



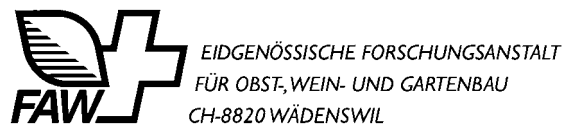
Cost Action 853

Agricultural Biomarkers for Array Technology

**Management Committee Meeting
and combined
Meeting of Working Groups 1 and 3**

September 26 – 28, 2002

Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture
Wädenswil, Switzerland



Agenda

Thursday, September 26

13:30 Registration and Welcome

14:00 Meeting of Working Group 3 “Bio-Informatics and Information Dissemination”

14:00 – 14:30 **Zlatko Trajanoski**

Information management systems for functional genomics

14:40 – 15:10 Expert 1: **Hubert Charles**

ROSO: A software to search optimized oligonucleotidic probes for microarrays

15:20 – 16:00 Expert 2: **Ulrich Wagner**

Concepts for biocomputing in a multiuser environment

16:00 – 16:30 Coffee Break

16:30 – 17:00 **Jürg E. Frey**

Towards the Random Chip – A Biologist’s Approach to Bioinformatics

17:10 – 17:30 **Patrick DeMarta**

Oligonucleotide probe development for diagnostic microarrays based on sequence alignments

17:40 – 18:00 Discussion

18:00 End of Working Group 3 Meeting

18:00 – 19:00 Management Committee Meeting, Part 1 of 3:

1. Opening and welcome by the Chairman Jürg E. Frey
2. Introduction of new Delegates
3. Approval of the MCM Draft Agenda
4. Approval of the Minutes of the first MCM held in Brussels, 7/8 March 2002

19:00 Closing

20:30 Dinner at the “alti Fabrik”

Friday, September 27

08:30 – 08:40 Meeting of Working Group 1 “Nucleic-Acid based Microarrays”; Peter Bonants

08:40 – 09:00 **Cor Schoen**

Use of a novel 3D microarray flow through system for plant pathogen multiplex detection

09:00 – 09:20 **Levente Bodrossy**

Diagnostic microarrays in microbiology

09:20 – 09:40 **José E. Perez-Ortin**

Microarray methods for the detection of pathogenic bacteria and viruses in plants

09:40 – 10:00 **Jens Sobek**

Towards the random chip: Establishing the practical parameters for a new versatile microarray

10:00 – 10:20 Coffee Break

10:20 – 10:50 Expert 1: **Ralph Schlapbach**

Microarraying in the context of functional genomics

10:50 – 11:20 Expert 2: **Giuseppe Firrao**

Exploring new strategies in loading, attachment and detection to lower the costs of DNA array work

11:30 – 13:30 Lunch at the University of Applied Sciences Wädenswil

13:30 – 14:00 Expert 3: **Wolfgang Ludwig**

ARB, a graphically oriented softwarepackage comprising various tools for sequence database handling and data analysis

14:00 – 14:20 **Neil Boonham**

Detection of potato viruses using microarrays

14:20 – 14:40 **Knut Rudi**

Application of sequence-specific labelled DNA probes in combination with array hybridization for fingerprinting and microbial community analyses

14:40 – 15:00 **Peter Bonants**

Multiplex detection of plant pathogens by microarrays: An innovative tool for plant health management

15:00 – 15:15 **Gustavo Nolasco**

Typing of Citrus tristeza virus strains by plate hybridization with panel of probes

15:15 – 15:30 **Mogens Nicolaisen**

DNA chip diagnostics: related work at Danish Institute of Agricultural Sciences

15:30 – 15:50 Coffee Break

15:50 – 16:05 **Quirico Migheli**

Identification of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *A. parasiticus* by RT-PCR

16:05 – 16:20 **Miroslav Sip**

Parallel detection of potato pathogens: Possibilities and problems

- 16:20 – 16:35 **Joanna Pulawska**
Identification of some economically important pathogens of fruit trees, shrubs and ornamental plants
- 16:35 – 16:50 **Karel Petrzik**
The most important fruit tree viruses in the Czech Republic
- 16:50 – 17:05 Expert 4: **Jana Boben**
Exploring Real Time PCR and microarray in plant virus research
- 17:05 – 17:30 Discussion
- 17:30 End of Meeting of Working Group 1
- 17:30 – 18:30 Management Committee Meeting, Part 2 of 3:
5. Report of the WG-Coordination on their activities since our last meeting
 - a. Nucleic-Acid Based Microarrays, Peter Bonants
 - b. Protein-Based Microarrays, Ian Barker
 - c. Bio-Informatics and Information Dissemination, Peter von Rohr
 - d. Chip Production and Analysis, Dietmar Blohm
 - e. Microarray Technology for Environmental Monitoring, Xavier Nesme
- 18:30 Closing

Dinner on your own

Saturday, September 28

08:30 – 12:30 Management Committee Meeting, Part 3 of 3

MCM Draft Agenda

6. Information on planned activities for the next 12 months period
7. Status of the Action
8. Short Term Scientific Missions
9. New research activities
10. Place and date of next meeting
11. Other topics

12:30 End of Management Committee Meeting of COST Action 853

For those interested:

15:30 – 16:20 Boat trip to Rapperswil

Abstracts

INFORMATION MANAGEMENT SYSTEMS FOR FUNCTIONAL GENOMICS

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While the principles underlying microarray expression analysis are relatively simple, the challenges associated with examining expression levels for thousands of genes in a single experiment, and of presenting those data in a usable form are substantial. Further, the true value of the data lies not in the result of any single experiment, but rather, in examining correlations across multiple experiments. Developing an accurate interpretation of expression levels from microarrays requires the development of genomic information management system capable of effectively capturing the data as well as tools to make that data accessible to the laboratory scientist. To that end, we suggest a software development program aimed at creating a system that makes microarray expression analysis accessible to a wide range of users. Primary development is in Java so that, to the greatest extent possible, the software has cross-platform compatibility. The components of the system are:

- 1) Analytical pipeline including modules for image processing, cluster analysis, promoter analysis, as well as tools for functional predictions.
- 2) Data warehouse including tools for storing and retrieving microarray data.

In order to fully exploit and leverage the data generated by expression profiling using microarrays, it is further necessary to integrate expression data with phenotype, genotype, and information including tissue distribution and time course expression data gleaned from previous studies. Currently used tools and methods will be highlighted as well as future developments and challenges.

ROSO: A SOFTWARE TO SEARCH OPTIMIZED OLIGONUCLEOTIDIC PROBES FOR MICROARRAYS

Nancie REYMOND¹, Hubert CHARLES¹, Guillaume BESLON², Laurent DURET³ and Jean-Michel FAYARD¹.

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Microarrays are powerful tools for analyzing and understanding genome functionality. To run microarray experiments, one needs to calibrate many consecutive steps in order to avoid variability and to allow experiment comparisons. In this work, we have developed ROSO (“logiciel de **R**echerche et **O**ptimisation de **S**ondes **O**ligonucléotidiques”), a software devoted to design optimal oligonucleotide long probes (> 30 bp) for microarrays.

ROSO allows users to choose the type of probes, their size and localization on the gene target, the number of probes per gene target, as well as different hybridization parameters such as ion concentrations, melting temperature range and threshold for secondary structure rejection. ROSO is a web tool accessible at the following URL (<http://pbil.univ-lyon1.fr/roso/loadWkgFiles.php>).

Probe optimization process is based on four successive key steps:

1. **Probe specificity.** Specificity is calculated by comparing probes to the overall studied set of genes (internal genes), but also by comparison with an external set of genes (any genes user want to avoid any cross-hybridization with). Specificity is calculated with Blast program. Blast parameters were estimated to detect a minimal homology of 70 % on 20 nucleotides length.
2. **Probe secondary structures** (hairpin and homoduplex). Stability of secondary structures is calculated with the thermodynamic model of nearest-neighbor.
3. **Probe melting temperature (Tm).** Tm is calculated with the thermodynamic model of nearest-neighbor. When at least one probe is found for each gene, ROSO keeps the set of optimal probes with the smallest possible Tm variability.
4. **Stabilizing criteria.** When multiple probes are found for one target gene, stability criteria (GC rate, GC clamp...) are calculated and allow to find the best probe. Moreover, it allows users to calculate Tm of control probes with mismatches.

Different kinds of validation were performed. First, simulated data have allowed for the comparison with the reference software Oligo6® and Mfold. ROSO estimations of Tm and secondary structures were found to be equivalent or better than Oligo6® and Mfold estimations, for probes size comprised between 15 and 70 nucleotides. Second, ROSO was used to design two sets of 541 and 609 probes for specific bacterial microarrays corresponding to *Buchnera aphidicola* and *Ralstonia solanacearum*. Human and murine probe sets were also designed.

The work is conducted in collaboration with UMR 5558 (UCB Lyon) and with support of the Genopôle Rhône-Alpes.

A few other web tools...

Tm calculation

<http://www.cbs.dtu.dk/services/DNAarray/probewiz.html>
<http://www.nwfsc.noaa.gov/protocols/oligoTmcalc.html>
<http://www.anachem.co.uk/public/new-products/hybsimulator/default.asp>
http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
<http://bioweb.pasteur.fr/seqanal/interfaces/melting.html>

Probe and primer design:

<http://berry.engin.umich.edu/oligoarray/index.html> (JM Rouillard)
<http://arrayit.com/Services/ArrayDesign/arraydesign.html>
<http://ural.wustl.edu/~lif/probe.pl>
<http://www.labvelocity.com/jellyfish/index.jhtml>

Secondary structures:

<http://bioinfo.math.rpi.edu/~zukunft/>

CONCEPTS FOR BIOCOMPUTING IN A MULTIUSER ENVIRONMENT

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With the extremely fast development of the microarray technology, the amount of gene expression and other functional gene data resulting from microarray experiments is increasing in a likewise speedy manner. In spite of the fact that important discoveries have already been made with the use of microarray experiments, there is a lack of standards for the representation and even more importantly for the exchange of microarray data. One of the reasons for this can be seen in the fact that research that is carried out making use of microarray experiments is a relatively new field. Furthermore, gene expression data are rather complex, as they only make sense when being seen in connection to the experimental conditions. Thus, it has taken some time to gather experience on the levels of importance of the different aspects of microarray experiments.

In a research environment with a multitude of users, on the one hand there exists also a multitude of annotation schemes for the respective data, which are published in different formats. On the other hand, there exists a natural interest to exchange data in order to e.g. complement own research aspects. Therefore, it is highly important to achieve a general agreement on the minimal amount of information that is needed in order to correctly interpret the microarray data and to check the derived results. The Microarray Gene Expression Data group (MGED) has proposed the document MIAME (Minimum Information About a Microarray Experiment) to define this minimum information and to give an answer to the need of comprehensive annotation necessary to interpret microarray data. Besides a general agreement on the nature of information it is also highly important to agree on a definition of a controlled vocabulary (e.g. by adapting to certain ontologies) as well as a standardized data format (e.g. by adapting to XML-derived formats like MAGE-ML) that is needed to efficiently exchange data and even to create standardized data bases like the *Arrayexpress* repository for microarray data designed by the EBI. So far, the MIAME document has been proposed as a starting point for a broader community discussion and hopefully will result in a general agreement.

TOWARDS THE RANDOM CHIP – A BIOLOGIST'S APPROACH TO BIOINFORMATICS

Jürg E. FREY

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The microarrays designed to use for diagnostic purposes are generally based on well described sequences of nucleic acid. They exploit the variation within this DNA/RNA-fragment that was previously shown to be low within a taxon and high between different taxa of interest. This approach has disadvantages in that it requires developing new probes for new groups of organisms and that it only samples information from a small fraction of the genome.

The idea of the random chip is to use many short oligonucleotides of random sequence and to check the DNA/RNA of each of the taxa for presence or absence of the corresponding inverse sequence of each of the short oligonucleotides. The composition of short oligonucleotides with respect to presence/absence is different for different taxa and this generates a taxon-specific pattern on a chip upon hybridisation with labelled primers.

To test if the idea of a random chip can actually work requires software to query the genomes of several fully sequenced organisms. Furthermore, to develop a random chip requires generation of large numbers of short primers and probes with random DNA sequence that must comply with several requirements such as identical T_m , no inverse sequences, no hairpin formation etc. I describe how I addressed these challenges and which problems remain to be solved.

ALISCAN. AN INTERACTIVE TOOL TO ASSIST THE DESIGN OF SEQUENCE ALIGNMENT-BASED PROBES

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The use of DNA arrays for diagnostic purposes introduces problems which were unknown to expression studies. Probes should be taxon-specific rather than gene-specific. The presence of within-taxon variability requires that probes are developed in regions which are conserved within each taxon in order to provide consistent detection of every individual.

However, such regions should be variable enough to allow clear-cut differentiation between the taxa to be discriminated. Intensive work on sequence alignments is therefore needed in order to identify the regions which are the most suitable for probe development.

AliScan is an interactive and flexible tool developed to assist the design of oligonucleotide probes from sequence alignment. The user is requested to input an alignment in FASTA format and to arrange sequences into different groups. The software helps the user to identify the regions in an alignment which are the most promising for group-specific probe development, using colour codes to show scores associated to each nucleotide of the sequence alignment. Scores are calculated by formulas which could be completely user defined and which can take into consideration the consensus within the group, the presence of a given base in other groups, base ambiguities, probe degeneracy and gaps.

USE OF A NOVEL 3D MICROARRAY FLOW THROUGH SYSTEM FOR PLANT PATHOGEN MULTIPLEX DETECTION

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Detecting harmful organisms in plant propagation material is necessary in order to ensure safe and sustainable agriculture. If different pathogens need to be detected simultaneously, this approach is difficult and costly.

The newest development in analysis of nucleic acids is the microarray technology, in which many different oligonucleotides can be spotted on little more than one square mm. In this way various target molecules can be detected in the same sample with increased specificity and meet the demands for multiplex detection methods of different plant pathogens. The use of these micro-fabricated DNA analysis tools will provide the next generation of inexpensive DNA diagnostics to measure different pathogens in a massively parallel manner on a single chip.

Recently PamGene B.V. has developed a revolutionary porous capillary solid phase microarray. This 3D array combines the unique properties of a two dimensional platform with the additional benefits of a third dimension, precise fluid manipulation through the test area. This new platform is designed to provide rapid, highly sensitive testing capability for diagnostic research.

The 3D array offers various advantages, including:

- 1) improved responsiveness and dynamic range due to the increased surface area relative to a flat surface geometry;
- 2) reduced assay times of only 15 minutes instead of 18 hours, due to dynamic fluid delivery to the chip;
- 3) more uniform probe deposition and higher array densities due to wetting properties of microporous materials; and
- 4) Temperature controlled hybridisation followed in real-time, for each individual oligo spotted on the matrix.

The 3D array has been shown to significantly improve reaction signals and increase test kinetics making this assay an extremely fast, sensitive and high performance microarray alternative to current available platforms. Characteristics of this hybridisation system will be highlighted.

DIAGNOSTIC MICROARRAYS IN MICROBIOLOGY

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The full potential of DNA microarray technology in high-throughput detection of bacteria and quantitative assessment of their community structures is widely acknowledged but not fully realized yet. A generally applicable set of techniques based on readily available technologies and materials was developed for the design, production and application of diagnostic microbial microarrays in molecular ecology, in clinical, veterinary and plant microbiology as well as in food quality control. A microarray targeting the particulate methane monooxygenase (*pmoA*) and ammonia monooxygenase (*amoA*) genes was developed for the detection and quantification of methanotrophs, nitrifiers and functionally related bacteria.

The microarray consists of a set of 59 probes and covers the whole known diversity of these bacteria and was validated with a representative set of strains and environmental clones. Initial tests assessing the quantification potential of this system showed very good correlation with 26% average standard deviation from the expected results.

As the cornerstone of diagnostic microbial microarrays, the design and behaviour of the oligonucleotide probes does not differ too much between different platforms, most of the techniques and guidelines presented here will be easily transferable to emerging novel technologies.

MICROARRAY METHODS FOR THE DETECTION OF PATHOGENIC BACTERIA AND VIRUSES IN PLANTS

José E. PEREZ-ORTIN

Laboratory of DNA Chips. Universitat de València. Spain.

Microarrays are capable of analyzing hundreds of different loci simultaneously in a short period of time. However, most microchip arrays require large amounts of template DNA, or RNA, for efficient rapid, passive hybridization. The oligonucleotide capture probes should be designed to maintain uniform stringency conditions for each hybridization reaction. Multiplex amplification is a possible method of obtaining high concentration of input DNA but often leads to large decreases in amplification efficiency. Microelectronic chips are potentially able to circumvent these problems. Electronically controlled microelectrode arrays are an interesting option for the DNA analysis as a diagnostic tool. In contrast to the passive hybridization environment of other methods, these devices offer the ability to actively transport DNA to, and to hybridize at, discreet locations on the microelectrode array surface. We are currently adapting the microelectronic devices to the plant pathogen detection problem.

A project in collaboration with IVIA (València, Spain) is being developed on microarrays for detection of viruses and phytopathogenic bacteria in potato plants.

TOWARDS THE RANDOM CHIP – ESTABLISHING THE PRACTICAL PARAMETERS FOR A NEW VERSATILE MICROARRAY

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A new method is presented that allows the characterization of genomes based on oligonucleotide hybridization patterns using microarrays. The method requires no information on the genome to be characterized. Identification of organisms on all taxonomic levels will be possible, and the method also allows to screen for molecular markers for genetically based characteristics and the identification of single nucleotide polymorphisms (SNPs).

One of the practical problems is the collective hybridisation of a large amount of oligonucleotides on the chip. Due to differences in their melting temperatures it is difficult to find appropriate hybridisation conditions. Attempts are presented to obtain proper conditions. Additionally, we describe a simple method for the production of a highly versatile polyethylene glycol coated glass slide, the determination of its properties and its use in hybridisation experiments.

MICROARRAYING IN THE CONTEXT OF FUNCTIONAL GENOMICS

Ralph SCHLAPBACH

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With the establishment of DNA microarrays as a research tool for a broad range of questions in vastly diverse biological systems, researchers are facing large amounts of complex data generated by this new and important technology. To avoid the risk of misinterpretation of microarray data and their significance for the understanding of the biological system of interest, it is essential to understand the potential and limitations of microarray data among other technologies. Using highly parallel and sensitive detection methods, even today biological questions still have to be split up according to the complexity of the question, the molecule classes to be investigated, the practicability of technologies and the resources needed to implement these systems on site.

A summary of the main aspects of microarray experiment design, production setup and data evaluation is put in the context of a broader range of current high throughput screening technologies in the areas of genomics, transcriptomics and proteomics.

EXPLORING NEW STRATEGIES IN LOADING, ATTACHMENT AND DETECTION TO LOWER THE COSTS OF DNA ARRAY WORK

Giuseppe FIRRAO

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Although the DNA arrays are regarded as a promising technology, their application to diagnostics is severely limited by cost considerations. It is expected that the cost will be reduced by some recent technical innovations, but nevertheless the array-based diagnostics may remain too expensive to reach the agriculture routine diagnostic lab.

In this talk I will summarise the major reasons of expense in the conventional array technology and will report the work carried out in Udine aimed at the definition of a new low-cost platform. In detail, a method for the parallel production of medium density DNA arrays to up to 6144 locations will be presented. According to this method, several individual flow-through arrays are piled, allowing communications between channels corresponding to the same location in different arrays. Each group of corresponding locations of all arrays is loaded with a single sample delivery of a volume in the microliter range.

It will be also shown how the use of electrical potential to link probes to supports and to detect nucleic acids by Ru(bpy) electrochemiluminescence can be applied in a cost effective manner using the technology provided by the printed circuit board (PCB) industry.

ARB, A GRAPHICALLY ORIENTED SOFTWARE PACKAGE COMPRISING VARIOUS TOOLS FOR SEQUENCE DATABASE HANDLING AND DATA ANALYSIS

Wolfgang LUDWIG

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The ARB (arbor, latin, tree) project is an interdisciplinary bioinformatics initiative of the Lehrstuhl für Mikrobiologie and the Lehrstuhl für Rechnertechnik und Rechnerorganisation of the Technical University of Munich. A comprehensive software package for sequence data as well as databases for genes and genomes are available at www.arb-home.de.

Initially, ARB was designed for ribosomal RNA data, however, can also be used for protein data and currently is adapted for handling genome data.

According to the ARB concept processed sequences and any other data assigned to the individual sequences are stored in an hierarchically structured highly compressed central data base and accessible by various directly cooperating software tools for visualisation and analysis. Sequence and other descriptive data can be imported and exported in commonly used flat file formats. These and any user provided data are stored in individual data base fields linked to the respective sequence or accessible in external databases via internet connection. Flexible search tools can be used for visualisation, selection, and extraction of the database entries. Automated aligners and powerful primary and secondary structure editors allow processing and visualisation of the sequences as well as sequence derived profiles and filters. The most commonly applied treeing approaches for phylogenetic analyses are implemented. A special version of a maximum parsimony based algorithm allows to reconstruct, and evaluate big trees (more than 30.000 sequences) as well as phylogenetic analyses of heterogeneous data sets (i.e. full and partial sequences). The trees can be used for database structuring as well as data access and visualisation. Any user defined selection of database field entries can be visualised at the terminal nodes of a tree. The ARB_PT-server (positional tree) allows rapid identification of sequence similarities or peculiarities. It is used by tools for finding the next relatives, automated alignment as well as design and evaluation of specific probes. The probe tools search for diagnostic sequence stretches for user defined organisms or phylogenetic groups. The potential target sites are further evaluated according to criteria of (hybridisation) technical relevance. Tools for the automated design and evaluation of comprehensive probe sets for micro arrays according to the multiple probe concept as well as for the interpretation of chip hybridisation patterns are currently under development.

Further ongoing software development concerns ARB-Genome which allows the comparative analysis of annotated genomes and – according to the ARB integrated database concept – the combination of the sequences with experimental data from differential cultivation, expression and proteome studies.

Processed ARB databases are maintained for evolutionary conserved genes or gene products such as rRNAs, ribosomal proteins, elongation factors, ATPase subunits, RNA polymerases, DNA Gyrase, heat shock proteins, recA, aminoacyl tRNA synthetases.

DETECTION OF POTATO VIRUSES USING MICROARRAYS

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Currently most diagnostic methodology is geared towards detection of a very specific target species. In order to test for a long list of unknowns a number of assays need to be run in parallel to reach a single result. The methods that are available for virus testing using a generic approach, namely electron microscopy, test plant inoculations and more recently MALDI TOF all give an identification to the genus level, follow up testing using another method is needed to get identification to the species level. The microarray method described in this paper addresses this problem by presenting a technology that can be used to test for a large number of targets using a completely generic technology. In the context of virus detection, the issues of specificity and sensitivity have been examined. The method has been shown to be able to discriminate sequences with less than 80% sequence identity, and hence useful for discriminating at the species level, but with broad specificity being able to detect sequences with greater than 90% sequence identity, thus the method should be able to cope well with the intrinsic variability found within the genomes of RNA viruses. The sensitivity of the assay was found to be comparable with ELISA.

APPLICATION OF SEQUENCE-SPECIFIC LABELED DNA PROBES IN COMBINATION WITH ARRAY HYBRIDIZATION FOR FINGERPRINTING AND MICROBIAL COMMUNITY ANALYSES

Knut RUDI

MATFORSK Norwegian Food Research Institute, Ås, Norway

Analyses of complex microbial communities are becoming increasingly important. Bottlenecks in these analyses, however, are the tools to actually describe the biodiversity. Novel approaches for a DNA array based analyzes of microbial communities are presented. In these approaches, the specificity obtained by sequence-specific labelling of DNA probes is combined with the possibility of detecting several different probes simultaneously by DNA array hybridization. Examples from both fingerprinting and 16S rDNA community analyses will be given.

MULTIPLEX DETECTION OF PLANT PATHOGENS BY MICROARRAYS: AN INNOVATIVE TOOL FOR PLANT HEALTH MANAGEMENT

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Detecting harmful organisms in plant propagation material is necessary in order to ensure safe and sustainable agriculture. If different pathogens need to be detected simultaneously, this approach is costly. The multiplicity of assays available for a specific pathogen leads to a lack of consistency among the various testing agencies in Europe and hampers standardisation.

The newest development in analysis of nucleic acids is the microarray technology, in which different oligos can be spotted on little more than one square mm. The use of these micro-fabricated DNA analysis tools will provide the next generation of inexpensive DNA diagnostics to measure different pathogens in a massively parallel manner on a single chip. To develop the microarray technology for diagnostic purposes generic DNA/RNA extraction and generic pre-amplification methods to increase sensitivity have to be developed.

Recently PamGene B.V. has developed a revolutionary porous capillary solid phase microarray. The capacity of this 3D-array to bind oligonucleotides is higher than that of a 2D-glass array resulting in a higher sensitivity. Moreover, the porous solid phase allows flow through measurements, resulting in fast hybridisation times of only 15 minutes instead of 18 hours as on glass. Using a temperature control system hybridisation can be adjusted instantaneously and together with the use of real-time monitoring, hybridisation kinetics and melting temperature can be determined for each individual oligo spotted on the matrix. By using different probes per pathogen the specificity can be increased even further. Generic amplification of different targets coupled to array detection makes this system a useful tool in multiplex detection of plant pathogens. Recent data to detect different plant-pathogens (viruses, bacteria, fungi and nematodes) in this multiplex setting will be discussed.

TYPING OF CITRUS TRISTEZA VIRUS STRAINS BY PLATE HYBRIDIZATION WITH A PANEL OF PROBES

Gustavo NOLASCO

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Citrus tristeza virus causes the most economically damaging viral disease of citrus. Natural infections are caused by diverse strains whose economic effects may be very different. In the last years important efforts have been made to develop tools for quick typing of strains. Sequence data from about 150 coat protein gene accessions obtained in the Genbank, University of Florida and our Laboratory was aligned and 7 groups of strains were defined based on nucleotide diversity. Interestingly, the groups so defined depicted a good relationship with the symptomatology produced. Parsimonious informative sites were located in each group and a panel of probe sequences designed in such way that the sequences of each group had a characteristic signature of reaction with the panel of probes. These probes have been used as capture probes in asymmetric PCR ELISA assays for typing isolates from different origins. In these assays the rate of substrate hydrolysis is measured and, by comparison with the reaction of typical strains of each group, the mixture of strains composing each isolate is determined. Besides epidemiological studies, from a practical point of view, this provides a very convenient way to detect isolates which may hide severe strains.

**DNA CHIP DIAGNOSTICS: RELATED WORK AT
DANISH INSTITUTE OF AGRICULTURAL SCIENCES**

Mogens NICOLAISEN

*Danish Institute of Agricultural Sciences
Dept. of Plant Protection
Research Centre Flakkebjerg*

A short overview of initiatives on diagnostic DNA chips in Denmark will be given. Work in our lab on the genomic characterization of plant pathogens (virus and fungi) providing a contribution to the basis of DNA chips will be presented. A project on the expression profiling and detection of mycotoxin producing *Fusarium* will be described.

IDENTIFICATION OF AFLATOXIN-PRODUCING AND NON-PRODUCING ISOLATES OF *ASPERGILLUS FLAVUS* AND *A. PARASITICUS* BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

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Aflatoxins are polyketide secondary metabolites that are produced by foodstuff- and animal feed-contaminating members in the *Aspergillus* section *Flavi*, particularly *A. flavus*, *A. parasiticus* and *A. nomius*. Due to the toxic and carcinogenic properties of aflatoxins, there is a need to develop rapid and sensitive methods to detect the presence of aflatoxigenic *Aspergilli* in contaminated foods and feeds. However, not all *Aspergillus* strains are able to produce aflatoxins, and this prompted the adoption of multiple screening techniques to ascertain the real toxigenic potential of contaminating molds.

Conventional methods used to distinguish among toxigenic and non-toxigenic isolates in the *A. flavus* group involve culturing the fungus in suitable inducing media, extracting aflatoxin with organic solvents and monitoring their presence by chromatographic techniques.

To meet the need for methodologies which may be applicable to screen large numbers of strains in a reasonable time, alternative methods were developed. These are either based on the use of complex media to detect the natural fluorescence of aflatoxins released by the growing mycelium or rely on multiplex PCR detection of genes involved in the aflatoxin biosynthetic pathway.

The generally accepted pathway for aflatoxin biosynthesis involves over 20 enzymatic reactions, and most of the corresponding genes have been now isolated and characterised. Recent studies suggest that regulation of aflatoxin biosynthesis in *Aspergillus* spp. involves a complex pattern of positive- and negative-acting transcriptional regulatory factors, which are affected by physiological response to both external and internal stimuli. Therefore, while rapid and accurate, the screening methods based on PCR detection of key genes in the biosynthetic pathway of aflatoxins may fail to distinguish true aflatoxigenic isolates from the complex of *Aspergillus* spp. contaminating food and feed.

Our aim was to test the reliability of the RT-PCR technique in differentiating between aflatoxigenic and non-aflatoxigenic isolates of the *A. flavus* group. In the present study, new sets of primers matching a series of key genes in the aflatoxin biosynthetic pathway were designed and used in RT-PCR experiments to distinguish, among a collection of well-characterized isolates of *A. flavus* and *A. parasiticus*, those potentially dangerous by correlating the expression of genes involved in aflatoxin biosynthesis and the production of aflatoxins in inducing and non-inducing media measured by high-performance liquid chromatography (HPLC)/mass spectrometry. The transcription of some key genes was correlated to the production of aflatoxin determined by HPLC/mass spectrometry. These results open the perspective to adopt RT-PCR as well as other cDNA-based micro- and macroarray techniques to rapidly identify toxigenic isolates of *Aspergillus* spp.

PARALLEL DETECTION OF POTATO PATHOGENS: POSSIBILITIES AND PROBLEMS

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Parallel detection of potato viruses is planned using the new microarray technology. This approach consists in anchoring an array of virus-specific sequences on a glass plate and subsequent hybridization with labelled samples of nucleic acids from the tested plants.

The importance of potato as nutrition source makes a reliable screening of its viral pathogens an economical necessity. In our project, we focus on the seven most widely distributed potato viruses: PVY and PVA potyviruses, PVX potexvirus and PVS and PVM carlaviruses, potato leafroll luteovirus and the newly detected potato mop-top potamovirus.

To achieve this goal, viral RNA was isolated from infected indicator plants. Primers for amplification of conservative as well as variable regions of the virus genomes were prepared and RT-PCR was performed. 400 - 600 bp DNA amplicons were cloned to pBSK(+) vector. The corresponding *E. coli* clones are kept in the LB medium containing 15% of glycerole.

The clones will be used for design of a DNA-chip. Selected sequences will be immobilized on a glass plate and used for parallel detection of the above-mentioned viruses.

In the present phase of the development extensive testing of selected sequences is being performed to achieve high selectivity and to avoid false positive results.

IDENTIFICATION OF SOME ECONOMICALLY IMPORTANT PATHOGENS OF FRUIT TREES, SHRUBS AND ORNAMENTAL PLANTS

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Diverse research on molecular detection of plant pathogenic viruses, bacteria and fungi is conducted in Research Institute of Pomology and Floriculture. One of the purpose of these studies is to develop DNA markers suitable for detection of bacterial plant pathogens: tumorigenic *Agrobacterium* - crown gall agent and *Erwinia amylovora* - -fire blight agent. Another purpose is to discover DNA - based system for identification strains belonging to different taxons of genus *Agrobacterium*. In case of fungal pathogens, attention is directed to three *Phytophthora* species: *P. citricola*, *P. cryptogea*, *P. cinnamomi* and *P. ramorum*, which are devastating pathogens for ornamental nurseries.

A set of markers will be designed to recognize and identify the isolates collected in the Polish nurseries - in microbiological cultures, infected plant material and substrates using probes existing in literature. The aim of the research project on viruses and virus-like pathogens is to provide DNA probes suitable for their detection.

The work is concentrated on following viruses and phytoplasmas: plum pox virus (PPV), prunus necrotic ringspot virus (PNRSV), apple stem pitting virus (ASPV), blackcurrant reversion virus (BRV), apple chlorotic leaf spot virus (ACSLV), apple stem grooving virus (ASGV), prune dwarf virus (PDV), pear decline MLO (PD-MLO). Most of potential probes are already available as cloned cDNAs. All molecular markers are predicted as probes for microarray technology.

THE MOST IMPORTANT FRUIT TREE VIRUSES IN THE CZECH REPUBLIC

APPLE MOSAIC, PRUNE DWARF, PRUNUS NECROTIC RINGSPOT ILARVIRUSES & PLUM POX POTYVIRUS

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Nucleotide sequence analysis of the capsid protein genes of the four viruses revealed regions of high and low variability suitable for specific and/or non specific primer design. Apple mosaic ilarvirus (24 sequences) revealed three highly variable regions between nt 70 and 290. There still exists 35nt long region after nt 414 with two variable position only. Quite similar features possess the PNRSV, where the most variable region was located between nt 120 and 300 and the distal part of the gene was the most conservative. The movement protein-CP intergenic region of PNRSV is also highly variable and copy the phylogenetic relationships of the CP gene. In PDV (15 sequences) there was not so distinct variable hotspot in the proximal part.

This is the only ilarvirus, where conservative sequence longer than 20 nt was found (23nt after nt 470). The RNA4 subgenomic promoter sequence was successfully PCR tested for PDV isolate-independent amplification. The variability of the plum pox virus (56 analysed sequences) CP reflects the known secondary arrangement of the protein: highly variable proximal part but several more-than 20nt long stretches in the distal part of the gene.

EXPLORING REAL TIME PCR AND MICROARRAY IN PLANT VIRUS RESEARCH

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The research work of the Department of Plant Physiology and Biotechnology focuses mainly on studies of the plant – pathogen interactions. Lately, Real Time PCR and microarray have also been incorporated into different research projects.

Microarray technique was used in physiological studies of defence response in potato to potato virus Y, NTN strain (PVY^{NTN}). Libraries of induced and repressed genes of potato in response to the infection with PVY^{NTN} were prepared, using a subtraction hybridisation method. Microarrays were printed and used to detect differences in gene expression following the infection, and to identify the differences in response to viral infection among potato cultivars with different susceptibility to PVY^{NTN}. Currently, the gene expression kinetics of selected genes in different *Solanum tuberosum* cultivars following the PVY^{NTN} infection is studied, using Real Time PCR method.

We have been using Real Time PCR method for detection of genetically modified organisms (GMOs) to distinguish the virus from the transgene by combine detection of virus CaMV promoter and coat protein (Cp). In research of plant-virus interaction, Real Time PCR was used to detect the level of PVY^{NTN} accumulation in sensitive and resistant potato cultivars and in determination of PVY^{NTN} coat protein expression in transgenic potato plants.

Currently, the work is focused on a model virus, a Tomato mosaic virus (ToMV), where primers and hybridisation probe were designed for Real Time PCR quantification in applying a new chromatographic method using monolith disks for virus concentration. Real Time PCR is also planned to be explored for detection of economically important plant pathogenic viruses which occur in plants in very low concentrations, which are hard to detect by conventional methods, such as Rupestris Stem Pitting associated Virus 1 (RSPaV-1).

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Train schedule

Getting from Wädenswil to Zürich HB and Zürich Airport

From Wädenswil	To Zürich HB (main station)	From Zürich HB	To Zürich Airport
6 ¹⁶	6 ⁴⁶	7 ⁰⁷	7 ¹⁶
6 ²⁸	6 ⁵⁰	7 ¹⁰	7 ²⁰
6 ⁴⁶	7 ¹⁶	7 ¹⁷	7 ²⁸
6 ⁵⁸	7 ²²	7 ²⁶	7 ³⁸
7 ¹⁶	7 ⁴⁶	7 ³³	7 ⁴³
...		7 ⁴⁰	7 ⁵⁰
		7 ⁴⁷	7 ⁵⁹
		8 ⁰⁷	8 ¹⁶
		...	
Every hour from 5 ⁴⁶ to 23 ⁴⁶		Every hour from 6 ¹⁷ to 23 ³³ (last train: 0 ¹⁵)	
Valid on Friday and Saturday			

Getting from Rapperswil to Zürich HB and Zürich Airport (after the boat trip)

From Rapperswil	To Zürich HB	From Zürich HB	To Zürich Airport
6 ¹⁰	6 ⁴⁹	7 ⁰⁷	7 ¹⁶
6 ²⁹	7 ⁰⁴	7 ¹⁰	7 ²⁰
6 ⁵⁹	7 ³⁴	7 ¹⁷	7 ²⁸
7 ¹⁰	7 ⁴⁹	7 ²⁶	7 ³⁸
...		7 ³³	7 ⁴³
		7 ⁴⁰	7 ⁵⁰
		7 ⁴⁷	7 ⁵⁹
		8 ⁰⁷	8 ¹⁶
		...	
Every hour from 6 ¹⁰ to 23 ⁴⁶		Every hour from 6 ⁴⁰ to 23 ¹⁰ (last train: 0 ¹⁵)	
Valid on Friday and Saturday			
Only on Saturday: Direct trains from Rapperswil to Zürich Airport			
From Rapperswil	To Zürich Airport		
5 ³⁰	6 ²⁸		
6 ³⁰	7 ²⁸		
...			
Every hour from 5 ³⁰ to 21 ³⁰			

For more information please contact Moni Pfunder