

# Differential Gene Expression is a Promising Tool for Understanding Host–Pathogen Interactions

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## ABSTRACT

Expression of genes is essential for normal development and pathological processes. Therefore, discovery of differentially expressed genes is critical for the understanding of the molecular mechanisms involved in normal and pathological states, as well as providing new insights for discovery of novel molecular genes. To date, a number of techniques such as microarray, suppression subtractive hybridization (SSH) and differentially displayed - reverse transcriptase - polymerase chain reaction (DD-RT-PCR) have been developed for novel gene discovery. Microarray analysis is one of the fastest-growing new technologies in the field of genetic research. Scientists are using microarrays that allow us to look at thousands of genes at one time and determine which are expressed in a particular cell type or under certain conditions. The SSH is a method that is used for the comparison of two RNA populations that differ in expression in response to a particular stimulus or environment, such as an infection by a pathogen, can identify differences in the abundance of specific transcripts that vary in a population dependent manner. DD-RT-PCR is a method based on PCR that allows systematic comparisons of expressed mRNA in the cells, so, is becoming more efficient nowadays to isolate and characterize genes differentially expressed among cells, tissues or individuals. In this mini-review, we will describe the techniques mentioned above that have been widely used in the field of molecular biology in the last decade to identify differentially expressed genes in many biological systems, including our own work in Fusarium head blight disease of wheat.

**Keywords:** differential display, functional genomics, microarray, suppression subtractive hybridization, transcriptome pattern

**Abbreviations:** cDNA-AFLP, cDNA amplified fragment length polymorphism; DD-RT-PCR, differentially displayed - reverse transcriptase - polymerase chain reaction; ESTs, expressed sequence tags; SAGE, serial analysis of gene expression; PCR, polymerase chain reaction; SSH, suppression subtractive hybridization

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## PLANT-PATHOGEN INTERACTIONS USING DNA MICROARRAYS

The interaction between a plant and a pathogen activates a wide variety of defense responses. The recent development of microarray-based expression profiling methods, together with the availability of genomic and/or ESTs (expressed sequence tags) sequence data for some plant species, has allowed significant progress in the characterization of plant pathogenesis-related responses (Fig. 1). The small number of expression profiling studies completed to date has already identified an amazing number of genes that had not previously been implicated in plant defense. Some of these genes can be associated with defense signal transduction or antimicrobial action, but the functional contribution of many others remains uncertain. Initial expression profiling

work has also revealed similarities and distinctions between different defense signaling pathways, and cross-talk (both overlap and interference) between pathogenesis-related responses and plant responses to other stresses (Harmer and Kay 2001; Kazan *et al.* 2001). Potential transcriptional *cis*-regulatory elements upstream of co-regulated genes can also be identified. Whole-genome arrays are only now becoming available, and many interactions remain to be studied (e.g. different pathogen species, plant genotypes, mutants, time-points after infection). Expression profiling technologies, in combination with other genomic tools, will have a substantial impact on our understanding of plant-pathogen interactions and defense signaling pathways.

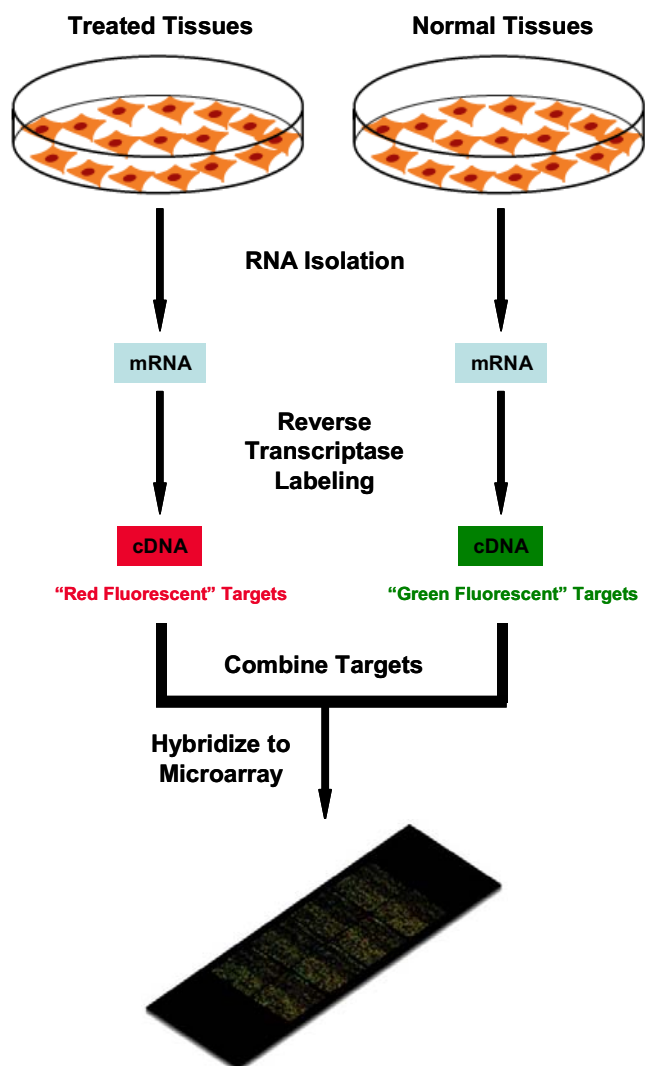


Fig. 1 Schematic representation of a microarray experiment.

### DNA microarrays are an excellent tool for identifying potential defense-related genes

The technology of DNA microarrays is constantly evolving, but widely used methods can be grouped into two basic types: cDNA microarrays and oligonucleotide-based arrays. Potential users of DNA microarrays are cautioned that the techniques are prone to a number of methodological and interpretive pitfalls (Duggan *et al.* 1999; Eisen and Brown 1999; Lipshutz *et al.* 1999; Brazma and Vilo 2000; Lockhart and Winzler 2000; Sherlock 2000; Wisman and Ohlrogge 2000; Kazan *et al.* 2001; Aharoni and Vorst 2001; Donson *et al.* 2002; Finkelstein *et al.* 2002). There are other methods for transcript profiling that may also be used to study plant-pathogen interactions, such as differential display, SAGE (serial analysis of gene expression), and cDNA amplified fragment length polymorphism (cDNA-AFLP) (Donson *et al.* 2002). However, DNA microarray techniques are particularly suitable for monitoring gene expression changes in plants during plant-pathogen interactions, due to their relative simplicity, comprehensive sampling capacity and high throughput (Kazan *et al.* 2001). The most attractive feature of DNA microarray techniques is that they allow researchers to examine the responses of hundreds or thousands of genes simultaneously during a given treatment. Using these expression profiles, it is possible to identify differentially present mRNA species and to hypothesize potential defense-associated function based on this differential expression.

Recently, a small number of DNA microarray experiments have identified an amazing number of potential

defense-related genes. Although some of these genes have previously been implicated in plant defense responses, most have not. The derived amino-acid sequences of some of these genes have significant similarity to known proteins, but many of the genes encode hypothetical or unknown products.

A few examples are illustrative. Using a maize DNA microarray representing 1,500 maize genes, Baldwin *et al.* (1999) identified 117 genes that consistently showed altered mRNA expression in maize 6 h after various treatments with the fungal pathogen *Cochliobolus carbonum*. Using a related microarray, Nadimpalli *et al.* (2000) identified nearly 70 genes having a two-fold or more change in mRNA abundance in the lesion mimic maize mutant, *Les9*. *Les9* is characterized by numerous spontaneous chlorotic to necrotic lesions that occur by the 9- to 14-leaf stage, and shows enhanced resistance to *Bipolaris maydis* and elevated expression of defense-related proteins. Many of the differentially expressed genes identified in the *Les9* mutant are defense-related, while the others are unknowns or are not generally understood to be defense-related. One of them, *Zm-hir3*, is implicated in plant cell death through ion channel regulation.

*Arabidopsis* is a particularly well-developed experimental system that has been utilized in a number of studies. In an important early study that examined gene expression changes in *Arabidopsis* under 14 different SAR-inducing or repressing conditions, including a notable focus on plant mutants, Maleck *et al.* (2000) identified 413 ESTs that appeared to be associated with SAR. This study used a cDNA microarray containing 10,000 ESTs representing approximately 7,000 genes, or 25-30% of all *Arabidopsis* genes. The number of SAR-regulated genes present on their arrays may actually be larger or smaller; unfortunately, no replications of individual treatments were conducted. The researchers applied a threshold of 2.5-fold change in two or more related treatments to identify relevant genes and compensate for this lack of replications. Experimental replication is costly, but is now widely viewed as essential. In particular, "biological replication" is preferred that uses not only separate chip hybridizations but also independent RNA samples obtained from separate plants grown on different dates or in different locations. The related issue of how to calculate the significance threshold (for concluding difference in mRNA level between experimental treatment and control), and at what level of stringency, is common to nearly all microarray studies. There is a clear need to make the primary data for microarray experiments available so that the same data can be analyzed by others using different criteria. Ready availability of raw data will facilitate maximum capture of value from both publicly and privately funded research expenditures.

Schenk *et al.* (2000) used a targeted microarray containing 2,375 *Arabidopsis* genes, identified 705 genes that were responsive to the fungal pathogen *Alternaria brassicicola* or to the defense-activating signaling molecules SA, methyl jasmonate (MeJ) or ethylene. These 750 genes included 106 genes with no previously described function or homology, along with putative defense-related genes. Sasaki *et al.* (2001) identified 41 jasmonate-responsive *Arabidopsis* genes of which 5 genes were JA biosynthesis genes, 3 genes were involved in other signaling pathways (ethylene, auxin, and salicylic acid), while others had some known defense association, but most were functionally unknown genes. Desikan *et al.* (2001), using a cDNA microarray representing approximately 30% of the *Arabidopsis* genome, studied regulation of the transcriptome during oxidative stress and identified 175 non-redundant ESTs that are regulated by H<sub>2</sub>O<sub>2</sub>. A substantial proportion of these ESTs have predicted functions in cell rescue and defense processes. Scheideler *et al.* (2002) used a particularly impressive custom cDNA microarray representing 13,000 randomly chosen ESTs, monitored changes in *Arabidopsis* transcript levels after attempted infection with an avirulent *Pseudomonas syringae* strain at different time points. They

found significant changes in the steady state transcript levels of around 680 genes 10 min after inoculation and, by 7 h, a massive shift in the expression pattern of around 2,000 genes representing many cellular processes.

Zhu and Wang (2000) used the Affymetrix *Arabidopsis* GeneChip oligonucleotide array representing around 8,200 *Arabidopsis* genes. Chen *et al.* (2002) monitored mRNA levels of 402 distinct *Arabidopsis* transcription factors under different environmental stresses. They found 74 transcription factor genes whose expression was altered by bacterial pathogen infection and was reduced or abolished in mutants with defects in SA, MeJ, or ethylene signaling. Some of these transcription factors seem very likely to play a role in plant defense signaling pathways. Many other genes responsive to bacteria, fungi, oomycetes or viruses were also identified in this study (Chen *et al.* 2002). Wan *et al.* (2002) identified by using Affymetrix *Arabidopsis* arrays approximately 300 genes regulated by one or more *avr* treatment (*avrRpt2*, *avrRpm1*, *avrPphB* and *avrRps4*). Many of these genes encode unknown proteins or are genes with no previously reported defense functions. The differences observed between the four *R/avr* interactions, all arising in the context of the same *Arabidopsis* host genotype and isogenic *P. syringae* pathogen genotypes, emphasize that each *R/avr* combination can elicit a somewhat different host response.

Notably, many workers have used custom-made arrays that were the best available technology choice at the time. The quality of the data derived from these custom arrays is likely to be even more variable than it already is with any given standardized technology. For example, on many EST-based arrays 5% or more of the spotted DNAs are mis-identified. In addition, even for arrays that cover the same organism, different and only partially overlapping sets of genes are being analyzed by different research groups when custom arrays are used. This, together with differences in the technology platform and differences in gene annotation, has made comparison of results between studies a substantial challenge that has not yet been adequately addressed.

Swidzinski *et al.* (2002) investigated the gene expression during programmed cell death in *Arabidopsis thaliana* using a custom microarray analysis, demonstrating that certain oxidative stress-related genes, including CSD1, CSD3, and GPX, in addition to cysteine proteinases, some transcription factors, and HR-related genes may serve as markers of a core plant cell death program. Additionally they observed a down-regulation of the mitochondrial adenine nucleotide transporter and suggested that this may be an early event in the execution of plant PCD. In 2004, Moy *et al.* found that it is possible to use a single microarray to simultaneously probe gene expression in two interacting organisms. In evaluation of thin films of agarose on glass for hybridization of DNA to identify plant pathogens with microarray technology, Koch *et al.* (2005) revealed that thickness of the agarose layer and length of the sample DNA were important factors affecting hybridization efficiency of immobilized probe to PCR product. Microarray also provides the greatest tool for plant viruses detection compared with biological indexing, electron microscopy, antibody-based detection, including enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR). Additionally, they can be used to detect individual, or combinations of viruses providing the greatest sensitivity among the listed techniques (Boonham *et al.* 2007). Genome-wide microarray approach was also used to distinguish between bacterial pathogens of potato (Aittamaa *et al.* 2008). Recently, Zahariev *et al.* (2009) found efficient design of barcode oligonucleotides that can lead to significant cost reductions in the manufacturing of DNA arrays. The oligonucleotides or polymorphism locations identified as species or clade specific by the new algorithm were refined and screened further for hybridization thermodynamic properties with third party software.

From these studies, it is apparent that the number of genes involved in pathogenesis-related responses is in hun-

dreds. One can foresee that many new defense-related genes will be identified in plants as more genomes and ESTs are sequenced, and as DNA microarrays become less expensive and more accessible to researchers.

In the near future, whole genome arrays will be available for some plant species. Whole-genome arrays may offer more analytical capacity than researcher's desire for some studies, but will reduce the possibility of a misleading bias toward gene sets that are already known. Standardized whole-genome arrays will also enhance the capacity for meta-analyses across different studies. However, cost, gene availability and other issues will continue to force use of custom arrays in many future studies.

The above examples demonstrate that DNA microarrays are a very powerful tool for simultaneously identifying and hypothesizing functions for many genes that may be involved in a complex process, such as plant-defense signaling.

## SUPPRESSION SUBRACTIVE HYBRIDIZATION

Understanding of molecular mechanisms underlying host-pathogen interactions is of primary importance in devising strategies to control diseases. For this purpose, differentially expressed genes analysis is particularly applied. One of the most powerful techniques for such analysis is Suppression Subtractive Hybridization (SSH) (Fig. 2).

In higher eukaryotes, biological processes such as cellular growth and organogenesis are mediated by programs of differential gene expression. To understand the molecular regulation of these processes, the relevant subsets of differentially expressed genes of interest must be identified, cloned, and studied in detail.

### SSH: A method for generating differentially expressed genes

SSH has been a powerful approach to identify and isolate cDNAs of differentially expressed genes (Hendrick *et al.* 1984; Duguid and Dinauer 1990; Hara *et al.* 1991). Numerous cDNA subtraction methods have been reported. In general, they involve hybridization of cDNA from one population (tester) to excess of mRNA (cDNA) from other population (driver) and then separation of the un-hybridized fraction (target) from hybridized common sequences. Because of the excess of driver cDNA after hybridization, the cDNA representing genes expressed at similar levels in both pools are present as heterodimers (hybridization between complementary driver and target cDNA) and homodimers (hybridization between complementary driver cDNA). The method consists of two main stages, the normalization step that equalizes the abundance of cDNAs within the target population and the subtraction step that eliminates the common sequences between the target and the driver populations (Diatchenko *et al.* 1996). This dramatically increases the probability of obtaining low-abundance differentially expressed cDNA or genomic DNA fragments (several molecules per cell) by more than 1,000-fold (Diatchenko *et al.* 1996), and simplifies analysis of the subtracted library (Rebrikov *et al.* 2004). Therefore, SSH greatly reduced the number of clones to be screened to a more manageable size, significantly reducing screening work and cost (Bernardo *et al.* 2007). The latter step is usually accomplished by hydroxylapatite chromatography (Hendrick *et al.* 1984), avidin-biotin binding (Duguid and Dinauer 1990; Sargent and Dawid 1983), or oligo(dT)<sub>30</sub>-latex beads (Hara *et al.* 1991). Despite the successful identification of numerous important genes by these methods, they are usually inefficient for obtaining low abundance transcripts. These subtraction techniques often require greater than 20 µg of poly(A)<sup>+</sup> RNA, involve multiple or repeated subtraction steps, and are labor intensive.

Recently, a new PCR-based technique, called representational difference analysis, has been described that does not require physical separation of single-stranded (ss) and

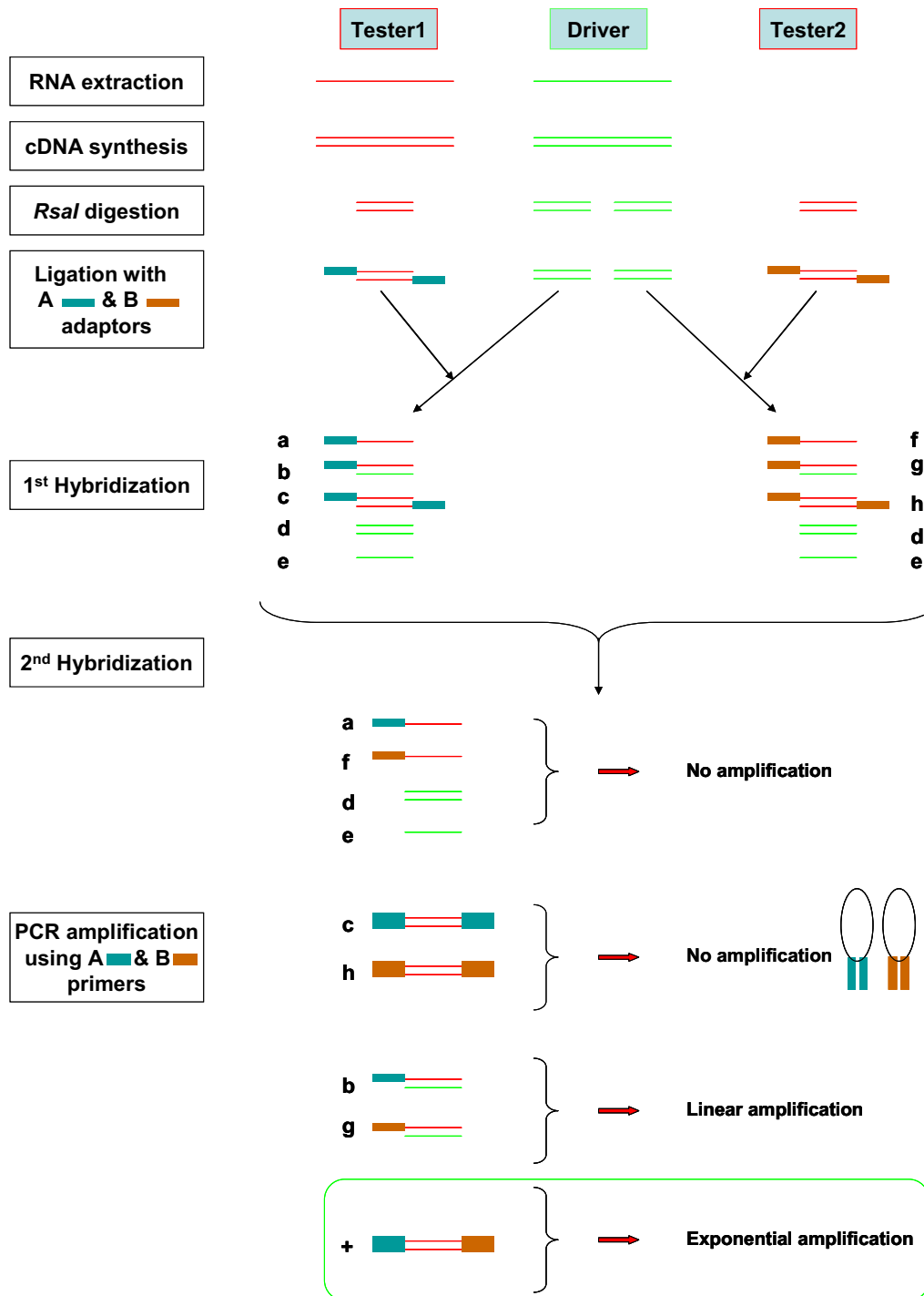


Fig. 2 Schematic illustration of the suppression subtractive hybridization technique.

double-stranded (ds) cDNAs. Representational difference analysis has been applied to enrich genomic fragments that differ in size or representation (Lisitsyn *et al.* 1993) and to clone differentially expressed cDNAs (Hubank and Schatz 1994). However, representational difference analysis does not resolve the problem of the wide differences in abundance of individual mRNA species. Consequently, multiple rounds of subtraction are still needed (Hubank and Schatz 1994). The mRNA differential display (Liang and Pardee 1992) and RNA fingerprinting by arbitrary primed PCR (Welsh *et al.* 1992) are potentially faster methods for identifying differentially expressed genes. However, both of these methods have a high level of false positives (Bauer *et al.* 1994; Sompayrac *et al.* 1995), biased for high copy number mRNA (Bertioli *et al.* 1995) and might be inappropriate in experiments in which only a few genes are expected to vary (Sompayrac *et al.* 1995). Therefore, SSH procedure can be modified to increase the possibility of

identifying quantitatively regulated transcripts between the tester and driver cDNA populations. Under the standard conditions, the driver cDNA would have eliminated most of the common sequences between the tester and driver cDNA samples during the first hybridization step. However, quantitatively different cDNA species may still remain in the tester populations. To further eliminate common sequences, excess fresh driver cDNA needs to be added to the samples in the second hybridization step, thereby further subtracting quantitatively different but common sequences between the tester and driver populations. Hence, to retain the representation of the quantitatively different cDNAs in the final SSH products, the driver cDNA can be omitted in the second hybridization step, thereby allowing quantitatively regulated cDNAs in the tester samples to anneal and form hybrid that are amplifiable in subsequent PCR.

One potential disadvantage of the SSH technique is the fact that under the standard procedure, a few micrograms of

poly(A)<sup>+</sup> RNA from the two cell populations are needed. In some special cases, such quantity of RNAs may be difficult to obtain. To circumvent this problem, an amplification step for both the driver and tester cDNAs can be incorporated to generate sufficient quantities of both cDNA samples before initiating the SSH procedure. In such cases, separate adapter/primers will be ligated to the cDNA fragments and subsequently used for the PCR amplification (Diatchenko *et al.* 1996). Nevertheless, we believe that avoiding the pre-amplification step is desirable because it may result in the loss of some sequences.

SSH technique is applicable to many comparative and functional genetic studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes, as well as for the recovery of genomic DNA fragments distinguishing the samples under comparison (Diatchenko *et al.* 1999). The traditional subtractive hybridization methods have been used successfully in many cases, but several rounds of hybridization are required for this method. Therefore, this technique can be used to compare two mRNA populations and obtain cDNAs representing genes that are either over-expressed or exclusively expressed in one population as compared to another. Moreover, rare messages are not easily identifiable (Sargent and Dawid 1983; Hedrick *et al.* 1984; Davis *et al.* 1984; Duguid and Dinauer 1990; Hara *et al.* 1991). SSH is a unique method based on selective amplification of differentially expressed sequences, which can overcome the technical limitations of the traditional subtraction methods (Diatchenko *et al.* 1996). Using SSH, differentially expressed genes, which have high, middle, low, or rare abundance transcripts, can be cloned with equal probability. Additionally, only 0.5–2 µg of poly(A)<sup>+</sup> mRNA is required over a period of 3–4 d, and it is not necessary to physically separate single- and double stranded DNA molecules (Duguid and Dinauer 1990; Sargent and Dawid 1983; Hedrick *et al.* 1984). The suppression PCR prevents undesirable amplification while the enrichment of target molecules proceeds.

### SSH: A versatile method for identifying differentially expressed genes

The first application of SSH in the study of plant-microorganism interactions was for the isolation of potato genes that are up-regulated in the hypersensitive response to *Phytophthora infestans* (Birch *et al.* 1999). Xiong *et al.* (2001) identified 34 distinct immediate early defense-related rice genes from 768 subtracted clones that are induced by jasmonic acid, benzothiadiazole, and/or blast infection using SSH method. The SSH method has been used in many systems for identifying genes with altered expression levels (Gepstein *et al.* 2003; Louie *et al.* 2003; Veena *et al.* 2003; Guilleroux and Osbourn 2004; Wang *et al.* 2005). While no method of differential gene expression can identify all regulated genes, SSH is a reasonable choice given the lack of knowledge available for the sugar beet genome that does not benefit from large scale sequencing (e.g. *Arabidopsis*, rice, maize) and has major genetic differences within and between varieties (De Riek *et al.* 2001). Fabritius *et al.* (2002) identified eight genes that are up-regulated during sexual development in the heterothallic oomycete, *Phytophthora infestans*. SSH has been used also to isolate potato genes that are up-regulated in the incompatible (Avrova *et al.* 1999; Birch *et al.* 1999) and compatible (Dellagi *et al.* 2000) interactions with *P. infestans*. Moreover, SSH-derived cDNA populations enriched for sequences expressed specifically at early (15 h post-inoculation) and late (72 h post-inoculation) stages of infection have been generated and used as probes to screen the *P. infestans* BAC library constructed by Whisson *et al.* (2001).

In rice an ABC transporter gene was isolated from a SSH study designed to identify early immediate defense genes. This gene was induced by both bensothiadiazole (BTH; a functional analog of salicylic acid) and by the rice-

blast fungus *Magnaporthe oryzae* (Xiong *et al.* 2001). Ricardo and Dean (2002) identified 18 gene differences between a citrus variegated chlorosis (CVC) strain and a Pierce's disease of grape (PD) strain of *Xylella fastidiosa*. The results were validated as being highly representative of actual differences by comparison of the completely sequenced genome of a CVC strain with that of a PD strain. Bittner-Eddy *et al.* (2003) characterized only 25 *Peronospora parasitica* (At) genes from 1345 clones expressed during infection of *Arabidopsis thaliana*. Thara *et al.* (2003) isolated at least 21 rust PIGs induced in *planta* encoding a variety of functions and at least three wheat genes that were induced by rust infection. SSH was also used for the identification of genes expressed after application of the non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 to the phyllosphere of the apple scab-susceptible cultivar *Malus domestica* cv. 'Holsteiner Cox', where 157 expressed sequence tag (EST) clones were obtained. Moreover, the application of suppressed subtracted hybridization technology enabled the isolation of a significant number of organ-specific sunflower ESTs and allowed the identification of novel sequences from a relative small number of analyzed sequences (Fernández *et al.* 2003). Because the majority of florally expression-related genes are low-abundance-expressed, SSH has been proven a useful method to isolate the florally expression-related genes (Hu *et al.* 2003). However, it should be pointed out that, like other PCR-based methods, SSH might produce false positives when it is applied to isolating subtracted expressed genes. Therefore, a combination of SSH and cDNA microarray can be a complementary way to identify subtracted expressed genes (Yang *et al.* 1999).

Zhao *et al.* (2006) found that a total of 29 MADS-box transcription factors were members of the *APETALA3/AP3* subfamily, while nine others were putative MADS-box transcription factors that formed a cluster with MADS-box genes isolated from *Amborella*, the basal-most angiosperm, and those from the gymnosperms. Shi *et al.* (2006) used two complementary approaches, SSH and microarray-based expression profiling to isolate and identify candidate genes in isogenic lines for *bm* mutants maize finding 53 expressed sequence tags (ESTs). The expression patterns of transcriptomes from wheat spikes of fusarium head blight (FHB) resistant cultivar Ning 7840 and susceptible cultivar Clark were monitored during a period of 72 h after inoculation (hai) with *Fusarium graminearum*. SSH technique coupled with microarray analysis identified 44 significantly differentially expressed genes between cv. 'Ning' 7840 and cv. 'Clark'. More differentially expressed genes were identified from susceptible libraries than from resistance libraries (Bernardo *et al.* 2007). Also in 2007, David and Ann identified more than 150 genes with SSH following SBRM feeding on both SBRM susceptible and moderately resistant sugar beet lines. While no quantitative data can be obtained from this procedure, the ease of the SSH technique and rapid completion provides a list of potentially regulated genes which can then be more extensively investigated to identify those responsible for resistance (Ramalingam *et al.* 2003). In addition, in this study the authors compared this strategy with a differential screening performed on a non-differential cDNA library. They found that the SSH allowed the detection of medium-low abundant genes such as protein kinases and transcription factors whilst the differential screening technique detected mostly abundant transcripts such as PR genes (Xiong *et al.* 2001). A defense gene has been identified by Letousey *et al.* (2007) in LR1 genotype, which is correlated with *Orobanche* resistance using SSH method. Additionally, Alavi *et al.* (2008) identified pathogenicity gene candidates for *X. axonopodis* pv. phaseoli and *X. fuscans* subsp. *fuscans* strains and a putative type III secretion apparatus that is usually not found in plant pathogenic bacteria. Recently, Egusa *et al.* (2009) examined the expression of multiple defense-related genes in Japanese pear leaves inoculated with nonpathogenic *Alternaria alternata* using SSH.

In the same context, we are right now investigating the

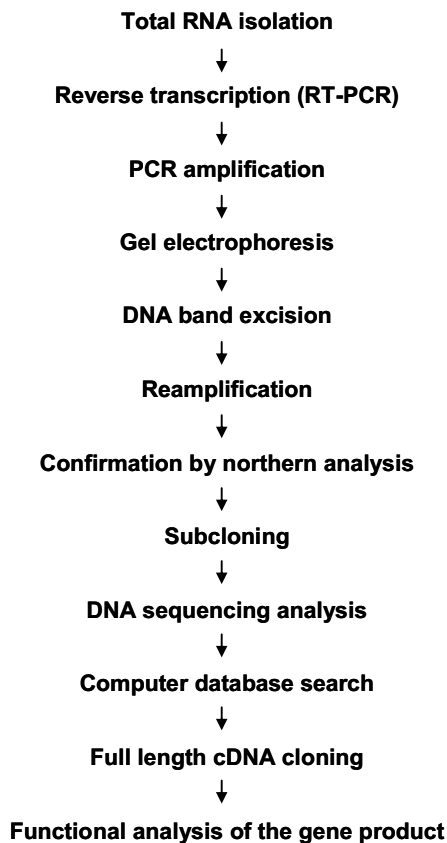


Fig. 3 Schematic illustration of differential displayed - reverse transcriptase polymerase chain reaction.

transcriptome pattern of wheat (*Triticum aestivum* L.) spikes that have been infected with *Fusarium graminearum* isolate Fg2 which produces 3 acetyl deoxynivalenol (3ADON) using SSH technology. The differentially expressed probes would be used in quantitative real-time PCR (Q-RT-PCR) to explore the expression levels of genes that expressed under 3ADON comparing with 15ADON-expressed genes for the same cultivar (Al-Taweel *et al.* unpublished data).

To our knowledge, the application of this technique in cloning differentially expressed genes in *Fusarium* and comparing their expression levels between 3ADON and 15ADON-infected wheat have not been done.

### DIFFERENTIAL DISPLAYED - REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Screening for differentially expressed genes is a straightforward approach to study the molecular basis of a biological system. In the last 10 years, differential screening technology has evolved rapidly and currently high-throughput tools for genome-wide transcript profiling, such as expressed sequence tags, microarray analysis and differential display method are becoming widely available. Here, an overview to emphasis on the differential display - reverse transcriptase polymerase chain reaction method (DD-RT-PCR) (Fig. 3), which for many years has been the preferred technique of scientists in diverse fields of research, and been adopted by a large number of laboratories as an important tool being applied for both *in vitro* and *in vivo* systems.

#### DD-RT-PCR

Although a gene must not necessarily be up- or down-regulated to play a key role in a certain process, screening for differentially expressed genes is one of the most straightforward approaches to unravel the molecular basis of a

biological system. The difficulty of isolating differentially expressed genes, particularly low-abundance ones, has been underestimated by some, albeit experienced by many. To fully recognize this, it is important to take into account the complexity of a cell's transcriptome. A eukaryotic cell contains ~15000–30000 distinct mRNAs with a prevalence ranging from one to several thousands in a total mass of ~100000 mRNAs. About 50% of the transcript population is made up of a relatively small number (some hundreds) of abundant transcripts, representing only 1% of the different mRNA species. The other half contains the 'rare' mRNAs (Wan *et al.* 1996). Not surprisingly, the difficulty of fishing out a gene responsible for a specialized function in a certain biological program often originates from the fact that the gene is expressed at low levels whereas the bulk of a cell's mRNA is made up of highly abundant transcripts.

In traditional screening methods, such as differential hybridization, the hybridization pattern of the total content of a cDNA library is compared between two samples (Maniatis *et al.* 1982). The fact that the abundant transcripts are also displayed implies high redundancy of non-relevant clones and thus very low labor efficiency. This problem has been solved partly by normalization and subtraction (Hedrick *et al.* 1984); even then, many interesting low-abundance differentially expressed genes are missed because of the low amplification of the hybridization signal (Wang *et al.* 1991). Other drawbacks are the limitation to pairwise comparisons and the fact that the techniques are mainly qualitative because of the relative insensitivity of the hybridization (Bauer *et al.* 1993).

#### DD-RT-PCR: Advantages, disadvantages and evolutions

With the availability of PCR, low-abundance transcripts could be amplified. One of the first differential screening methods that used this possibility was the differential display technique described by Liang and Pardee (1992). By combining 3' anchored oligo(dT) primers and short 5' arbitrary primers, subsets of the transcriptome are amplified, the resulting cDNA fragments are separated on a denaturing polyacrylamide gel and visualized autoradiographically. Original statistics indicated that 80 primer combinations would be sufficient to cover the whole transcript mass (Liang *et al.* 1993). The expected advantages were numerous: the method would be fast, producing band patterns in 2 days; it was based on simple, well established and widely accessible techniques, making it easily applicable for most researchers; compared with previous methods the sensitivity had been increased dramatically, resulting in a good detection of low-abundance genes; both expressed and suppressed genes could be detected and more than two samples could be compared, making it highly versatile; furthermore, only a small amount of starting material was needed (Liang and Pardee 1992).

It soon became clear from numerous reports that the differential display technique represented a major contribution to the molecular biology toolbox. At the same time, however, investigators experienced drawbacks and limitations and over the years, many variations of the original protocol were published (Debouck 1995; Liang *et al.* 1995; McClelland *et al.* 1995; Liang 1998; Matz and Lukyanov 1998; Appel *et al.* 1999).

Among the major criticisms, one was the questioned ability of the technique to identify rare mRNAs. Some studies suggested that competition for substrates between the PCR products, particularly dNTPs, would be the limiting factor for amplification, so that only abundant transcripts would be amplified to a detectable level by the time dNTPs were depleted (Bertioli *et al.* 1995). Other experiments, however, gave a more optimistic evaluation of the sensitivity (Wan *et al.* 1996). By comparing untreated HeLa cells with cells treated with interferon- $\gamma$ , the calculated prevalences of the isolated clones ranged from 1/214 to ~1/200 000 with a median of ~1/20 000. The apparent contradiction

between both reports could be explained, at least partially, by a dramatic change in gene expression in the latter system, with the fraction of differentially expressed genes reaching levels of at least 6.6%. A relatively high total number of (rare) differential transcripts increase the probability of detecting some of the low-abundance ones (Matz and Lukyanov 1998).

Once a candidate band has been eluted from the gel, been re-amplified and cloned, gene expression analysis tools, such as northern blot, RT-PCR or RNase protection, are applied to confirm the expression pattern and to attribute it to the correct clone. Indeed, most often, cloning of a band from the gel results in a mixture of cDNAs because it is difficult to avoid contamination from neighboring bands during band excision and because one band may consist of several clones, the observed pattern being the additive result of overlapping expression patterns (Li *et al.* 1994; Callard *et al.* 1994). Thus, the downstream part of the differential display procedure reveals a major drawback, i.e. the frequency of false positives, which may be as high as 50–75% of the excised bands (Liang *et al.* 1993; Debouck 1995; Wan *et al.* 1996).

The most significant source of artifacts, however, might be inherent to the design of the differential display method. The combination of short primers and low annealing temperatures during PCR results in non-specific and inefficient amplification (Zhao *et al.* 1995). Another factor that may generate false positives is the competition for primers by transcripts of different abundance. Experiments showed that increased amounts of a spiked RNA species resulted in increasing amounts of the corresponding PCR product. Concomitantly, the signal intensity of several unrelated bands decreased, questioning the quantitative aspect of the technique (Debouck 1995). The number of false positives might also increase in some cases because of extrinsic factors, such as the systems that are compared and care that is taken in experimental design (e.g. PCR tubes, internal controls; Liang 1998).

When the workload of the downstream processing of differential display candidates is considered, another important issue is redundancy. Decamer primers often mismatch and hybridize to distinct regions of the same cDNA (Bertioli *et al.* 1995; Wan *et al.* 1996). Consequently, different positive clones might correspond to the same gene. This observation not only implies a reduced screening efficiency, it also means that a lot more primer combinations than theoretically calculated should be used to cover the complete transcriptome. Depending on the design of both 5' arbitrary and 3' anchored primer and the cycling program, the proposed amount of primer combinations varies greatly (Liang and Pardee 1992; Bauer *et al.* 1993; Wan *et al.* 1996; Appel *et al.* 1999).

Most of the published technical modifications and refinements aim to reduce the amount of artifact bands and improve the sensitivity of the differential display. Alternative primer designs have been proposed, such as longer, more specific arbitrary primers, eventually in combination with an altered cycling program (Zhao *et al.* 1995; Liang *et al.* 1994; Graf *et al.* 1997). By re-amplifying a subset of the original fingerprint with nested primers, which carry one or more extra selective nucleotides, more reliable results have been reported (Ralph *et al.* 1993). Other improvements included use of stronger radioactive labels (Tokuyama and Takeda 1995), implementation of automatic sequencing machines (Bauer *et al.* 1993), use of end-labeled 3' primers (Hadman *et al.* 1995), combination with a subtraction procedure (Fuchs *et al.* 2000; Kang *et al.* 1998) and many other minor modifications (Appel *et al.* 1999). A multicolor fluorescent differential display protocol has been developed that allows digital analysis and the reduction of false positives by inherent signal proofreading (Cho *et al.* 2001).

Considering the rate of false positives and redundancy, differential display seemed much less attractive than originally presented. The downstream verification process is not only labor intensive, it also requires significant amounts

of RNA, which compromises one of the major advantages of the method. Many different approaches have been proposed to meet the necessity of large-scale screening of candidate cDNA fragments with low amounts of RNA, such as reverse Northern with Southern-blotted (Consalez *et al.* 1996), slot (Vögeli *et al.* 1996) or dot-blotted (Mu *et al.* 1994) clones, eventually in combination with the use of amplified RNA as a probe (Poirier *et al.* 1997). Another important factor that adds to the post-display effort is intrinsically linked to the design of the technique. Because the cDNA fragments obtained from differential display are short (typically 100–500 bp) and correspond to the 3' end of the gene that represents mainly the 3' un-translated region, they usually do not contain a large portion of the coding region. Unless a model system is studied for which a significant amount of sequence information is available in public databases, labor-intensive full-length cDNA screening is needed before significant sequence homology, informative for gene classification and prediction of function, can be obtained. Recently, this problem has been tackled by a number of authors by integrating the protocol for long-distance PCR into differential display. By combining the use of longer primers, higher dNTP concentrations, hot-start PCR at higher stringency and thermostable enzyme mix, bands of up to 2 kb could be displayed (Diachenko *et al.* 1996). The long-distance differential display-PCR (LDD-PCR) produced similarly band patterns of cDNAs ranging from 150 bp to 2 kb. Furthermore, the alterations to the protocol resulted in lower redundancy, reduced amounts of artifact bands and detection of both abundant and rare transcripts.

### DD-RT-PCR: The method of choice for plant host-pathogen interaction

Transcriptome analysis is a common way of discovering differences in gene expression because regulation of gene activity occurs primarily on transcription level. Numerous low-cost, simple methods are available for gene discovery projects, providing a limited set of transcripts more or less randomly selected from a pool of genes expressed differently between two samples (for example treated sample/control or diseased/healthy tissue). RT-PCR differential display (Liang and Pardee 1992) has been widely used to isolate genes whose expression profiles have been altered under different abiotic or biotic cues because of its technical simplicity and lack of requirement for previous genomic information of the species of interest (Kuno *et al.* 2000; Basse 2005, Lang *et al.* 2005). Therefore, DD has been the method of choice for identifying the differentially expressed genes involved in the plant host-pathogen interaction.

In 1997, Truesdell and Dickman used DD to isolate a full-length (*SRG1*) and a partial (*SRG2*) alfalfa cDNA induced during infection with the fungal pathogen *Colletotrichum trifolii*. The deduced amino acid sequences are similar to each other and resemble plant defense-related proteins. The same technique was used to isolate cDNA clones corresponding to genes, which were expressed in soybean hypocotyls by *Phytophthora sojae* f.sp. *glycines* infection (Yi and Hwang 1998). A new pathogenesis-related gene, also induced by 2,4-D, SA and wounding, was cloned and correlation between its expression and the resistance of sunflower to *Plasmopara halstedii* was found (Mazeyrat *et al.* 1998). Soybean cell suspension cultures inoculated with the pathogenic bacteria *Pseudomonas syringae* pv. *glycinea* were responded with a hypersensitive reaction (HR) when the bacteria express the avirulence gene *avrA*. A mRNA differential display was applied to allow the identification of genes induced during the HR. Six PCR-fragments from the differential display analysis were identified, which are induced during the HR (Seehaus and Tenhaken 1998). Timmusk and Wagner (1999) addressed changes of plant gene expression following inoculation by the root invading plant-growth-promoting rhizobacteria (PGPR) *Paenibacillus polymyxa*. A gnotobiotic system was used to show that *P.*

*polymyxa* isolates confer resistance to biotic (*Erwinia carotovora* challenge) and abiotic (drought) stress, and genes were identified whose expression level was altered upon treatment with the PGPR by DD. Chen and Chen (2000) isolated two tobacco WRKY genes, *tWRKY3* and *tWRKY4*, that are rapidly induced in resistant tobacco plants after infection by tobacco mosaic virus (TMV). In the same year, Hermsmeier *et al.* used the technique of differential display of mRNA to analyze mRNA abundance as a first indicator of gene expression changes reporting the identification of *Arabidopsis thaliana* cDNA clones that correspond to mRNA species that change abundance specifically around the developing syncytium, and also identified *Heterodera schachtii* cDNA clones that correspond to mRNA abundance changes occurring during the transition from the pre-parasitic (i.e., before root penetration) to the parasitic (i.e., after root penetration) life style.

To find out the interaction between potato and *Phytophthora infestans* at the molecular level, Collinge and Boller (2001) screened for genes induced early after infection using mRNA differential display. Among the twenty cDNA clones recovered in the screen, two were found to represent plant genes whose transcript levels increased during infection of intact plants. A differential display of mRNAs was used again to isolate periwinkle cDNAs differentially expressed following infection with one of three mollicutes: *Spiroplasma citri*, *Candidatus Phytoplasma aurantifolia*, and stolbur phytoplasma. Twenty-four differentially expressed cDNAs were characterized by Northern blots and sequence analysis (Jagoueix *et al.* 2001). DD was also used to identify alterations of gene expression in young *Arabidopsis thaliana* root galls caused by *Meloidogyne incognita*. Six genes were confirmed as plant genes by DNA gel blot hybridizations. A full-length cDNA, *PPRG2*, representing a gene highly expressed in dodder (*Cuscuta trifolii* Bab et. Gibs)-infected alfalfa (*Medicago sativa* L.) stems was isolated using the same method (Borsics and Lados 2002). Differential display analysis has been carried out to detect changes in gene expression in giant cells induced in tomato roots by *Meloidogyne javanica*, using mRNA isolated directly from mature giant cell cytoplasm, compared to non-infected root tissue. A total of 81 differential displayed bands were generated, 73 of which were up-regulated and 8 were down-regulated (Wang *et al.* 2003). The messenger RNA (mRNA) differential display technique was applied to the identification and isolation of genes whose transcription was altered in leaves of *Prunus armeniaca* infected by European stone fruit yellows (ESFY) phytoplasma belonging to ribosomal subgroup *16SrX-B*. Four genes whose steady-state levels of expression significantly changed in response to phytoplasma infection were isolated and identified (Carginale *et al.* 2004). Promoter activity of *ABI3* and of three *LEA* genes was monitored in *Arabidopsis* transgenics infected with *Heterodera schachtii* and *Meloidogyne incognita*, using DD (Meutter *et al.* 2005). In 2006, Ada and Ilan isolated *hydrophobin*-like clone (*TasHyd1*) during a PCR differential mRNA display analysis conducted on *Trichoderma asperellum* mycelia interacting with plant roots. In addition, DD was employed to isolate anoxic and/or hypoxic genes whose expression responded to short, low-oxygen regimes. This approach led to the isolation, cloning, successful sequencing, and bioinformatic analysis of 98 transcripts from *Citrus* flavedo tissues that were differentially expressed in DD gels in response to 0, 0.5, 3, and 21% O<sub>2</sub> for 24 h (Pasentsis *et al.* 2007). In the same year, Ansari *et al.* (2007) used DD to demonstrate that fusarium-derived mycotoxin deoxynivalenol (DON) affects transcript levels of a few specific host wheat genes in roots, including peroxidase genes. The analyses of ESTs indicated that not only tandem repeats existed in ESTs but also tandem repeats differentially presented in different organ or tissue specific ESTs within and between the species (Ince *et al.* 2008). Saima *et al.* (2008) identified 17 differentially expressed transcripts using non-radioactive DDRT-PCR including some reported fiber development specific genes and two

new transcripts that appear at late stages of fiber development.

## SUMMARY

Application of differential display was used to investigate gene expression in plants. A substantial number of articles report the isolation and profiling of various genes expressed in cells using this technique. Genes involved in physiological events, stress responses (biotic and abiotic stress), signal transduction and secondary metabolism have been isolated and characterized. Some of the isolated genes encode transcription factors, membrane proteins, resistant genes, and rare enzymes that were previously difficult to purify. These results suggest that differential display is a powerful tool used to investigate the rare genes involved in the plant life cycle without using information from proteins.

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