from mRNA from arbuscular mycorrhizal-colonized tomato roots (Tahiri-Alaoui and Antoniw, 1996). Two different cDNA clones were isolated, which after partial sequencing showed 94% similarity to an inorganic phosphate transporter from potato and 86% similarity to the ATP phosphoribosyl transferase from *Escherichia coli* and *Salmonella typhimurium* (A. Tahiri-Alaoui and J.F. Antoniw, unpublished work).

13.3 Conclusions

PCR techniques have made a large impact on research into plant–fungal interactions, as they have done in many other areas of biological research. PCR-based methods have been developed which allow the detection of many plant pathogens and symbionts on or in their hosts more rapidly and reliably than before and for a few fungi these methods are quantitative. PCR has also simplified the isolation of genes involved in plant–fungal interactions. Once a protein or nucleotide sequence is available for one gene, primers can be designed to allow related genes to be isolated very quickly. However, PCR can also be used to isolate genes involved in plant–fungal interactions without prior knowledge of the proteins involved. These methods detect differences at the RNA level that correlate with fungal infection or differences at the DNA level that correlate with a trait such as plant resistance or fungal pathogenicity.

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