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DDRT–PCR for Screening of Fungal Gene Expression

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3.1 Introduction

Every biological process results from selective expression of the genome. Processes such as cell division and proliferation, differentiation, ageing and response to stress situations are governed by modulation of the expression of specific genes or sets of genes. Studies on differential gene expression have therefore attracted much interest and research effort. Until recently, the isolation of differentially expressed genes has largely relied on differential hybridization (Sargent, 1987) and subtractive hybridization procedures (Lee *et al*., 1991). These methods are laborious and time-consuming, and usually require relatively large amounts of starting material. Furthermore, their sensitivity is limited, only allowing the detection of relatively abundant mRNAs. In 1992 a new procedure for differential gene expression analysis was described: 'differential display reverse transcriptase–polymerase chain reaction' (DDRT–PCR) (Liang and Pardee, 1992). This method makes it feasible to display the full set of mRNAs in a particular cell type and to compare expression patterns, either with other cell types or with cells subjected to other experimental conditions. DDRT–PCR possesses several advantages over subtractive and differential hybridization procedures. Firstly, it is quicker because probes for Southern and Northern blot analysis as well as partial sequence information of differentially expressed genes may be made available in a few days. Secondly, only a small amount of biological material is required, making this methodology particularly useful when the amount of starting material is limiting (Zimmermann and Schultz, 1994). Thirdly, the application of PCR increases the sensitivity and thereby allows the detection of low abundance mRNAs (Liang and Pardee, 1992). These

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features all contribute to the power and utility of this method. Since it was first described, refinements have been introduced and the procedure has been applied in a wide variety of biological systems (reviewed by Liang and Pardee, 1995).

We have applied DDRT–PCR to study gene expression in *Botrytis cinerea*, a ubiquitous fungal plant pathogen causing important economic losses in many agricultural crops (Jarvis, 1977). The aim of this research is to investigate fungal gene expression *in planta* during the interaction of *B. cinerea* with the host plant, tomato (*Lycopersicon esculentum*). We assume that mutual signalling during the interaction results in changes in gene expression both in the fungus (for activation of mechanisms for penetration and colonization of the host tissue) and in the plant (for activation of defence responses). Among the fungal genes whose expression is induced during the interaction, it is likely that some of the genes are essential for pathogenesis. To this end, it was decided to undertake a systematic comparison of gene expression of the fungus *in planta* and during growth in liquid culture. When studying such a system, the limiting factor is the small amount of fungal biomass in the interaction material, particularly during early stages of the infection process. Therefore a highly sensitive method, such as PCR, is required.

In this chapter we summarize the principles of DDRT–PCR and experimental parameters that were considered during the development of the methodology. Furthermore we describe its application to the analysis of *B. cinerea* gene expression *in planta*. Our results illustrate the high sensitivity and versatility of the method.

3.2 Principles of DDRT–PCR

The differential display procedure is designed for the identification and isolation of genes that are differentially expressed in various cell populations under defined conditions. It combines techniques that are commonly applied in molecular biological research: reverse transcription, PCR and electrophoresis in polyacrylamide gels (Fig. 3.1). In the first step, a subpopulation of mRNAs is reverse transcribed into cDNA by using an 'anchored' 3′ primer, taking advantage of the presence of a poly(A) tail in most eukaryotic mRNAs. The anchored primer consists of a number of thymidine residues (usually 11), which anchor it to the poly(A) tail of the mRNA, plus two additional nucleotides at its 3′-extreme which provide the specificity of the annealing. Such a primer anneals selectively to mRNAs containing two nucleotides complementary to those at the 3′-end of the primer immediately upstream of the poly(A) tail. In total, 12 anchored primers can be designed (12 different combinations of the last 3′ nucleotides omitting thymidine at the penultimate position). Thus, 12 cDNA subpopulations need to be generated if the entire mRNA population is to be analysed. The reduction of template

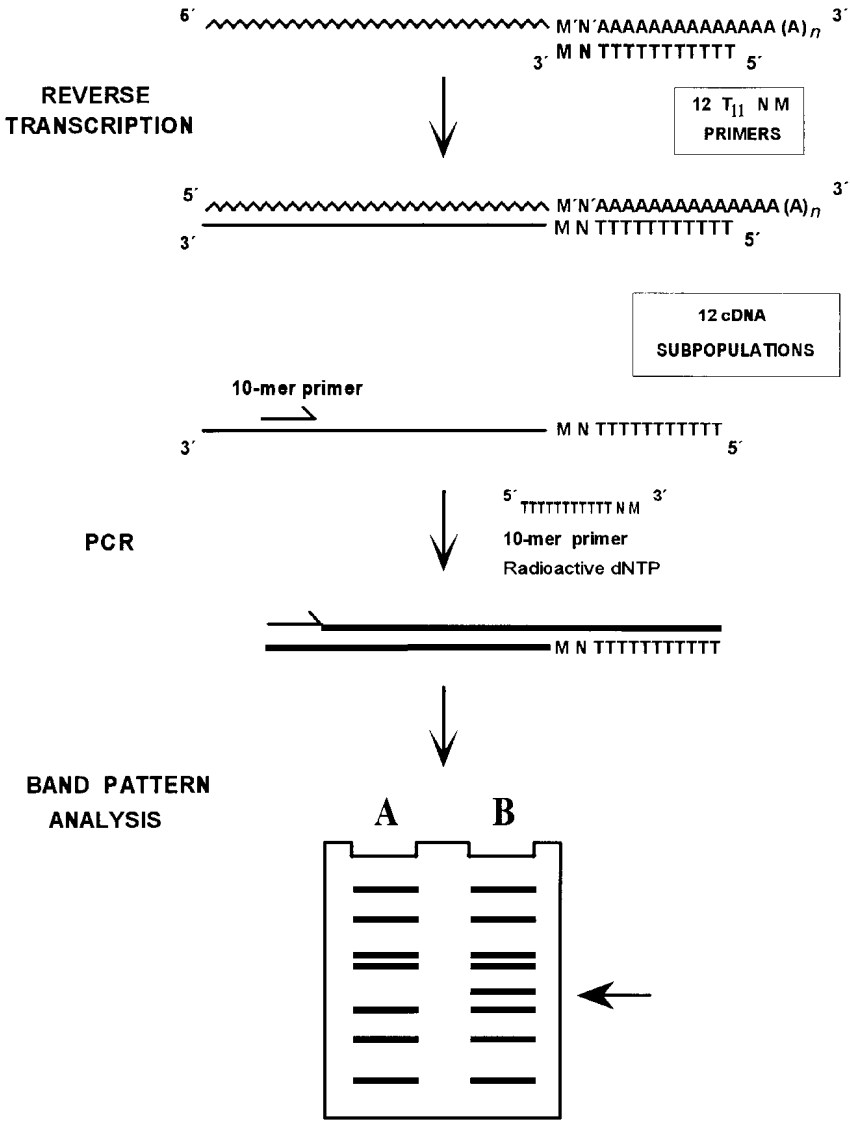

Fig. 3.1. Principles of DDRT–PCR (N = adenosine, guanosine or cytosine; M = any nucleotide).

complexity by using selective anchored primers for cDNA synthesis is an essential step in the procedure.

PCR is subsequently performed on each of the cDNA subpopulations, using the same anchored primer applied in cDNA synthesis in combination

with a small (10-mer) upstream primer. Any cDNA species is suitable for amplification by PCR if the distance between the poly(A) tail and the position at which an upstream primer anneals is smaller than 2–3 kb. For a 5′ primer of arbitrary nucleotide sequence, annealing positions (if present) are distributed randomly upstream of the poly(A) tail. The resulting amplified products represent only parts of a particular mRNA species (3′) and products derived from various mRNAs will differ in length. If PCR is performed in the presence of a labelled nucleotide (usually [α-^{33}P]dATP), the amplified products can be resolved in a polyacrylamide gel and visualized as discrete bands by autoradiography. The band patterns of samples derived from different cell types or from cells cultured in different conditions are analysed in parallel. Fragments of cDNA representing differentially expressed mRNAs can be recovered from the gel and used to further characterize the corresponding genes.

3.2.1 Considerations for experimental parameters

Since it was first described in 1992, the differential display methodology has been applied to a variety of biological systems. Many groups have adapted the original protocol to their particular systems and requirements. Although general guidelines can be given, it is our experience that the optimal conditions must be found by each user when applied to a particular system. Below, we will discuss briefly the main variations that can be considered in each step of the differential display procedure and point out the conditions that we have found most appropriate in our system.

Starting material and reverse transcription

The most critical factor influencing DDRT–PCR analysis is the quality of the starting material. Protocols for RNA isolation yielding undegraded and highly purified RNA should be used (guanidinium thiocyanate-based protocols usually give good results). Both total RNA and poly(A)$^+$-RNA can be used as template for reverse transcription and nearly identical band patterns are obtained (Liang *et al*., 1993). For simplicity, total RNA has been used in many cases. In our hands, however, better results were obtained using poly(A)$^+$-RNA, in terms of both the number of bands produced and reproducibility.

The original protocol made use of anchored primers with two selective nucleotides for the cDNA synthesis. More recently, protocols have been described which use anchored primers with two selective nucleotides in which the penultimate position is degenerate (Liang *et al*., 1993), or anchored primers with only one selective nucleotide (Liang *et al*., 1994). Such primers have allowed a significant reduction of the number of cDNA synthesis reactions required for each RNA sample. However, the use of such primers will generate cDNA populations of higher complexity that serve as templates in the subsequent PCR step, resulting in bands occupying almost every

position of the gel. This makes the analysis of the band patterns more complicated. Additionally, as pointed out by Bertioli *et al.* (1995), a reduction of template complexity will contribute to an increase in the sensitivity of the DDRT–PCR procedure for any particular cDNA species.

Polymerase chain reaction

The experimental conditions used for PCR amplifications of complex templates in the differential display procedure are different from conditions applied in standard PCR applications. In the original protocol (Liang and Pardee, 1992), 13 nucleotide-long anchored primers were used in combination with decamers of arbitrary sequence. Low annealing temperatures in the PCR profile are required for annealing of short primers but will also allow mismatches. This appears to be essential for the priming of at least a fraction of the templates. In our hands, temperatures higher than 42°C resulted in a drastic reduction in the number of bands detected while temperatures lower than 40°C produced bands occupying nearly every position in the lane. It was experimentally determined that the specificity of the amplification increases when the dNTP concentration is decreased from 200 μM to 2 μM (Liang and Pardee, 1992). A low dNTP concentration was also essential for labelling the PCR products to a specific activity high enough for detection on an autoradiogram.

An average of 120 bands in the size range of 100–600 nucleotides are consistently detected per primer combination when applied to a higher eukaryotic system. Assuming that about 15,000 genes are expressed in a higher eukaryotic cell, theoretical considerations indicate that at a confidence level of 0.95 the entire mRNA population is displayed, when 12 anchored primers with two selective nucleotides are used in combination with at least 25 upstream primers (Bauer *et al.*, 1993).

Liskens *et al.* (1995) reported the use of 5′-extended primers as a means of enhancing the reproducibility of the differential display technique. Primers with a length of 22 nucleotides were used in a PCR profile including a few initial cycles at low annealing temperatures. At low temperatures, these primers behave in a similar way to the decamer primers used in the original differential display protocol, since the sequence at the 3′-extreme determines the priming specificity. Subsequent PCR cycles are performed at higher annealing temperatures, resulting in very efficient amplification of the products obtained during the first cycles. This procedure should improve the signal-to-noise ratio of individual bands. The extended primers can be designed so that they contain a restriction site at their 5′-end, making subsequent manipulations easier.

Separation and retrieval of bands

Polyacrylamide gels provide the resolution needed to analyse the complex mixtures of amplified cDNAs after PCR. Denaturing polyacrylamide gels have been used in most applications, although for fragments smaller than 200

nucleotides the band patterns generated are more complex. Many of the bands appear as doublets representing both strands of each fragment as a result of the denaturing conditions. Non-denaturing polyacrylamide gels have been used to reduce the complexity of the band patterns (Bauer *et al*., 1993). We have found that a non-denaturing system gives reproducible patterns and is particularly useful when very complex cDNA mixtures are to be analysed (Benito *et al*., 1996).

The bands of interest can easily be recovered from the gel by a simple boiling method (Zimmerman and Schultz, 1994). They should be reamplified in reactions containing higher dNTPs concentrations (20 μM) to produce enough DNA for isolation from agarose gels and subsequent cloning. Ampli*Taq* has most frequently been the enzyme of choice. Because this enzyme adds a single (non-encoded) adenosine to the 3′-end of most of the reaction products, linearized vectors containing 3′-T overhangs are commonly used to clone the PCR products. Cloning strategies based on blunt-end ligation of PCR products are also used. The cloned fragments can be used as probes in Northern and Southern blot analyses for the isolation of the full length cDNA and genomic copies of the gene from libraries. Furthermore, the sequence of the cloned fragments can yield information about the nature of the differentially expressed mRNAs that were detected.

Reproducibility and reliability

The DDRT–PCR procedure generates highly reproducible band patterns with the same RNA sample. More than 95% of the bands are detected in common in duplicate reactions and most of the differences observed concern the intensity of the bands (Bauer *et al*., 1993). To maximize the reproducibility and accuracy of the method in a given experiment, the samples to be analysed should preferably be processed in parallel and in an identical manner. This must be done for every step in the procedure, from RNA isolation to electrophoresis. The same buffers and reagents and, whenever possible, the same batch of enzymes should be used for the entire procedure.

In spite of all the precautions taken, false-positive bands are frequently detected. These include differential bands which fail to detect any mRNA on Northern blots or bands representing non-induced mRNAs. These bands are usually non-reproducible and can be generated as a result of the large number of PCR cycles at low stringency temperatures (Liskens *et al*., 1995). Performing the analysis of every RNA sample in duplicate may help to discriminate between true- and false-positive bands (Liang *et al*., 1993). Another source of false-positive bands is the presence of contaminating chromosomal DNA in the RNA sample co-purified during the extraction procedure. This is less of a problem when poly(A)$^+$-RNA is used instead of total RNA for the DDRT–PCR analysis. A DNaseI treatment can be included in the protocol to remove traces of chromosomal DNA (Liang *et al*., 1993). However, this extra treatment may lead to partial RNA degradation, since DNaseI preparations are never completely free of RNase activity.

3.3 Principles of Differential Display in a Plant–Fungal Pathogen Interaction

We have applied the DDRT–PCR methodology to the analysis of differential gene expression in a complex plant–fungus interaction system, *Lycopersicon esculentum–B. cinerea*. Since two organisms are present in the interaction, cDNAs from both will be detected by differential display (Fig. 3.2). Most of these cDNAs represent fungal or plant genes which are constitutively expressed. Their origin can be discriminated if the expression pattern of both organisms displayed during the interaction is compared with the expression pattern of the fungus grown *in vitro* and with the expression pattern of a non-infected tomato plant. Interaction-specific cDNAs are also detected which represent either fungal genes induced *in planta* or plant genes induced in response to the pathogen. Both categories can be discriminated if samples from control infections with a second pathogen are included in the comparative gene expression analysis.

3.3.1 The pathosystem B. cinerea *–tomato*

To investigate the infection process of *B. cinerea* on detached tomato leaves we first established a standardized inoculation procedure. Experimental conditions were designed that determine high infection efficiency by inducing high germination rates of conidia on the leaves and synchronicity of the infection in different lesions. Synchronous infection is essential for a successful temporal analysis of fungal gene expression *in planta*. Under the experimental conditions used, the first symptoms are detectable 20 h post-inoculation (h.p.i.). At this time-point small necrotic lesions start appearing over the leaf surface. The lesions become darker during the following 48 h but neither the number of lesions nor their size increases. At 72 h.p.i., about 1–5% of the total number of lesions start to expand and from these lesions the fungus is able to colonize the whole leaf. At about 120–140 h.p.i. total leaf necrosis is observed and the fungus sporulates on the surface of the necrotized plant tissue. We analysed gene expression of the fungus at two different stages of the infection process: at 16 h.p.i. when no symptom is yet visible but the fungus has already penetrated the host cells, and at 72 h.p.i. at the onset of the formation of spreading lesions.

3.3.2 Sensitivity of the DDRT–PCR procedure

In order to determine whether it is possible to detect fungal gene expression *in planta* at these time-points, we estimated the proportion of fungal biomass in leaves during the infection process over time. *B. cinerea* was inoculated on tomato leaves which were sampled at different times after inoculation. Equal amounts of RNA extracted from these samples were electrophoresed, blotted and hybridized with a probe derived from the *B. cinerea* β-tubulin (*tubA*) gene,

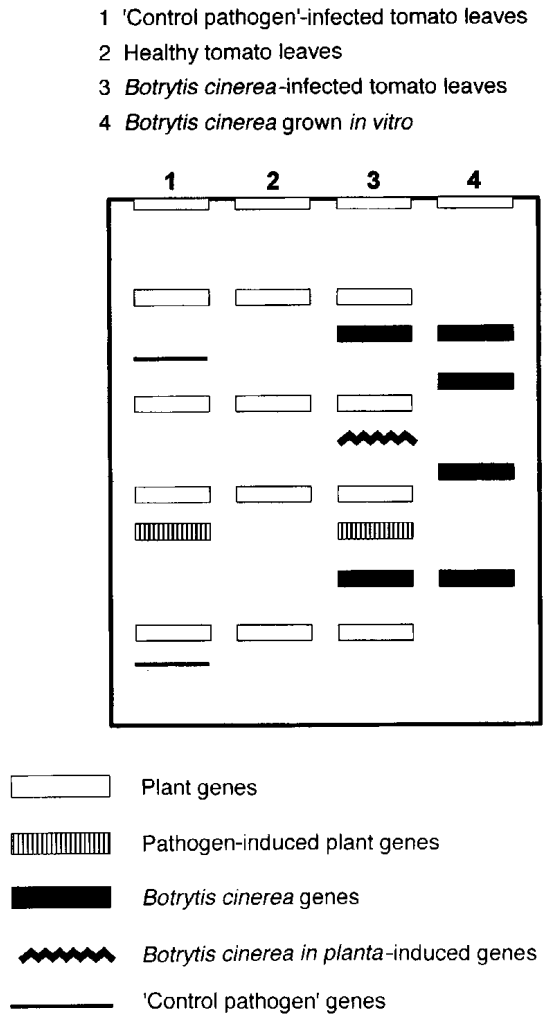

Fig. 3.2. Schematic representation of a 'differential display' gel indicating the origins of the bands expected when applying DDRT–PCR to the analysis of a plant–fungal pathogen interaction.

which is assumed to be expressed constitutively (E.P. Benito and J.A.L. van Kan, unpublished results). The intensities of the signals detected at different time-points were compared with the intensity of the signal obtained on RNA extracted from a *B. cinerea* liquid culture *in vitro*. This intensity ratio provides an estimation of the proportion of fungal RNA/interaction RNA, and therefore of fungal biomass in the interaction material, during the infection. As shown in Fig. 3.3A, this proportion is about 3–5% at the time-points analysed.

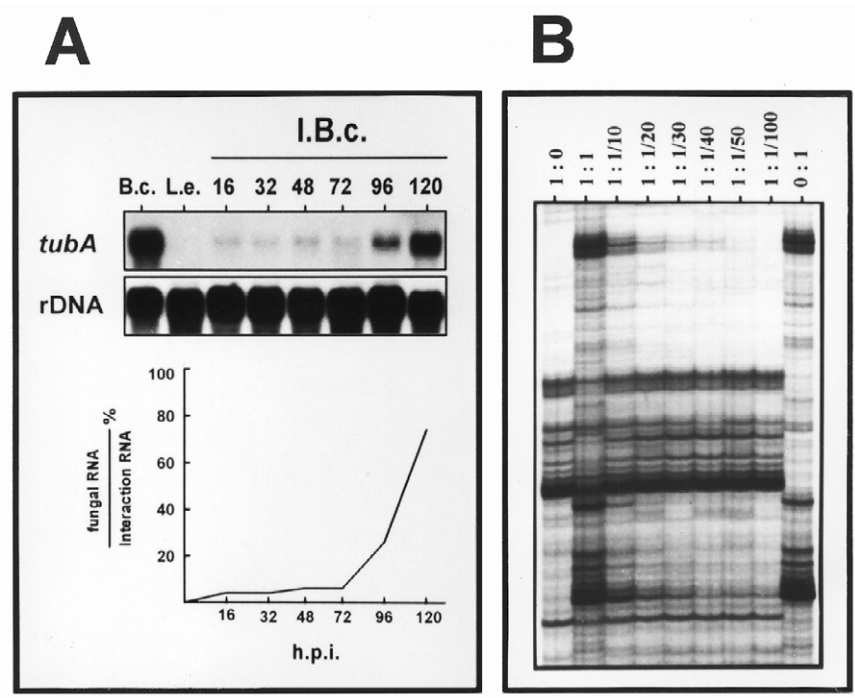

Fig. 3.3. (A) Estimation of the proportion of fungal biomass on infected tomato leaves during the infection process. Lanes: B.c., total RNA extracted from *B. cinerea* cultured *in vitro*; L.e., non-infected tomato leaves; I.B.c., *B. cinerea*-infected tomato leaves sampled at the indicated time-points (h.p.i.) 0.20 pg of each RNA sample were separated by acrylamide gel electrophoresis, blotted on to a nylon membrane and hybridized with a *B. cinerea* β-tubulin (*tubA*) gene. The values used to generate the graph in the lower part of the figure represent the relative intensities of the signal obtained with each time-point sample in comparison with the signal obtained with the *B. cinerea in vitro*-cultured sample. The same filter was probed with a radish 18S rDNA probe (clone pRG3) (Grellet *et al.*, 1989) to demonstrate equal loading. (B) Sensitivity of the DDRT–PCR procedure. cDNAs synthesized with primer RT3 on mRNAs from healthy tomato leaves and *B. cinerea* grown *in vitro* were mixed and subjected to PCR amplification using the same anchored primer in combination with the 10-mer primer P35. Each lane shows the amplified products obtained using different proportions (ratios indicated above each lane) of tomato–*B. cinerea* cDNA mixtures as templates. Reproduced from Benito *et al.* (1996) by permission of Kluwer Academic Publishers.

Using DDRT–PCR, is it possible to detect and discriminate fungal mRNAs within such a complex mRNA mixture containing only 3–5% of fungal mRNA? We investigated this question by performing a reconstruction experiment. mRNA from *B. cinerea in vitro* liquid cultures and from healthy tomato leaves were reverse transcribed using anchor primer RT3. The plant

cDNA and serial dilutions of the fungal cDNA were mixed and PCR was performed. As shown in Fig. 3.3B, most of the bands detected using cDNAs from healthy leaves (lane 1:0) and cDNAs from *B. cinerea* grown *in vitro* (lane 0:1) as template in PCR, are also detected in a single lane when equal amounts of both cDNAs are mixed and used as template (lane 1:1). When cDNA from *B. cinerea* grown *in vitro* is diluted in relation to cDNA from healthy leaves, the intensity of the bands representing *B. cinerea* cDNAs decreases with increasing dilution, while the intensity of the bands representing plant cDNAs remains constant (lanes 1:1/10–1:1/100). Even at the highest dilution tested, several *B. cinerea* cDNA-derived bands are still detectable. In the sample with the 1/30 dilution factor a large proportion of bands representing *B. cinerea* cDNAs are detected. Since the amount of fungal cDNA in this sample is comparable to that from an infected leaf in which 3% of the interaction mRNA is from *B. cinerea*, we assumed that the DDRT–PCR procedure was sensitive enough to apply to our experimental system. However, it must be noted that the weakest bands detected in the *B. cinerea in vitro*-grown sample are not detected in the reconstruction sample, suggesting that some fungal mRNAs expressed at a low level *in planta* may be overlooked.

3.3.3 Analysis of the expression pattern in the B. cinerea *–tomato interaction*

We initiated the analysis of *B. cinerea in planta* gene expression. As mentioned above, samples from control interaction systems are required to discriminate fungal genes induced *in planta* from plant genes induced in response to the pathogen. A useful control interaction in our experimental approach should mimic as much as possible the plant defence response induced by *B. cinerea*. For this purpose, we used two different pathogens: the fungus *Phytophthora infestans* and tobacco necrosis virus (TNV). On tomato leaves, *P. infestans* induces necrotic spots that appear in the centre of water-soaked areas at about 50–55 h.p.i., whereas TNV induces small necrotic spots at 70 h.p.i. For both control pathogens, samples were collected shortly before symptoms became detectable: 40 h.p.i. for *P. infestans*-infected leaves and 60 h.p.i. for TNV-infected leaves.

A first set of experiments was performed in order to determine the best conditions for DDRT–PCR analysis and to test the validity of our experimental approach on a limited number of samples: *B. cinerea*-infected tomato leaves collected at 16 h.p.i.; *B. cinerea* cultured *in vitro*; non-infected tomato leaves; and *P. infestans*-infected tomato leaves.

Figure 3.4A shows a representative example of a differential display gel obtained. In all experiments duplicate reactions were included of the *B. cinerea*–tomato interaction samples (using cDNAs derived from two independent reverse transcriptase reactions) to reduce the number of false-positive bands. An average of 100–120 bands were detected in lanes from

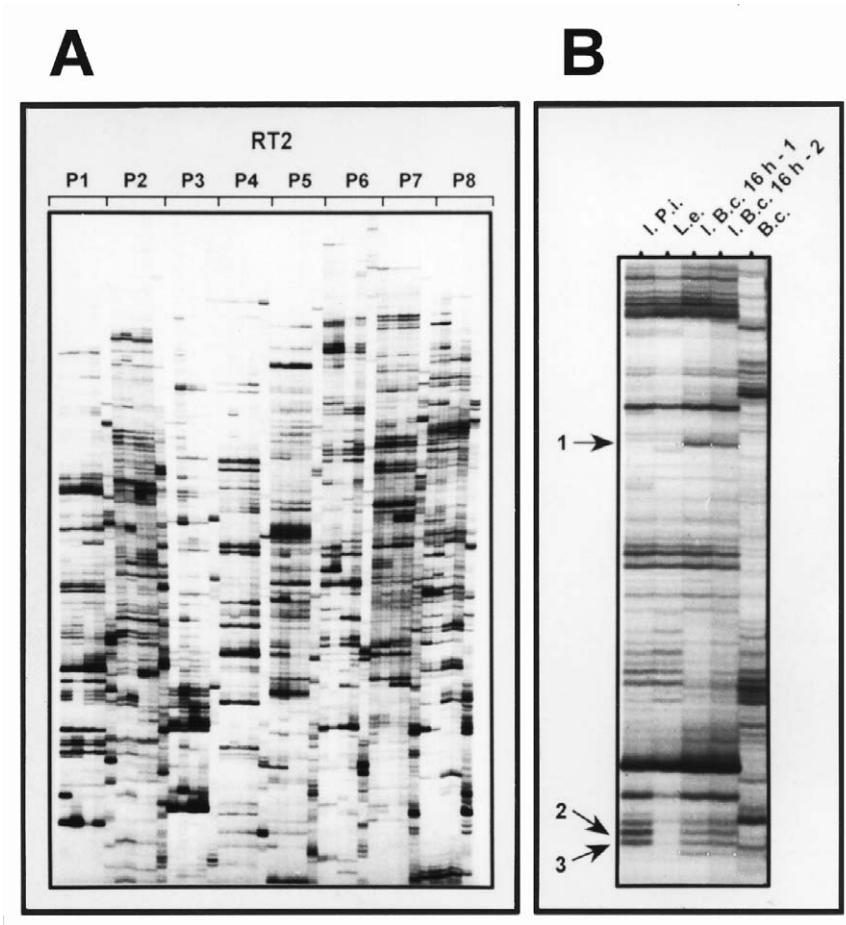

Fig. 3.4. (A) Example of a 'differential display' gel. mRNA samples were reverse transcribed with anchored primer RT2 and PCR amplifications were performed using the same anchored primer in combination with eight different upstream primers (P1–P8). For each primer combination samples are as indicated in (B). (B) Detail of the band patterns obtained by 'differential display' with primers RT2 and P1 using cDNAs derived from mRNAs obtained from: I.P.i., *P. infestans*–tomato interaction 40 h.p.i.; I.B.c., *B. cinerea*–tomato interaction 16 h.p.i. (duplicate reactions, 16 h-1 and 16 h-2); L.e., healthy tomato leaves; B.c., *B. cinerea* grown *in vitro*. The arrows indicate interaction-specific bands. Reproduced from Benito *et al.* (1996) by permission of Kluwer Academic Publishers.

plant cDNA samples, while 70–80 bands were detected in samples derived from *B. cinerea* grown *in vitro*. About 15–20% of the latter bands were also detected in the *B. cinerea*–tomato interaction lane for most of the primer combinations. Figure 3.4B shows in detail the patterns obtained with a given primer combination. In addition to the bands representing constitutively

expressed mRNAs from *B. cinerea* and tomato, novel bands are detected in samples derived from *B. cinerea*-infected tomato leaves (indicated by arrows). Some of these bands are also detected in samples from *P. infestans*-infected tomato leaves (arrows 2 and 3) and probably represent plant defence genes induced in response to both pathogens. Other bands are apparently specific to the *B. cinerea*–tomato interaction and are candidates to represent *B. cinerea* mRNAs induced *in planta* (arrow 1).

In view of the fact that interaction-specific bands were detected, the analysis was extended including mRNA samples derived from *B. cinerea*-infected tomato leaves collected 72 h.p.i. and from TNV-infected tomato leaves as a second control interaction system. In total, 52 primer combinations were used (two anchored primers and 26 upstream primers) in these experiments and 22 *B. cinerea*–tomato interaction-specific fragments were detected. About 4000 *B. cinerea* cDNA fragments derived from genes expressed *in vitro* were displayed, of which 700–800 were also detected in interaction samples. Since we have examined only two out of the 12 possible cDNA subpopulations, the entire analysis should yield about 4000–5000 *B. cinerea* cDNA fragments derived from genes expressed *in planta*.

3.3.4 Further characterization of bands of interest

Further characterization of 'differential' bands detected by DDRT–PCR includes confirmation of differential expression by Northern blot analysis and cloning to obtain sequence information. In an interaction between two organisms, the origin of the differentially displayed bands needs to be determined. To this end, these bands were recovered from the gel, reamplified, labelled and hybridized to a Southern blot containing *Hin*dIII digests of genomic DNA from tomato and from *B. cinerea* (we have found that labelling by PCR is a much more effective procedure for labelling small DNA fragments such as those obtained in DDRT–PCR analysis than labelling by 'random priming'). Four fragments hybridizing with genomic DNA of *B. cinerea* were selected for further analysis. It is commonly observed in differential display analysis that single bands represent several molecular species of the same size (Callard *et al*., 1994; Li *et al*., 1994). To test this possibility the fragments hybridizing with *B. cinerea* genomic DNA were cloned and inserts from individual plasmids were used to confirm the hybridization pattern on Southern blots. Only inserts reproducing the hybridization pattern originally obtained were used for *in planta* gene expression analysis on a time-course Northern blot. Two of the four fragments tested appeared to be homogeneous while the other two were mixtures of at least two different DNA fragments: only one of the fragments reproduced the initial hybridization pattern whereas the second one hybridized neither to *B. cinerea* nor to plant genomic DNA. Northern blot analysis confirmed differential expression *in planta* for three of the cDNAs detected and these are currently characterized in detail. The fourth one did

not detect any signal on the Northern blot, probably due to a low level of expression.

When large numbers of 'differential' fragments are to be tested, alternative screening procedures based on 'mass' analysis can be performed (Mou *et al*., 1994). Duplicate filters containing equal amounts of the cDNA fragments of interest are probed with labelled total genomic DNA to determine the origin of the cDNAs isolated and with labelled cDNA synthesized using mRNAs isolated from the sources under investigation. Many fragments can be processed simultaneously in this way. However, the method has limitations since frequently, as mentioned above, differential bands represent a mixture of DNA sequences. In this situation, positive cDNAs expressed at low levels can be overlooked.

Sequencing of the cloned fragments can provide information about the nature of the fragments detected through database searches. According to the principle of the differential display procedure, the fragments displayed represent the 3′ extreme of the mRNA molecules. Usually the two primers used to amplify a particular fragment are found at the extremes, thereby providing information about the orientation of the fragment. A limitation of the procedure is that the amplified fragments are relatively small and only represent a (largely untranslated) part of the mRNA, thus restricting the usefulness of the sequence information obtained (Sompayrac *et al*., 1995). Furthermore, fragments often arise as a result of amplification primed by only one of the two primers used in the PCR (Guimarães *et al*., 1995). Such fragments are likely to represent internal regions of the cDNA molecules. In either case, the reamplified fragments can be used as probes to isolate the full length cDNA and genomic copies of the genes of interest.

3.4 Perspectives

As predicted by Liang and Pardee in 1992, the differential display methodology has found a broad range of applications. The potential of the methodology is enormous. As originally described, the method is only qualitative (Liang and Pardee, 1992), but modifications have been proposed which would make the method semiquantitative, with potential applications in diagnostic procedures (Bauer *et al.*, 1993; Guimarães *et al.*, 1995). Moreover, it is not limited to the comparison of two mRNA samples, like subtraction-based methods. Instead it allows the comparison of multiple mRNA samples, for instance in the analysis of spatial or temporal gene expression (tissue-specific expression, different developmental stages) (Utans *et al*., 1994; Guimarães *et al*., 1995). Furthermore, its sensitivity is high, although this is controversial. Bertioli *et al*. (1995) presented experimental data suggesting that differential display shows a strong bias towards high copy number mRNAs. Guimarães *et al*. (1995), on the contrary, demonstrated that DDRT–PCR is able to detect low abundance

mRNAs. Our results support the high level of sensitivity of the method, since induced mRNAs were detected within a subpopulation of mRNAs which constitutes only 3–5% of the total infected leaf mRNA population (Benito *et al*., 1996).

Differential display has become the method of choice to detect and isolate differentially expressed genes in many biological systems, mainly higher eukaryotes. Both basic and applied (clinical and medical) areas of research have benefited from DDRT–PCR in mammalian systems (Utans *et al*., 1994; Zimmermann and Schultz, 1994; Liskens *et al*., 1995; Yeatman and Mao, 1995) and several groups have reported its successful application in plants (Goormachtig *et al.*, 1995; Sharma and Davis, 1995; van der Knaap and Kende, 1995; Wilkinson *et al*., 1995). Our work presents one of the first reports on the application of DDRT–PCR to the analysis of differential gene expression in a lower eukaryote, such as a phytopathogenic fungus. Fungi have been used as model systems in fundamental (genetics, biochemistry) and applied (biotechnology, phytopathology, biomedicine) areas of research in the last decades. Information derived from fungal systems has provided significant contributions to the understanding of more complex higher eukaryotic systems. Undoubtedly, DDRT–PCR will be a useful tool when applied to the analysis of differential gene expression in processes of basic interest like mating, morphogenesis and dimorphism. Appleyard *et al*. (1995) have demonstrated the potential of the differential display methodology for the isolation of genes of biotechnological interest from a filamentous fungus, *Gibberella fujikuroi*. Chen *et al.* (1996) used the method to examine the alteration of gene expression of the chestnut blight fungus (*Cryphonectria parasitica*) when infected with a virulence-attenuating hypovirus. We have successfully applied this methodology to the analysis of differential gene expression in a plant–fungus interaction and demonstrated that it possesses enough sensitivity to analyse fungal gene expression *in planta*. Provided the appropriate controls are included, plant defence genes induced by a pathogen can also be identified (Benito *et al*., 1996), demonstrating the versatility of the methodology. Such a procedure enables the study of several aspects of plant–pathogen interactions. It is foreseeable that the DDRT–PCR methodology will find numerous applications in mycology.

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