Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes

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Abstract: Invention of polymerase chain reaction (PCR) technology by Kary Mullis in 1984 gave birth to real-time PCR. Real-time PCR — detection and expression analysis of gene(s) in real-time — has revolutionized the 21st century biological science due to its tremendous application in quantitative genotyping, genetic variation of inter and intra organisms, early diagnosis of disease, forensic, to name a few. We comprehensively review various aspects of real-time PCR, including technological refinement and application in all scientific fields ranging from medical to environmental issues, and to plant.

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BACKGROUND

The invention of polymerase chain reaction (PCR) by Kary Mullis in 1984 was considered as a revolution in science. Real-time PCR, hereafter abbreviated RT PCR, is becoming a common tool for detecting and quantifying expression profiles of selected genes. The technology to detect PCR products in real-time, i.e., during the reaction, has been available for the past 10 years, but has seen a dramatic increase in use over the past 2 years. A search using the key word real-time and PCR yielded 7 publications in 1995, 357 in 2000, and 2291 and 4398 publications in 2003 and 2005, respectively. At the time of this writing, there were 3316 publications in 2006. The overwhelming majority of the current publications in the field of the genomics have been dealing with the various aspects of the application of methods in medicine, with the search for new techniques providing higher preciosity rates and with the elucidation of the principal biochemical and biophysical processes underlying the phenotypic expression of cell regulation. Series of RT PCR machines have also been developed for routine analysis (Table **1**) [1].

The advancements in bioscience during the last century help in comprehensive understanding of information about interacting network of various gene modules that coordinately carry out integrated cellular function in somewhat isolated fashion, i.e., the molecular mechanism of phenotypic expression of genotype. The function of a major part of the genome is still unknown and the relationship between enzymes, signaling substances and various small molecules is still rather limited. In order to fully understand the regulation of metabolism and to alter it successfully more information of gene expression, recognition of DNA by proteins, transcription factors, drugs and other small molecules is required.

Gene expression profile has been widely used to address the relationship between ecologically influenced or disease phenotypes and the cellular expression patterns. PCR–based detection technologies utilizing species specific primers are proving indispensable as research tools providing enhanced information on biology of plant/microbe interactions with special regard to the ecology, aetiology and epidemiology of plant pathogenic microorganisms.

In general, laboratory experience with nested PCR for diagnostics on presence of microbial DNA in extracts from a diverse range of plant matrices (including soils) offers improved sensitivity and robustness, particularly in the presence of enzyme inhibitors. In order to meet consumer and regulatory demands, several PCR-based methods have been developed and commercialized to detect and quantify mRNA in various organisms. Most of them are based on the use of internal transcribed spacer regions within the nuclear ribosomal gene clusters as these are particularly attractive loci

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Table 1. Real-Time Cyclers Available in the Market and their Characteristics

Cycler	Source	Detector	Applications	No. of Samples	Footprint
ABI Prism 7000	Tungsten-halogen	CCD camera	SYBR, FAM, HEX, TET, TAMRA, VIC	96	39×51 cm
Bio-Rad iCycler iQ	Tungsten-halogen	CCD camera	SYBR, FAM, HEX, TE, TA, VIC	96	33×62 cm
Cepheid Smartcycler	LED	Silicon detectors	SYBR, FAM, TET, ROX, Cy3, Cy5	16	30×25 cm
Corbett Research Rotor-Gene 3000	LED	PMT	SYBR, FAM, HEX, TET, TAMRA, VIC	72	38×48
MJ Research DNA Engine Opticon 2	LED	2 PMTs	SYBR, FAM, HEX, TET, TAMRA, VIC	96	34×47 cm
Roche LightCycler 2	LED	Fluorimeters	SYBR, FAM, HEX, VIC, LightCycler Red Stains	32	30×45 cm
Stratagene Mx3000P	Tungsten-halogen	1 PMT scanner	SYBR, FAM, HEX, TET, TAMTA, VIC	96	33×46 cm
Techne Quantica	Halogen	PMT	SYBR, FAM, HEX, TET, TAMRA, VIC	96	45×50 cm

for the design of PCR–based detection assays. These clusters are readily accessible using universal primers and typically present in high copy number in the cell, whilst often exhibiting sufficient inter-specific sequence divergence for the design of species specific primers. The limit of detection is usually a few alien molecules even in the presence of very high levels of background DNA.

The high sensitivity and specificity of RT PCR allow it to be the first choice of scientists interested in detecting dynamics of gene expression in plant/microbe associations (Table **2**).

Table 2. Obligate Pathogen Detection Using Real-Time PCR

Pathogen	Reference
Fungi	
Melampsora medusae	Boyle *et al*., 2005 [152]
Synchytrium endobioticum	van den Boogert *et al*., 2005 [153]
Bacteria	
Chlamydia pneumoniae	Kuoppa *et al*., 2002 [154]
Ehrlichia species	Doyle *et al*., 2005 [155]
Burkholderia species	Ulrich *et al*., 2006 [156]
Coxiella burnetii	Klee *et al*., 2006 [157]
Neisseria gonorrhoeae	Tobiason and Seifert, 2006 [158]
Mycobacterium	
Mycobacterium leprae	Groathouse *et al*., 2006 [159]

The RT PCR allows quantitative genotyping and detection of single nucleotide polymorphisms and allelic discrimination as well as genetic variations when only a small proportion of the sample carrying the mutation. The use of multiplex PCR systems using combined probes and primes targeted to sequences specific to counterpartners of plant/microbe associations is becoming more important than standard PCR, which is proving to be insufficient for such living systems.

The multiplex RT PCR is suitable for multiple gene identification based on the use of fluorochomes and the analysis of melting curves of the amplified products. This multiplex approach showed a high sensitivity in duplex reactions and is useful alternative to RT PCR based on sequence-specific probes, e.g., TaqMan chemistry (Table **3**).

Although RT PCR is a powerful technique for absolute comparison of all transcripts within the investigated tissue, it has a few problems as it depends critically on the correct use of calibration and reference materials. Successful and routine application of PCR diagnostics to tissues of plant/microbe consortium is often limited by the lack of quality template due to inefficient RNA extraction methodologies, but also the presence of high levels of unidentified, co-precipitated PCR inhibitory compounds, presumably plant polyphenolics and polysaccharides (Table **4**).

The sampling procedures are of great importance towards the validation of analytical methods for analysis. The largest single source of error in the analysis of plant/microbe associations is the sampling procedure (Fig. **1**). Sampling risks can be managed by choosing an appropriate sample size for analysis. The extraction and purification of nucleic acids is a crucial step for the preparation of samples for PCR. Current methods for gene expression studies typically begin with a template preparation step in which nucleic acids are freed of bound proteins and are then purified. Many protocols for nucleic acid purification, reverse transcription of RNA and/or amplification of DNA require repeated transfers from tube to tube and other manipulations during which materials may be lost.

Of the range of protocols reported for the extraction of DNA/RNA from plant material, most are complicated and time consuming in application. The protocols should be perused case by case and to be adopted judiciously for a particular plant species. In this respect major variations exist in this step as compared to samples of mammalian origin. Isolation of RNA is particularly challenging because this mole-

Table 3. Multiplexing Using Real-Time PCR

Purpose	Reference
Simultaneous detection of mycorrhizal and pathogen DNA	Bohm *et al*., 1999 [160]
Detection and Quantification of Transgenes in Grains	Permingeat *et al*., 2002 [161]
Monitoring of host-pathogen dynamics	Hietala *et al.*, 2003 [162]
Mycotoxin producing fungi	Bluhm *et al*., 2004 [163]
Simultaneous detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*	Courtney *et al*., 2004 [164]
Discrimination of viral infections	Templeton *et al*., 2004 [30]
Heat-labile and heat-stable toxin genes in enterotoxigenic *Escherichia coli*	Grant *et al*., 2006 [165]
Pathogen colonization in the bark and wood of *Picea sitchensis*	Bodles *et al*., 2006 [166]
Detection of norovirus genogroups	Hoehne and Schreier, 2006 [167]

cule is sensitive to elevated temperatures and is degraded by RNAses, which therefore have to be immediately inactivated upon cell lysis. Design of species or race specific primers from inter-specific universal internal transcribed spacer primers is also needed.

Table 4. PCR Inhibitory Compounds

Factors Influencing Polymerase Chain Reaction	
Inhibitor	**Enhancer**
Hemoglobin, Urea, Heparin	DMSO, Glycerol, BSA, Formamide, PEG, TMANO, TMAC
Organic or phenolic compounds	Special commercial enhancers, Gene 32protein, TaqExtender, Perfect Matchr
Glycogen, Fats, Ca^{2+}	*E. coli* ss DNA binding
Tissue matrix effects	
Laboratory items, powder, etc	

There are numerous commercially available kits for PCR. The data output from certain RT PCR machines gives an immediate appreciation of the kinetics of the PCR occurring within the tube and, in addition, gives an instantaneous visual representation of the amount of PCR product present following each cycle. Following a single RT PCR, the data extracted give the type of information that was only previously inferable from multiple conventional PCRs. Detailed information is available from the respective companies' websites about the protocols and output information generated.

In this review, we highlight some of the general criteria and essential methodological components of PCR technologies, for rapid functional genomics. Examples are provided to illustrate the utility of results of plant pathology studies and validation of targets for mammalian studies.

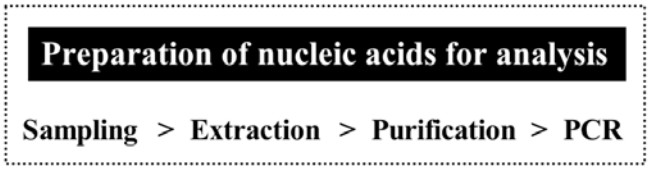

Fig. (1). Sampling procedures are of great importance towards the validation of analytical methods for analysis.

APPLICATIONS

Medical Science

Nucleic acid amplification techniques have revolutionized diagnostics. Current technologies that allow the detection of amplification in real-time are fast becoming clinical standards, particularly in a personalized diagnostic context [2]. On the way to personalized medicine, we may stepwise improve the chances of choosing the right drug for a patient by categorizing patients into genetically definable classes that have similar drug effects (as, for example, human races, or any population group carrying a particular set of genes) [3]. Adverse drug reactions (ADRs) are a significant cause of morbidity and mortality. The majority of these cases can be related to the alterations in expression of clinical phenotype that is strongly influenced by environmental variables [4]. Application of RT PCR combined with other molecular techniques made possible the monitoring of both therapeutic intervention, and individual responses to drugs. However, it is wise to expect that, even after we have reached the goal to establish personalized medicine, we will not have eliminated all uncertainties [5]. The needs in clinical application of molecular methods initiated important developments in diagnostics stimulating progress in other branches of science. The introduction of these new methods in fields of human practices induced rapid expansion of molecular approaches.

Cancer

Cancer arises from the accumulation of inherited polymorphism (SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes [6]. Despite

advances in diagnostic imaging technology, surgical management, and therapeutic modalities, cancer remains a major cause of mortality worldwide. Early detection of cancer and its progression is difficult due to complex multifactorial nature and heterogeneity [7]. A reliable method to monitor progress of cancer therapeutic agents can be of immense use. RT PCR, currently the most sensitive method to quantify the specific DNA makes it possible to detect even a single molecule and diagnostics become feasible with lower amounts of complex biological materials compared to traditional methods [8, 9]. Research has been well documented in cancer research [10, 11, 12]. Most of the commonly occurring cancers have been detected by measuring marker gene expressions or by using probes. The sensitivity of single-marker assays is not high enough for clinical applications [13]. Adopting a multigene panel for most common malignant diseases (carcinoma of bladder, breast cancer, colorectal cancer, endometrial carcinoma) significantly increased the accuracy of diagnosis that is extremely important as each of them had excellent prognosis if diagnosed at early stage [14]. The use of new technology and methodic developments has been intensively started with diseases of complicated diagnosis (Table **5**). During the first five years after introduction of RT PCR six of ten applications were made for detecting leukemias. Recently numerous kits are marketed for clinical tests, and these developments promoted the use of RT PCR in other fields of human practices.

Table 5. Time Course of Developments in Application of Real-Time PCR Used for Cancer Diagnosis

Implications of RT PCR	Reference
Molecular diagnosis of chronic myeloid leukemia	Menskin *et al.*, 1998 [168]
Molecular diagnosis of hematological malignancies	Morgan and Pratt, 1998 [169]
Molecular diagnosis of follicular lymphoma	Luthra *et al.*, 1998 [170]
Molecular diagnosis of non-Hodgkins lymphoma	Rambaldi *et al*., 2005 [171]
Diagnostics of acute lymphoblastic leukemia	Eckert *et al.*, 2000 [172]
Real-time quantitation of E2A-Pbx1 fusion gene; leukemia	Pennings *et al.*, 2001 [173]
Prostate-specific antigen detection	Straub *et al.*, 2001 [174]
Diagnosis of breast carcinoma cells in peripheral blood	Aerts *et al*., 2001 [175]
Quantification of human herpesvirus 8, Kaposi's sarcoma; multicentric Castleman's disease	Boivin *et al.*, 2002 [176]
Analysis of low abundant point mutations in K-ras oncogenes	Wabuyele *et al.*, 2003 [177]
Hematologic neoplasia, human cytomegalovirus	Ohyashiki *et al.*, 2003 [178]
Molecular diagnosis of neuroblastoma	Cheung *et al*., 2003 [179]
Quantitative analysis of methylated alleles, retinoblastoma	Zeschnigk *et al.*, 2004 [180]
Prostate cancer identification	Jiang *et al*., 2004 [181]
Diagnostics of minimal residual disease; chronic myeloid leukemia, acute lymphoblastic leukemia	Pongers-Willemse *et al*., 1998 [182]; Preudhomme *et al*., 1999 [183]
Chronic myeloid leukemia	Khalil, 2005 [184]
Lung cancer, oncogene mutations	Schmiemann *et al.*, 2005 [185]
Acute respiratory syndrome, chronic myeloid leukemia colorectal cancer	Bustin and Mueller, 2005 [186]
Cutaneous melanoma	Lewis *et al.*, 2005 [187]
ATP-binding cassette transporters; cystic fibrosis; familial HDL deficiency; recessive retinitis pigmentosa, acute myeloid leukemia	Schuierer and Langmann, 2005 [188]
Thyroid cancer	Hesse *et al*., 2005 [189]
Allelic discrimination in prenatal diagnosis, single nucleotide polymorphism, cytokine gene expression	Arya *et al.*, 2005 [190]
Cancer diagnostics, non-Hodgkin lymphomas, B-cell lymphoma, follicular lymphoma	Stahlberg *et al.*, 2005 [9]
Human papillomavirus	Molijn *et al*., 2005 [191]
Rapid detection of Hippel-Lindau disease	Hoebeeck *et al.*, 2005 [13]
Normalization of gene expression measurements in tumor tissues	de Kok *et al.*, 2005 [192]
Application of RT-PCR to intraoperative cancer diagnostics	Raja *et al*., 2005 [193]

Virology

Majority of research using RT PCR has been made for detecting or quantifying viruses from viral infected human specimens. Various studies have provided protocols for detecting and quantifying viruses especially related to human diseases [15]. Detection of HSV1 and HSV2 was achieved by using TaqMan probes and it was in many ways alternative to conventional nested PCR assays [16]. Recently, a detection, quantification and differentiation between HSV1 and HSV2 genotypes were achieved using primers and probes (Light cycler) targeting HSV DNA polymerase gene [17]. Furthermore, genital herpes, which is the most common sexually transmitted disease (STD) around the world, accounts for 20 % of the STDs in United States alone [18]. RT PCR detection of HSV of genital and dermal specimens has also been well documented [19-25]. RT PCR showed superior sensitivity in detecting varicella-zoster virus compared to cell culture assays in dermal specimens [21, 26, 27]. Further RT PCR has been standardized for studying the interactions between virus and the host, which in turn can provide a reliable means to study the efficacy of antiviral compounds or to determine the chronic conditions [28, 29]. Immunodeficient patients tend to harbor several co-infections; under this, detection of multiple pathogens is essential for therapy (Table **6**). RT PCR multiplex assays have been developed for viral genotype differentiation [17, 30].

Bacteriology

Traditionally, initial antibiotic therapy was based on identifying the Gram stain classification. High variability that existed in identification of bacterial pathogens by mere observations was corrected by use of conventional PCR-based methods; later, this was further fastened by use of RT PCR. Fluorescence hybridization probes allowed a fast detection of low amounts of bacterial DNA and a correct Gram stain classification [31]. RT PCR has been shown as advantageous over other techniques (immunoassay or culture method) for detecting the bacteria irrespective of type of clinical specimen and especially those which are difficult to culture or slow growing. A quicker conformation of the pathogen will facilitate early prescription of appropriate antibiotics. Published accounts indicate that RT PCR was faster and sensitivity was greater or equal in some cases when compared to conventional methods.

Identification of mycobacterial infections earlier on certain occasions lacked specificity and sensitivity while em-

Table 6. Application of Real-Time PCR for Virus Diagnosis

Implications of RT PCR	Reference
Detection of Herpesvirus in central nervous system, genital and dermal regions	Ryncarz *et al*., 1999 [19]
Highly sensitive detection of Varicella-zoster virus from dermal specimens	Epsy *et al*., 2000b [21]
Detection and quantification of cytomegalovirus	Aberle *et al*., 2002 [194]
Epstein barr virus	Niesters *et al*., 2000 [195]
Enterovirus	Verstrepen *et al*., 2001 [196]
Polymavirus	Whiley *et al*., 2001 [197]
Parovirus	Schmidt *et al*., 2001 [198]
West nile virus	Briese *et al*., 2000 [199]
Respiratory viruses	Ward *et al*., 2004 [200]
Poxviruses	Espy *et al*., 2002 [201]
BK virus	Leung *et al*., 2002 [202]
Hepatitis virus	Costa-Mattioli *et al*., 2002 [203]
Parapoxviruses	Nitsche *et al*., 2006 [204]
Dengue virus	Chien *et al*., 2006 [205]
HIV	Desire *et al*., 2001 [206]
Rift Valley virus	Garcia *et al*., 2001 [207]
Parainfluenza virus	Hu *et al*., 2005 [208]
SAR associated coronavirus	Keightley *et al*., 2005 [209]
St Louis encephalitis virus	Lanciotti and Kerst, 2001 [210]
Denge virus serotype detection	Shu *et al*., 2003 [211]
Influenza virus serotype detection	Templeton *et al*., 2004 [30]

ploying conventional methods [32]. Mycobacterium species of common interest and so far detected as well as quantified by RT PCR include *Mycobacterium tuberculosis*, *M. avium*, *M. bovis*, *M. bovis* BCG, *M. abscessus*, *M. chelonae* and *M. ulcerans* [33-40]. Further, detection of antitubercular resistant isolates that were usually detected by broth dilution method have been replaced by RT PCR targeting mutant genes isoniazid (*katG*), rifampin (*rpoB*) and ethambutol (*embB*) from culture or clinical specimens [41-45].

Bacteria represent the potential agents for biological warfare. Some RT PCR assays (Light Cycler) have allowed the use of autoclaved samples for immediate detection of *Bacillus* species causing anthrax [46-47]. However, clinical studies are required to determine the usefulness of these tests for the rapid identification of this pathogen directly from human specimens.

Fungi

Major fungi causing infections in humans are *Aspergillus* species (*A. fumigatus, A. flavus, A. niger, A. nidulans, A. terreus, A. versicolor*), *Candida* species (*C. albicans, C. dubliniensis*), and *Pneumocystis jiroveci*. The conventional methods developed for detection of these infectious fungi are culturing, histopathology/phenotypic assays/biochemicals/ microscopy, conventional PCR, nucleic acid probe, CFU quantification, broth dilution and staining followed by microscopic observations. The efficacy of these methods seems to be slower on many occasions. The RT PCR for detecting and measuring the same proved to be faster on many instances irrespective of the clinical specimen [48-53]. Quantitative or qualitative RT PCR assays have also been developed for other fungi such as *Coccidioides* sp., *Conidiobolus* sp., *Cryptococcus* sp., *Histoplasma* sp., *Pneumocystis* sp., *Paracoccidioides* sp., and *Stachybotrys* sp. [54-61].

Protozoa

Molecular biology (and particularly PCR) has been increasingly used for the diagnosis of parasitic protozoa of medical interest [62]. RT PCR and other technical improvements in the past decade permit precise quantification and routine use for the diagnosis facilitating the study of parasitic populations, although the use of this method for malaria remains limited due to high cost [62]. RT PCR assays for clinical application have been described for detecting amoebic dysentery [63], chagas' disease [64], cutaneous and visceral leishmaniasis [65], giardiasis [66], *Cyclospora cayetanensis* [66] causing prolonged gastroenteritis [67], toxoplasmosis in the amniotic fluid of pregnant women [68], and in immuno-compromised patients [69]. Protozoans cause several diseases, which are endemic in large parts of the world. Further genome sequencing efforts are requested as many parasitologists work on organisms whose genomes have been only partially sequenced and where little, if any, annotation is available [70].

Veterinary

Viruses

Animal models have served investigators from decades to understand several biological functions of humans including disease diagnosis and to take appropriate measures for therapy. The development of quantitative reverse transcription-PCR, such as RT RT-PCR techniques, approach theoretical limits of per reaction sensitivity, further increments in the sensitivity of measurements of the pathogens [71-72]. Infection of domestic cats with the feline immunodeficiency virus (FIV) results in a fatal immunodeficiency disease, and is similar to the human immunodeficiency virus 1 (HIV-1) in humans. This has helped the progress of in-depth research on this morphologically and genetically resembling virus especially in development of candidate vaccines. Highly sensitive detection and quantification assays have been developed by RT PCR methods for this virus [71, 73]. Simian immunodeficiency virus (SIV) detection was earlier done by branched-chain DNA assay that was quite expensive, but with low sensitivity (1500 viral RNA copies/ml). Leutenegger and co-workers developed a TaqMan RT RT-PCR assay which could detect with higher sensitivity (50 viral RNA copies/ml) [74]. Feline coronavirus (FcoV) is known to be more prevalent in cat population and is a fatal infectious disease. Control measures include detection as well as separation of infected populations or vaccination. A reliable absolute quantification real-time TaqMan probes were designed to detect important laboratory and field strains of FcoV by Gut and co-workers [75]. Further, tick-borne zoonotic pathogens are well known in many areas all over the world [76]. Clinical diagnosis of tick-borne diseases is difficult due to unusual clinical signs. Early diagnosis and treatment is necessary to prevent fatal infections and chronic damage to various tissues. A series of new projects in this area have yielded detection and quantification methods for important tick borne pathogens [77-79].

Other studies on various aspects of veterinary science have been performed using RT PCR for instance, effects of viral infections on neural stem cell viability [80], detection of several viruses [81-83], innate immune responses to virus infection [84], factors influencing viral replication [85], gene expression profiling during infection [86], characterization of viruses [87] are a few to mention.

Bacteria

Insects tend to harbor *Corynebacterium pseudotuberculosis* and are responsible for the disease spread in dairy farms [88]. An investigation on identification of insect vectors spreading *Corynebacterium pseudotuberculosis* by TaqMan PCR assay (*PLD* gene) supported the hypothesis that this pathogen may be vectored to horses by *Haematobia irritans*, *Stomoxys calcitrans*, and *Musca domestica*. The organism can be identified in up to 20 % of houseflies in the vicinity of diseased horses [89].

Mycoplasma

The prevalence, clinical manifestations, and risk factors for infection for all three feline hemoplasma species were performed by Willi and co-workers [90]. Diagnosis, quantification, and follow-up of hemoplasma infection in cats were performed using three newly designed sensitive RT PCR assays. Efficacy Marbofloxacin drug was studied in cats against *Candidatus Mycoplasma haemominutum*, which revealed decreased copy number of the pathogen and no correlation was evident on *Candidatus Mycoplasma haemominutum* in chronic FIV infection [91-92].

Food Microbiology and Safety

Mycotoxins are the major food contaminants and they have become a great concern worldwide due to their several ill effects [93]. In order to overcome this problem, a rapid, cost-effective, and automated diagnosis of food-borne pathogens throughout the food chain continues to be a major concern for the industry and public health. An international expert group of the European Committee for Standardization has been established to describe protocols for the diagnostic detection of food-borne pathogens by PCR [94]. A standardized PCR-based method for the detection of food-borne pathogens should optimally fulfill various criteria such as analytical and diagnostic accuracy, high detection probability, high robustness (including an internal amplification control [IAC]), low carryover contamination, and acceptance by easily accessible and user-friendly protocols for its application and interpretation [95]. RT PCR has the potential to meet all these criteria by combining amplification and detection in a one-step closed-tube reaction. A high throughput identification of *Fusarium* at genus level or distinguishing species [96-97] has been published. *Salmonella*, one of the most common causes of food-borne disease outbreaks due to its widespread occurrence and several sources have been known to harbor this pathogen [98]. A duplex real-time SYBR Green LightCycler PCR (LC-PCR) assay was developed for 17 food/water borne bacterial pathogens from stools by Fukushima and co-workers [99-100]. The pathogens examined were enteroinvasive *Escherichia coli*, enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Aeromonas* spp., *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus cereus*, *Plesiomonas shigelloides* and *Providencia alcalifaciens*. Further, detection assays for *Clostridium botulinum* applicable to both purified DNA and crude DNA extracted from cultures and enrichment broths as well as DNA extracted directly from clinical and food specimens were developed [101]. Similarly, RT PCR has been used to quantify the food-borne pathogen *Listeria monocytogenes* by first incorporating an IAC [102].

Food borne viral infections are one of the leading diseases in humans worldwide. Currently over two billion people have evidence of previous Hepatitis B virus infection and 350 million have become chronic carriers of the virus [103]. Successful detection of this virus from serum and plasma, by RT PCR has been developed. This method is useful for monitoring the efficacy of Hepatitis B virus therapy and screening human population in endemic areas. Other important food borne viruses quantified by this technique are Rotavirus [104] and gastroenteritis virus [105]. However, detection or quantification of these viruses directly from various types of food samples seems to be a difficult task.

Forensic Science

Advanced technologies for DNA analysis using short tandem repeats (STR) sequences has brought about a revolution in forensic investigations. One of the most common methods used is PCR, which allows accurate genotype information from samples. Forensic community relied on slot blot technique which is time consuming and labor intensive. RT PCR has become a well-recognized tool in forensic investigations. Improved amplification and quantification of human mtDNA was accomplished by monitoring the hypervariable region (HV1) using fluorogenic probes, and the same study was also extended to discriminate sex. A duplex RT qPCR assay was developed for quantifying human nuclear and mitochondrial DNA in forensic samples and this method also was efficient for highly degraded samples [106]. Repetitive *Alu* sequence based RT PCR detection has been developed and have proved to be advantageous compared with other methods with detection limits as low as 1 pg [107]. MGB Eclipse primers and probes as well as QSY 7-labeled primer PCR method have been designed for *Alu* sequence [108-109]. Similarly, RT PCR assays to quantify total genomic DNA and identify males from forensic samples with high efficiency have been standardized [110]. Recently, human DNA quantifier and qualifier kits have been developed and validated. The efficiency was either comparable or superior to methods available [111]. Forensic samples are often contaminated with PCR inhibitors and DNA extraction methods fail to exclude the contaminants. A computational method that allows analysts to identify problematic samples with statistical reliability was standardized by using tannic acid and comparing the amplification efficiencies of unknown template DNA samples with clean standards [112]. Further, methods have also been standardized for assessing the DNA degradation in forensic samples [113].

Environmental Issues

RT PCR is a convenient method for detection of the mobility of genetic elements. The worldwide increasing environmental pollution is pressing us to find new methods for elimination of undesirable chemicals. The application of microorganisms for the biodegradation of synthetic compounds (xenobiotics) is an attractive and simple method. Unfortunately, the majority of these pollutants are chemically stable and resistant to microbial attack. The isolation of new strains or the adaptation of existing ones to the decomposition of xenobiotics will probably increase the efficacy of microbiological degradation of pollutants in the near future. The widespread application of combined techniques using microbiological decomposition and chemical or physical treatments to enhance the efficacy of the microbiological decomposition can also be expected. The cloning and expression in *Escherichia coli* of an 'azoreductase' from various species have been reported (Table **7**). The exoenzymes of white-rot fungi have also been objects of genetic engineering. The laccase of various filamentous fungi was successfully transmitted into yeast. These manipulations enhanced the capacity of microorganisms to decompose polyaromatic compounds (PAC).

The expression of oxidases from higher plants augmented the catabolic potential of microbes [114] and in turn microbial genes straightened the tolerance of higher plant to Poly R-487 [115-116]. Plants tolerant to PACs may be useful in phytoremediation because they could provide a rhizosphere that was suitable for colonization by microbes that are efficient degraders of aromatic structures. Moreover, the plant derived compounds can induce production of fungal redox

Table 7. Improvement of Deteriorative Activity of Organisms by Interspecific Transfer of Genetic Elements

Organisms		Function	References
Donor	**Acceptor**		
Prokaryotes			
Clostridium perfringens	*Escherichia coli*	Azoreductase	Rafii and Coleman (1999) [212]
Bacillus sp.	*E. coli*	Azoreductase	Suzuki *et al.* (2001) [213]
Rhodococcus sp.	*E. coli*	Azoreductase	Chang and Lin (2001) [214]
Xenophilus azovorans	*E. coli*	Azoreductase	Blumel *et al.* (2002) [215]
E. coli	*Sphingomonas xenophaga*	Flavin reductase	Russ *et al.* (2000) [216]
Agrobacterium rhizogenes	*Mentha puligeum*	Tolerance to R–478	Strycharz and Shetty (2002) [115]
Eukaryotes			
Geotrichum candidum	*Aspergillus oryzae*	Peroxidase	Sugano *et al.* (2000) [217]
Ceriporiopsis subvermispora	*A. nidulans*	Peroxidase	Larrondo *et al.* (2003) [218]
C. subvermisopra	*A. oryzae*	Peroxidase	Larrondo *et al.* (2001a) [219]
Coprinus cinereus	*Saccharomyces cerevisiae*	Laccase	Cherry *et al.* (1999) [220]
C. cinereus	*A. oryzae*	Laccase	Schneider *et al.* (1999) [221]
Coriolus versicolor	*Nicotiana tabacum*	Peroxidase	Iimura *et al.* (2002) [116]
Phanerochaete chrysosporium	*A. nidulans*	Peroxidase	Larrondo *et al.* (2001b) [222]
Pycnoporus cinnabarinus	*Pychia pastoris*	Laccase	Otterbein *et al.* (2000) [223]
P. cinnabarinus	*A. niger*	Laccase	Record *et al.* (2002) [224]
Pleurotus sajor-caju	*P. pastoris*	Laccase	Soden *et al.* (2001) [225]
Trametes versicolor	*S. cerevisiae*	Laccase	Larsson *et al.* (2001) [226]
T. versicolor	*P. pastoris*	Laccase	O'Callaghan *et al.* (2002) [227]
T. versicolor	*P. pastoris*	Laccase	Hong *et al.* (2002) [228]
Armoracia rusticana	*S. cerevisiae*	Peroxidase	Morawski *et al.* (2001) [114]

enzymes. The C-hydroxylation of aromatic rings by mammalian monoxygenases facilitates subsequent microbial degradation. Human cytochrome P450 enzymes are now routinely expressed as recombinant proteins in many different systems [117-118]. The capacity of such recombinants to catabolize PACs has been tested. It is clear that complexity of association involved in the complete degradation should be increased with increasing complexity of the chemical structure of xenobiotics. The genetically engineered microorganisms can accomplish degradation of xenobiotics, which persist under normal natural conditions. In natural habitats, complex microbial/macrobial communities carry out biodegradation. Within them, a single organism may interact through inter-specific transfer of metabolites. This co-metabolic potential may be complementary so that extensive biodegradation or even mineralization of xenobiotics can occur [119]. In this respect, deterioration of industrial and municipal effluents in constructed wetlands with multi-site catabolic potential is a promising possibility. Mobilizing specific genes, encoding nonspecific multifunctional degradative sequences, may decisively increase the degradative potential of natural synthropic community against synthetic pollutants and persisting natural toxins. The use of recombinants that harbor deteriorating determinants from other species can essentially enhance the capacity of remediation technologies. However, the widespread use of genetically modified organism needs continuous survey of gene transmission, and for that RT PCR is a plausible and rapid method.

Plant

Validation of Microarray Results

RT PCR has been employed to study the gene expression patterns during several stresses leading to activation of genes relating to signal transduction, biosynthesis, and metabolism. Nitrogen deprivation response in *Arabidopsis* was analyzed by profiling transcription factors using Affymetrix ATH1 arrays and a RT RT-PCR platform [1, 120]. The results revealed large number of differentially expressed putative regulator genes. In this study, MapMan visualization soft-

ware was used to identify coordinated, system-wide changes in metabolism and other cellular processes. Similarly, Czechowski and co-workers have profiled of over 1,400 *Arabidopsis* transcription factors, and revealed 36 root and 52 shoot specific genes [121]. Further, gene expression studies have been made in the direction of stress signaling during biotic and abiotic stress conditions in plants [122-127]. Standardization of house-keeping genes for such studies has been made in potato. Among the seven common genes tested, *ef1alpha* was the most stable gene during biotic and abiotic stress [128]. Furthermore, the data obtained by microarray analysis are questioned on few instances and confirmation is achieved by RT PCR (or conventional PCR in some instances). The expression levels observed in microarray is generally higher compared to measurement by RT PCR [129]. In general, studies made so far reveal a good relationship between these two techniques, and for this reason RT PCR is considered as confirmatory tool for microarray results [130].

Plant-Microbe Interaction

Host plant and associated microbes form a special consortium where the parasite is an alien element. Early diagnosis of the pathogens can provide rapid and suitable measurements for limiting the epidemics and selection of appropriate control measures. Molecular diagnostics is a rapidly growing area in plant pathology especially for detection and quantification of commercially important crop pathogens. As a novel methodology, adoption of RT PCR technique is of growing interest due to its rapidity and sensitivity as well as its ability to detect minute amounts of the pathogen's DNA from infected plant tissues and insect vectors [131]. Simultaneous detection of several pathogens can be achieved by multiplex PCR. The technique has aided detection of pathogens associated with serious diseases like *Fusarium* head blight, which is a prerequisite for reduction in the incidence by understanding of its epidemiology [97]. Several reports are available on detection and/or quantification of plant pathogens (Table **8**). Published literature reveals quantification of pathogens [132-133], determination of symbiotic microbes and pathogens [134], detection/quantification of seed borne pathogens [135], host resistance screening [136] and distinguishing between pathogen pathovars [137-138] using RT PCR.

Species Identification

In plants, the presence of such a large number of multiple copies within each gene family complicates the clear understanding of function of each member. Plant molecular biologists prefer RT PCR methods to other methods and the number of findings is increasing at high rate. The northern blotting determination of genes expressed at lower levels is difficult and closely related genes may cross-hybridize [139]. Both unique and redundant functions within a multigene family have been identified [140-142]. Expression analysis of all members (33 genes) encoding cell-wall enzymes in *Arabidopsis thaliana* using RT PCR revealed that most members exhibited distinct expression profiles along with redundant expression patterns of some genes [143]. Similarly, an expression profile for shaggy-like kinase multigene family during plant development has also been made using this technique [144]. Further, transformants with high number of copies lead to lower or unstable gene expression of inserted gene. Primary transformants are analyzed for randomly inserted gene copy number. A study using duplex RT PCR has also been described for determining the transgene copy number in transformed plants with high degree of correlation with southern blot analysis [145]. Likewise, many studies are available on detection of copy number using RT PCR in various crops [146-147].

CONCLUSIONS

RT PCR is becoming a common tool for detecting and quantifying expression profiles of desired genes. The review itself indicates that the technology to detect PCR products in real-time, i.e., during the reaction, has seen a dramatic leap in use and application over the past couple of years. The PCR based detection technologies utilizing species- specific primers are proving indispensable as research tools providing enhanced information on biology of plant-microbe interactions with special regard to the ecology, aetiology and epidemiology of plant pathogenic micro-organisms. The RT PCR allows quantitative genotyping and detection of single nucleotide polymorphisms and allelic discrimination as well as genetic variation. The use of multiplex PCR systems using combined probes and primes targeted to sequences specific to counterpartners of plant/microbe associations is becoming more important than standard PCR, which is proving to be insufficient for such living systems. Application of RT PCR combined with other molecular techniques made possible the monitoring of both therapeutic intervention and individual responses to drugs. Developments in bioinformatics helped to understand how the genome gives rise to different cell types, how it contributes to basic and specialized functions in those cells and how it contributes to the ways cells interact with the environment. RT PCR is a valuable methodic tool in clarifying such problems. The needs in clinical application of molecular methods initiated important developments in diagnostics stimulating progress in other branches of science. The introduction of these new methods in other fields of human practices induced rapid expansion of molecular approaches.

CHALLENGES

Plants and animals use small RNAs (microRNAs [miRNAs] and siRNAs) as guides for post-transcriptional and epigenetic regulation. The microRNAs (miRNAs) were initially considered a biological sideshow, the oddly interesting regulators of developmental timing genes in *Caenorhabditis elegans*. But in the past few years, studies have shown that miRNAs are a considerable part of the transcriptional output of the genomes of plants and animals. Therefore these miRNAs play important regulatory functions in widespread biological activities. Accordingly, miRNAs are now recognized as an additional layer of post-transcriptional control that must be accounted for if we are to understand the complexity of gene expression and the regulatory potential of the genome. Owing to this impressive progress in understanding the genomics and functions of miRNAs, we think this is an ideal time to examine the available evidence to see where this rapidly growing field is going. The small RNA repertoire in plants is complex, and few known about their function that constitute new challenges [148].

Table 8. Plant Pathogens/Pests Determined by Quantitative Real-Time PCR

Pathogen	Host	Reference
Clavibacter sepedonicus	Potato tubers	Schaad *et al*., 1999 [229]
Ralstonia solanacearum	Potato tubers	Weller *et al*., 2000 [230]
Acidovorax avenae subsp. citrulli	Watermelon	Randhawa *et al*., 2001 [231]
Agrobacterium strains	Several plants	Weller and Stead, 2002 [232]
Xylella fastidiosa	Grape vine	Schaad and Fredrick; 2002 [1]
Erwinia amylovora	Apple	Salm and Geider, 2004 [233]
Spongospora subterranea	Potato	van de Graaf *et al*., 2003 [234]
Synchytrium endobioticum	Potato	van den Boogert *et al*., 2005 [153]
Fusarium solani f. sp. phaseoli	Soil-french beans	Filion *et al*., 2003 [235]
Ophiosphaerella narmari	Bermuda grass	McMaugh and Lyon, 2003 [122]
Phytophthora infestans	Potato	Avrova *et al*., 2003 [236]
Verticillium dahliae	oliva tree	Mercado-Blanco *et al*., 2003 [237]
Alternaria brassicicola	*Arabidopsis*	Gachon and Saindrenan, 2004 [238]
Botrytis cinerea	*Arabidopsis*	Gachon and Saindrenan, 2004 [238]
Fusarium solani f. sp. glycines	Soybean	Gao *et al*., 2004 [239]
Phytophthora ramorum	Sudden oak	Hayden *et al*., 2004 [240]; Tomlinson *et al*., 2005 [241]
Tilletia spp.	Wheat	Eibel *et al*., 2005 [242]
Biscogniauxia mediterranea	Oak	Luchi *et al*., 2005 [243]
Fusarium oxysporum f. sp niveum	Melon and soil	Zhang *et al*., 2005 [244]
Mycosphaerella melonis	Melon and soil	Zhang *et al*., 2005 [244]
Oculimacula sps.	Wheat	Walsh *et al*., 2005 [245]
Thrips palmi	melon	Walsh *et al*., 2005 [246]
Candidatus Liberobacter species	citrus	Li *et al*., 2006 [247]
Heterobasidion annosum	Spruce	Bodles *et al*., 2006 [136]
Xanthomonas campestris	Brassicas	Berg *et al*., 2006 [137]
Phytophthora ramorum	*Parrotia persica*	Tomlinson *et al*., 2005 [241]
Biscogniauxia nummularia	*Fagus sylvatica* L.	Luchi *et al*., 2006 [248]
Puccinia coronata	Barley	Jackson *et al*., 2006 [249]
Candidatus Phytoplasma americanum	Potato	Crosslin *et al*., 2006 [131]
Potato yellow vein virus	Potato	Lopez *et al*., 2006 [250]

Research has focused on approaches to detect the presence of miRNAs and their impact on genomes, and the roles they play in regulating biological functions had been explored. Studies generally followed a progressive logic from discovery to target prediction to function to systems perspective and finally to organism perspective.

Plant and animal genomes have been shaped by miRNAs, as seen by the substantial number of conserved miRNAs that have accumulated through selection and the presence of miRNA target sites in genes of diverse functions. However, the true number of miRNAs and targets remains difficult to estimate. In plants, miRNAs and trans-acting (ta) siRNAs form through distinct biogenesis pathways, although they both interact with target transcripts and guide cleavage [149]. Developments in bioinformatics requested for correct definition a 'true' miRNA and the implications this definition will have for future studies. Approaches to the prediction of targets of miRNAs consider the case for combinatorial control of target expression by multiple miRNAs acting synergistically. Some of the fundamental goals of investigations into

genome function are to understand how the genome gives rise to different cell types, how it contributes to basic and specialized functions in those cells and how it contributes to the ways cells interact with the environment. RT PCR is a valuable methodic tool in clarifying such problems. One has to take a systems approach to conceptualize a network of interacting miRNAs and targets and might be supposed that miRNAs act to canalize developmental gene expression programs through ontogeny on both unicellular and multicellular organisms. The topology of this network resembles that mapped previously in yeast, reinforcing the idea that similar networks may underlie the genetic basis of complex human disease. Recent breakthrough discovery by Rigoutsos and co-workers of self-similar, repetitive elements (what they call "Pyknons") throughout the coding- as well as non-coding "Junk" DNA elevates the question how the novel findings relate to fractality of the DNA as well as opens question on fractal hierarchies of complex organization of genes and non-genes [150]. These unexpected findings suggest functional connections between the coding and noncoding parts of the human genome. Some recent data provide evidence for roles of miRNAs encoded in pathogen and host cell in influencing the cell-type specificity of their interaction. The miRNAs from an organismal perspective and other endogenous regulatory RNAs in plants might have diverse biological roles in realization of both developmental programs and stress responses. There are several instances of polymorphism influence on human disease progression but no definitive answer has yet to be obtained. However, no data was found in plant-microbe interactions. Most heritable traits, including disease susceptibility, are affected by interactions between multiple genes. However, we understand little about how genes interact because very few possible genetic interactions have been explored experimentally.

A genome-wide association approaches to map the genetic determinants of the transcriptome in established host/parasite complexes and microbial populations associated to plants. The concept, that genes and non-genes comprise fractal sets, determining the ensuing fractal hierarchies of complexity of biological processes undoubtedly helps to analyze enormous sets of data obtained by RT PCR on functional expression of genes. Although algorithms for discovery of generic motif in sequential data represent an extremely valuable tool for data analysis, the emergence of informatic market makes difficulties as patent applications back out of scientific disputation on these new methods in large scale [151]. Nevertheless, one can assume that application of this approach to plant-microbe interactions will accelerate evolution of our imaginations about this matter and initiates elaboration of new theory of plant pathology. Also, the organization of microbial consortia and their functional interaction with macrobial partners can be evaluated in whole complexity basing on this new concept.

The genes might also serve as therapeutic agents. The use of alien toxin as well as detoxifying enzyme-coding genes led to promising economic results in plant cultivation. Sequencing of the genomes of a number of model organisms provides a strong framework to achieve this goal. Several methods, among which gene expression profiling and protein interaction mapping, are being used on a large-scale basis, and constitute useful entry points to identify pathways involved in disease mechanisms. The requested time for clarification of these processes can be shortened by applying RT PCR.

The methods relying on the genetic manipulation of well-characterized and simple models of host/parasite systems (HPS) to reconstruct disease-associated pathways can pinpoint biologically-valid therapeutic targets on the basis of function-based datasets generated *in vivo*. The HPSs are strongly complementary to well-established complex models, and multiple ways exist to integrate these results into the early stage of the drug discovery process.

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