CHAPTER ONE

LITERATURE REVIEW

DEVELOPMENT AND ASSESSMENT OF MICROARRAY-BASED DNA FINGERPRINTING IN *EUCALYPTUS GRANDIS*

INTRODUCTION

Forest trees are essential components of the natural landscape and of many ecological systems. They also form the foundation of a global, multi-billion dollar wood products industry. The economic importance of forest trees has contributed to a worldwide focus on the selection of breeding material with increased resistance or tolerance to pests and diseases. The first attempts to select for improved genotypes were based on morphological characteristics (Eldridge et al. 1994). However, most forest tree species are characterized by long generation intervals and much time is needed before assessment of disease resistance can be carried out (Grattapaglia 1994). This limitation has precluded the rapid identification and selection of disease resistant trees. A maximum of three to four generations of breeding have been completed in most commercial forest tree species. This has not provided sufficient opportunity to introgress disease resistance genes into germplasm that have otherwise only been selected for growth and form (Byrne et al. 1996). Breeders are in need of selection systems that will allow early identification of resistant germplasm and that will furthermore facilitate the introgression of resistance factors into elite or improved breeding material.

The discovery of isoforms and allelic variants of proteins (isozymes and allozymes) provided the first genetic markers based on molecular variation in plant and animal genomes (Markert and Moller 1959, Tanksley and Orton 1983, Weeden 1988). In the early 1970s, protein variants were first studied in trees by Bartels (1971) and

Bergmann (1971). Since then, much progress has been made in experimental genetic studies of forest tree species using isozyme analysis (Strauss and Conkle 1986, Niebling et al. 1987, Shirasi 1998). Isozyme markers have been useful for the identification of varieties (Ashari et al. 1989, Huang et al. 1994), recognition of economically important genes (Mackill et al. 1993), construction of genetic maps (Bernatzky and Tanksley 1986) and detection of genetic introgression (William and Mujeeb-Kazi 1993). However, isozyme markers are often dependent on the environmental conditions under which the plant is grown and the developmental stage of plant tissues used for protein extraction (Winter and Kahl 1995). In addition, relatively few proteins are amenable to isozyme analysis, which limits the genome coverage that can be achieved with these markers. These problems have largely been overcome with the development of DNA-based molecular markers, and high-throughput, automated genotyping technology.

A number of DNA-based molecular marker technologies have been developed in the past two decades. Such marker technologies include restriction fragment length polymorphisms (RFLPs, Botstein et al. 1980, Neale et al. 1989), simple sequence repeats (SSRs, Weber and May 1989, Jarne and Lagoda 1996, Goldstein and Schloetterer 1999), random amplified polymorphic DNA (RAPD, Williams et al. 1990, Rafalski et al. 1991) and amplified fragment length polymorphisms (AFLPs, Vos et al. 1995, Blears et al. 1998). These molecular markers have become fundamental tools for forest geneticists. They have assisted tree breeders in improving the efficiency of classical breeding techniques (Smith and Beavis 1996).

In most plant species, molecular markers have been particularly useful in manipulating disease resistance genes (Michelmore 1995, Okubara et al. 1995, Wilcox et al. 1996, Harkins et al. 1998). Currently, the major limitation of these molecular marker methods is their dependence on gel electrophoresis (Jaccoud et al. 2001). This hampers the processing of a large number of samples or markers in parallel. There are difficulties in precisely correlating bands with allelic variants, and only one or a few loci can be studied at a time.

Jaccoud et al. (2001) demonstrated that these limitations can readily be overcome by using microarrays. DNA microarray technology has the potential to greatly increase the level of automation and thoughput of molecular marker technology (Schuchhardt et al. 2000, Foster and Huber 2001). Hundreds or thousands of loci can be analysed in a short time. This significantly decreases the time required to identify disease resistance loci and develop markers for breeding purposes. The efficiency of this process facilitates the release of new varieties that display durable resistance. Furthermore, microarray-based breeding has the potential to allow whole-genome selection, which will enable tree breeders to manipulate the complete genomes of trees, while selecting for individual, high value loci.

The focus of this review is on the exploitation of DNA microarrays as a method for developing disease resistance markers to be used in tree breeding programmes, and particularly those related to Chrysoporthe canker caused by *Chrysoporthe austroafricana* in South Africa.

THE IMPACT OF DISEASE ON FOREST TREES IN SOUTH AFRICA, WITH PARTICULAR REFERENCE TO *CHRYSOPORTHE*

The forestry industry in South Africa depends on a small number of fast-growing species, most notably in the genera *Eucalyptus* and *Pinus* (Evans 1992). These species have been developed in South Africa due to their rapid growth and easy nursery propagation. In recent years, planting *Eucalyptus* species has become increasingly important (Directorate of National Forestry Planning 1987, Eldridge et al. 1994). This interest in eucalypts and the trend towards propagation of clones from cuttings has prompted concern regarding the role that diseases, such as those caused by *Chrysoporthe austroafricana* Gryzenhout & M. J. Wingf. (Gryzenhout et al. 2004), could have on the sustainability of the industry (Conradie et al. 1990, Wingfield et al. 1991).

Chrysoporthe species, previously united under the single name *Cryphonectria cubensis* (Bruner) Hodges (Gryzenhout et al. 2004), are serious canker pathogens of commercially grown *Eucalyptus* spp. in plantations (Boerboom & Maas 1970, Hodges et al. 1979, Hodges 1980, Florence et al. 1986, Wingfield et al. 1989, Wingfield 2003). DNA sequence comparisons showed that isolates from South East Asia, South America and South Africa represented distinct phylogenetic groups (Myburg et al. 2002, 2003). Specimens of *Cryphonectria cubensis* from South East Asia and South America could, however, not be separated or distinguished morphologically from the type specimen, originating from Cuba (Gryzenhout et al. 2004). For the present, these specimens are thus collectively transferred to

Chrysoporthe as a single species, *Chrysoporthe cubensis* (Bruner) Gryzenhout & M.J. Wingf. (Gryzenhout et al. 2004). Specimens previously treated as *Cryphonectria cubensis* from South Africa could be distinguished morphologically from those representing *Chrysoporthe cubensis* and was thus described as *Chrysoporthe austroafricana*.

Chrysoporthe spp. have severely limited the development of plantations of susceptible *Eucalyptus* species, where climatic conditions favour disease development (Alfenas et al. 1982, Sharma et al. 1985, Florence et al. 1986). Thus, it has become important to implement an effective disease management strategy for these pathogens. There are several means to accomplish this goal. The most effective means to reduce losses due to *Chr. cubensis* and *Chr. austroafricana* is to plant disease tolerant species or hybrid clones of *Eucalyptus* (Alfenas et al. 1983, Wingfield 1990). This approach has been shown to be effective in various parts of the world (Campinhos et al. 1983, Wingfield et al. 1990).

Various strategies have been used to select for trees with highest resistance to infection by both *Chr. cubensis* and *Chr. austroafricana*. A study conducted by Van Zyl and Wingfield (1999), assessed the relative susceptibility to *Chr. austroafricana (as C. cubensis)* by using the capacity of *Eucalyptus* clones to close wounds through callus production. Results indicated that tolerant clones close wounds significantly faster than susceptible trees. Another method currently used for resistance screening related to this group of pathogens is by artificial

inoculation (Ferreira et al. 1977, Alfenas et al. 1983, Van der Westhuizen 1992). However, it is also important to undertake disease screening in the areas where the clones will be commercially propagated, due to the genotype x environmental (G x E) interaction observed for disease susceptibility (Van Heerden and Wingfield 2002).

In the longer term, the impact of Chrysoporthe canker could potentially be reduced via biological control though hypovirulence. Hypovirulence is associated with the presence of double-stranded RNA (dsRNA) (van Alfen et al. 1975, Elliston 1985, Nuss 1992, van Heerden et al. 2001). Van Heerden showed that hypovirulence did not lead to a significant reduction in canker size, but did alter the morphology of the canker. Further, morphology of the transfected *Chr. austroafricana (*as *C. cubensis)* isolate is characterized by significantly smaller lesions than those associated with virulent, virus-free isolate (van Heerden et al. 2001).

Another exciting prospect to reduce the impact of Chrysoporthe canker is by using DNA microarray technology. The availability of DNA microarray technology will enhance the rate of tree improvement. Molecular markers tightly linked to resistance genes will allow assessment of the population and evolutionary genetics of resistance to *Chrysoporthe cubensis* and *Chr. austroafricana* on *Eucalyptus*. These markers could be indispensable for map-based approaches to clone resistance genes against Chrysoporthe canker. Furthermore, the availabililty of molecular markers allow for similarity studies between species involved in a tree breeding

programme. A successful hybridization programme, particularly in search of a hybrid species, does require genotyping of sample individuals from a population. Genotyping assists in the discovery of positive traits required from parents.

OVERVIEW OF DNA MICROARRAY-BASED MARKER ANALYSIS

TECHNICAL FOUNDATIONS

The field of array technology has evolved from Southern's key insight that hybridisation between nucleic acids (one of which is immobilized on a matrix) provides a core capability of molecular biology (Southern 1975, Kafatos et al. 1979, Southern et al. 1992). Southern blotting was only a small step towards filter-based screening of clone libraries, which introduced the employment of a pure, single, labelled oligonucleotide or polynucleotide species in the liquid phase and complex mixtures of polynucleotides attached to a solid support (Giege et al. 1998, Lockhart et al. 2000).

More relevant to microarrays is the method using gridded libraries, stored in microtitre plates and stamped onto filters in fixed positions (dot blotting). Each clone can be uniquely identified and information about it, accumulated (Southern 1975). The next advance was the automation and reduction in size of the dots on dot blots. This resulted in the large-scale exploitation of data emerging from hybridisation experiments (Lennon and Lehrach 1991, Southern et al. 1992). Several scientists explored expression analysis by hybridising mRNA to cDNA

libraries gridded on nylon filters (Jaakola et al. 2001, Voiblet et al. 2001). Although the ideas for such arrays were sound, the implementation had yet to be clarified.

Interest in array technologies developed rapidly after Ekins patented miniaturised assays for protein and DNA-RNA measurements in the mid 1980s (Ekins 1987, Ekins et al. 1989). Stanford University was the first institution to use DNA microarrays (Shena et al. 1995). Since then, application of the technology has grown rapidly. The importance is illustrated by ambitious genomic programmes using microarrays (Baldwin et al. 1999, Richmond et al. 2000) and by the establishment of core microarray facilities in the United States (http://www.rana.stanford.edu, http://www.nhgri.nih.gov, http://www.tigr.org), Australia (http://www.cambia.org, http://www.agrf.org), Germany (http://www.mips.bichem.mpg.de) and Switzerland (http://www.unil.ch). Lander et al. (1999) stated that "it is safe to predict that, not long after the turn of the century, researchers will be able to purchase standardised oligonucleotides and cDNA arrays containing the complete sets of 100 000 human and mouse genes" (Reymond et al. 2001).

MICROARRAY TECHNOLOGY

The key principle behind microarray technology is a hybridisation event. One binding partner, the target, is robotically deposited at indexed locations on a suitable surface such as a glass microscope slide (Lemieux 1998, Worley et al.

2000, Reymond et al. 2001). Targets can be of several types, including proteins (Zhu and Snyder 2001, MacBeath 2002, Sydor and Nock 2003), carbohydrates (Mellet et al. 2003) and tissues (Bubendorf et al. 2001, Hedvat et al. 2002). However, this review focuses only on two general methods for producing arrays: DNA-fragment based and oligonucleotide-based chips (Schena et al. 1995, Schena et al. 1996, Lockhart et al. 1996, Duggan et al. 1999). Detailed descriptions of both techniques can be found in a number of reviews, covering technical and comparative aspects of the technologies (Lipschutz et al. 1999, Lockhart et al. 2000, van Hal et al. 2000).

DNA fragment-based microarrays are generally applicable to non-model organisms, as they require only that a large library of DNA is available as a source of clones to be arrayed (Gibson 2002). Clones to be arrayed can originate from a variety of sources including anonymous genomic DNA or cDNA clones, EST clones, or DNA amplified from open reading frames (ORFs) found in sequenced genomes (Schena et al. 1995). Currently, it is feasible to array up to 10 000 DNA fragments/ 3.24cm^2 on the slide (http://cmgm.stanford.edu/pbrown).

Once produced, the microarrays are hybridised with fluorescently labelled probes (Marshall and Hodgson 1998, Ekins and Chu 1999, Aharoni and Vorst 2001). The fluorescently labelled samples allow the detection of individual species at a threshold of one part in 100 000 (Ruan et al. 1998) to 1 part in 500 000 (Schena et al. 1996). Since confocal laser scanning microscopy is an extremely sensitive method for fluorescence measurement at high resolution (Cheung et al. 1999,

Southern et al. 1999, Worley et al. 2000), both weak and strong signals can be monitored. The technology involved in the fabrication and analysis of DNA arrays has recently been extensively reviewed (Brazma and Vilo 2000, Haab et al. 2001, Planet et al. 2001, Foster and Huber 2001).

The alternate oligonucleotide technology, pioneered by Affymetrix GeneChips® (Pease et al. 1994, Lockhart et al. 1996, Lipschutz et al. 1999, Brown and Botstein 1999), uses light-directed synthesis for the construction of high-density DNA probe arrays (Lockhart et al. 1996, Bowtell 1999). Affymetrix GeneChips are short oligonucletide arrays and consist of up to 25-mer oligonucleotides per gene. The 25-mer oligonucleotides utilise perfect match and mismatch pairs that hybridise specifically or non-specifically (Gibson 2002, Hoheisel 1997). Arrays are hybridised to a single biotinylated amplified sample, and the intensity measure for each gene is computed by an algorithm that calculates the difference between the match and mismatch measurements and averages over each oligonucleotide, rather than comparing ratios (Gibson 2002). This technology is expensive, but has wide application for model organisms such as yeast and *Arabidopsis*.

MICROARRAY MARKERS FOR GENETIC ANALYSIS OF FOREST TREES

MICROARRAY MARKERS FOR DNA FINGERPRINTING

An often overlooked aspect of microarrays is that the sequence or even the origin of the probes does not need to be known to make interesting observations (Cheung et al. 1999). The complex profiles, consisting of thousands of individual observations can serve as a fingerprint. Fingerprints can be used for either the identification of diagnostic markers suitable for developing new PCR-based detection assays or for the characterization of population dynamics (genetic distance, similarity, phylogeny) of different genotypes. Experimentally, associating a particular genotype with a phenotype is an important step towards dissection of complex traits such as those associated with disease resistance. Opportunities and challenges of microarray fingerprinting are highlighted in this review.

DNA microarrays as a diagnostic tool

DNA microarrays could potentially be used as tools to address multiple questions linked to species identification (Ye et al. 2001). The use of DNA microarrays for species identification and detection of rifampin resistance in microbes has for example, been described by Troesch et al. (1999). A total of 26 of the 27 species used in the study, as well as all rifampin-resistant mutants, were correctly identified. However, this approach can fail to fully reflect genetic potential of

many organisms. This failure could be overcome by comparative genomic methods with whole genome arrays of unknown sequences (Jaccoud et al. 2001).

For field applications, a portable system for sample preparation and oligonucleotide microarray analysis has been reported for microbial systems (Bavykin et al. 2001). This system has been developed by Nanogen (http://www.nanogen.com/technology). The company is addressing each step in the sample-to-result process on microfabricated chip-based devices (Sosnowski et al. 1997). This includes the integration of electronic cell separation, electronic sample transport, electronically accelerated hybridisation and electronic denaturation (Wang et al. 2000). The disposable LabChip has multiple interconnected reservoirs for samples, sizing ladder, sieving matrix and buffers (van de Goor 2003).

No portable system has yet been developed for DNA microarrays containing genomic regions with no sequence information. However, it is not difficult to envision the future construction of a DNA microarray that will contain unique rRNA, chloroplast DNA or other genomic DNA fragments of immobilised plant probes for identification purposes. These arrays could be useful in many applications. Borevitz et al. (2003) has already demonstrated repeatable and informative allelic variation scanning of the *Arabidopsis* genome. The same principle of the system could be used for the development for portable chips for the detection of resistant trees. However, there remains a lack of comparative data for

cDNA arrays versus long oligonucleotide arrays (Holloway et al. 2002). Care needs to be taken when changing from oligonucleotide arrays to cDNA arrays.

Genetic diversity studies

DNA microarray technology can also be used for genetic diversity studies. Genomic hybridisation of a whole genome array can be used to detect molecular variation between similar DNA regions in plants (Ye et al. 2001). The complex profiles, consisting of thousands of individual observations, can serve as 'fingerprints'. These fingerprints can be used to maintain genetic diversity within breeding populations with respect to disease.

Recently, Diversity Array Technology (DarT) has been used to assay for the presence (or amount) of a specific fragment in a representation derived from the total genomic DNA of rice (Jaccoud et al. 2001). DArT™ is a solid state fingerprinting technique based on AFLP and enables analysis of large numbers of marker loci without any DNA sequence information. Microarray-based genotyping as implemented in the DArT™ technique is a 2-dye approach and relies on the detection of DNA fragments in a complex mixture of selectively amplified restriction fragments. Reduction of complexity by selective amplification allows comparison of polymorphic fragments among genotypes. This is achieved by hybridising DNA to an array containing a large number of DNA fragments, derived from genomic representations of an organism. The study of Jaccoud et al. showed that DNA microarrays make it possible to resolve complex genomic samples into

respective components, offering a method to study genetic diversity at a genome-wide level.

At present, the major limitations of microarray technology as a marker system are cost and the difficulty of achieving both highly sensitive (proportion of polymorphism detected) and highly specific (proportion of true positives) marker identification. The ability of current efforts to satisfy the demand for one but not the other, resides in the difficulty of achieving the same hybridisation pattern for probes (Steinmetz 2000). It should also be noted that studies on genetic diversity based on molecular markers must be interpreted with caution. There are often low correlations with patterns of variation for disease resistance.

Inference of family relationships of mating systems

Inference of family relationships of forest trees has drawn increasing interest recently. This interest is a consequence of the development of hypervariable markers, such as microsatellites (Lexer et al. 1999) and AFLPs (Gerber et al. 1999, Wu et al. 2000). These highly polymorphic markers enable parentage analysis and thus the reconstruction of the mating system and/or gene flow (Dow and Ashley 1996, Ehm and Wagner 1996, Streiff et al. 1999). Furthermore, given additional genotyped family members, incorrect specification of relationship may be detected on the basis of apparent incompatibilities with Mendelian inheritance (Boehnke et al. 1997, Lexer et al. 1999). Within a family, unintentionally duplicated samples,

half sibs or unrelated pairs can be identified, if a high number of loci are considered (Goring et al. 1995, Streiff et al. 1999, Ziegenhagen et al. 1999).

To date, only microsatellite markers and AFLPs have successfully been adapted for segregation analysis of polymorphic bands (Wu et al. 2000). Since co-dominant (microsatellites) and dominant markers (AFLPs) can be used for the reconstruction of family relationships within forest tree populations, microarrays may be considered as an alternative. Microarrays provide a method to quickly genotype progeny at high resolution at the whole genome level (Borevitz et al. 2003). Parallel genotyping of a large number of markers permit linkage analysis. This, in turn, will be sufficient to allow accurate inference of relationships (Boehnke and Cox 1997).

Phylogenetic reconstruction

Fingerprinting data can be used for phylogenetic studies, if the genome studied fits the assumptions made by all methods of inference. This is, that organisms evolve mainly by drift and mutation under a bifurcating tree assumption (Swofford and Olsen 1991, Rzhetsky and Nei 1993, Allman and Rhodes 2004). Cho and Tiedje (2001) confirmed that this also applies to microarray data when using oligonucleotide arrays. These authors showed that cluster analysis of the hybridisation profiles revealed taxonomic relationships between *Pseudomonas* strains tested at species and strain level resolution. The results suggested that this approach is useful for the identification of bacteria as well as for determining the

phylogenetic relationships between the isolates. The overall topology of the dendrogram based on cluster analysis of similarity coefficient matrix was consistent with the phylogenetic tree obtained from 16S rDNA sequence data (Moore et al. 1996).

Oligonucleotide microarrays have been used to study phylogenetic relationships mainly in microbes and pathogens. However, Nouzava et al. (2001) used the DNA microarray technology to survey repetitive sequences. These authors devised a modified DNA-microarray-based technique to screen short fragment genomic libraries. The preliminary study showed that some of newly isolated repetitive sequences of *Vicia* species could be used as supportive molecular markers for phylogenetic reconstruction of this plant. A similar method can be used for microarray analysis of forest trees.

IDENTIFICATION OF MICROARRAYS LINKED TO SPECIFIC GENES OF INTEREST USING BSA

Since no near-isogenic lines (NILs) exist for forest trees, Bulked Segregant Analysis (BSA) provides an alternative method for identifying markers linked to resistance in forest trees (Michelmore et al. 1991, Weeden et al. 1994, Wilcox et al. 1996). The underlying principle of BSA is the grouping of informative individuals, so that a particular genomic region is studied against a randomised genetic background of unlinked loci. Informative individuals are representatives of

phenotypic extremes for a segregating trait and are bulked in two separate pools (Giovannoni et al. 1991, Lynch et al. 1997, Hill 1998). It is assumed that the individuals at the ends of the phenotypic distribution have opposing homozygous alleles for the trait in question. All the other loci forming the randomised genetic background are assumed to be in a heterozygous state due to the random contribution of these unselected alleles (Michelmore et al. 1991). Therefore, the polymorphisms identified are closely linked to the target locus (Asims 2002).

BSA can also be used to target multiple loci of highly heritable traits (Michelmore et al. 1991). This is especially important for disease resistance breeding in forest trees, because genetic variation for host resistance in endemic pathosystems typically appears continuous (Wilcox et al. 1996). With BSA, it is possible to determine the number of genes involved in resistance of a tree, or whether all trees harbour an inordinate number of susceptibility alleles (Mackay et al. 2000). At present, only a few discrete resistance genes are known in forest trees. Inheritance of disease resistance in forest trees has been commonly explained by polygenic models, where resistance is controlled by many genes, each with a small additive effect (Kinloch et al. 1970, Robinson 1987; von Weisenberg 1990, Kinloch and Walkinshaw 1991, Wilcox et al. 1996, Van Heerden et al. 2002).

BSA and three molecular techniques, namely restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs) and random amplified polymorphic DNA (RAPDs), have most commonly been used to

develop DNA-based molecular markers (Groover et al. 1994, Grattapaglia and Sederoff 1994, Bradshaw and Stettler 1995, Grattapaglia et al. 1995). However, the molecular techniques are constrained by their reliance on gel electrophoresis. This hampers the processing of a large number of samples in parallel (Smith and Beavis 1996). In contrast, the microarray technology holds the promise of parallel analysis of thousands of markers at the whole genome level. This will improve the candidate gene selection process. Further, microarrays may also be useful for BSA as the two bulks can be labelled with different dyes and incubated in a single microarray reaction. This would simplify comparisons considerably.

GENE MAPS AND QTL DETECTION

Genetic linkage maps can be used to locate genes affecting quantitative traits (Wayne and Mc Intyre 2002, Borevitz et al. 2003). Quantitative traits are usually controlled by many genes, termed quantitative trait loci (QTL). By using molecular markers linked to one or more QTL, information at the DNA level can be used for early selection. Molecular marker maps have been constructed for the major commercial genera of forest trees, such as eucalypts, pines and acacias. Updated information can be obtained at http://dendrome.uc.davis.edu/index.html (Groover et al. 1994, Bradshaw and Stettler 1995, Grattapaglia 1996).

Until recently, only microarray expression profile studies had been used to map genes (Winzeler et al. 1998, Cho et al. 1999, Brem et al. 2002, Wenzl et al. 2004).

However, high-density arrays can also be powerful new tools for parallel genotyping of a large number of markers of unknown sequence (Steinmetz et al. 2002). Informative spots obtained in this way can function as forensic fingerprints to assist selection in forest tree breeding (Samuel et al. 2003). The ability to use arrays for detection of variation lies in the specificity of DNA hybridisation, which allows the detection of polymorphisms within complex genomes. Polymorphisms are detected as DNA segments, which amplify from one parent but not the others, and can be used to construct genetic maps (Grattapaglia 1996).

Microarray technology, together with the large amount of sequence information, will allow marker-trait association studies in economically important forest tree species. Expressed gene sequences (ESTs), or full-genome sequences can be used to design probes for single nucleotide polymorphisms, or other types of polymorphisms. Such polymorphisms detected in segregating progeny can then be used to construct genetic maps. Borevitz et al. (2003) already demonstrated the use of microarrays to demarcate recombination events along chromosomes of recombinant inbred lines of *Arabidopsis*. Since chromosomal regions that have a measurable effect on economically important traits can be identified, microarrays hold the potential for the genome-wide selection of superior plant materials.

Although significant progress in marker genotyping with oligonucleotide arrays has been made in recent years, genotyping with microarrays is still in its infancy (Hardenbol et al. 2003, Borevitz et al. 2003, Wenzl et al. 2004). A great deal of

information must still be developed on the effective incorporation of markers. It is essential that clonally replicated experiments in several locations and genetic backgrounds are established (Schuchhardt et al. 2000). It is also important to understand that if a particular QTL allele is not detected in all genetic backgrounds, this does not imply that the locus is not present (Yanchuk 2002). Other better alleles at that locus occur in the population, which can be used for subsequent analysis (Byrne et al. 1997).

DEVELOPMENT OF SCARS

Sequence characterised amplified regions (SCARs) are also known as allele specific associated primers (ASAPs), or sequence tagged sites (STSs). A SCAR is a genomic DNA fragment at a single genetically defined locus that is identified by PCR amplification (Paran and Michelmore 1993). These markers are designed by cloning and subsequent sequencing a polymorphic fragment of interest. The unique nature of the marker system is determined by the sequence and spacing of the primer sequences, rather than by hybridisation. This makes the marker system ideal for routine screening of forest trees (Demeke and Adams 1994, Barreneche et al. 1998).

Current methods of SCAR-based marker discovery typically involve the detection of sequence variation using a marker technology. RAPD and AFLP markers are most commomly used to develop SCARs (Paran and Michelmore 1993; Adam-

Blondon et al. 1994) as they obviate the need for specific probe development, and are rapidly applied and technically simple. Although both marker systems have many advantages for SCAR development, they are restricted somewhat by their dominant nature, the sensitivity of the amplifications to precise reaction conditions and their dependence on gel electrophoresis. The necessity to run PCR products on a gel hampers the ability to process a large number of samples. This may be overcome by hybridisation-based microarrays, which would allow for the discrimination of allelic variation at several thousand loci in a single assay.

SCARs have successfully been used to obtain markers linked to brown spot needle blight resistance in longleaf pine (Nelson et al. 1994), and to the eastern filbert blight resistance genes in *Corylus avellana* (Mehlenbacher et al. 2004). These and other studies have shown that SCARs are reproducible, show low variability among different thermocyclers and when different DNA polymerises are used (Melotto et al. 1996, Schachermeyr et al. 1995). Therefore SCARs allow a level of reproducibility between laboratories that is independent of the method employed in the physical mapping. However, SCARs have no yet been developed from microarray markers.

MAP-BASED CLONING OF RESISTANCE GENES

The use of molecular markers has enabled geneticists to accelerate breeding programs (Shepherd et al. 1995, Xu et al. 1996, Yang et al. 1997). Using molecular

markers, tightly linked markers for particular genes of interest can be identified and cloned using map-based cloning procedures (Young 1990). Gene-flanking regions that contain at least one of two flanking markers are cloned and used to construct contigs spanning the genomic region defined by the molecular markers. By tagging economically important genes it is possible not only to select for presence of those genes in individuals, but also to isolate and introgress genes from one line to another.

Breeders expect marker-assisted selection to be useful, particularly in situations where trait heritability is low, typically in selection at the individual tree level (Grattapaglia 1996). With high heritability, the phenotype becomes a more efficient predictor of a genotype (Bradshaw and Grattapaglia 1994). However, an absolute requirement for the selection of traits based on map-based cloning of a gene is that markers must be closely associated with the gene (Winter and Kahl 1995, Feuillet et al. 1995).

Although map-based cloning has been successful in many species, the question that remains is whether there are real opportunities for incorporating microarrays as a genetic marker system in tree breeding programs. Microarrays might not be suitable for programs with small budgets and where significant gains can be achieved by implementing other marker systems or conventional selection procedures coupled to map-based cloning. The effective incorporation of

microarrays in breeding programems will require significant experimental work before they can be used as a marker system.

DATA ANALYSIS AND MANAGEMENT

During the course of the next few years, microarray technology is likely to become an integral part of molecular biology laboratories around the world (Kazan et al. 2001). Along with the rapid development of microarray technology, more data will be generated (Cole et al. 1998, Schena et al. 1998). The sensible storage, analysis, interpretation and management of this data will be a major challenge (Debouck and Goodfellow 1999, Wilson et al. 2002). Efforts to construct public repository databases for the management of information on the primary results of hybridisation and the construction of algorithms making it possible to examine the outputs from single and multiple experiments are clearly needed (Chen et al. 1997; Douglas et al. 1999).

Another requirement for microarray analyses is effective software for efficient handling of hybridisation-based DNA fingerprints or to extract binary scores from hybridisation data. The majority of available microarray software has been designed for expression profiling studies. For fingerprinting applications, the presence or absence of fragments (dominant scoring) or signal intensity (for co-dominant scoring) has to be determined to construct a fingerprint. Furthermore,

quality values must be assigned to each data point to evaluate the reliability of the combined fingerprint. Scoring markers remains tedious, and in many cases current programs fail to identify all fingerprints (Bassett et al. 1999).

At present, there is no standard solution for microarray data analysis and storage software. Successful interpretation of data will rely on integrating experimental data with external information resources, such as those encompassed by NCBI's Entrez system (Mc Entyre 1998, Schuler et al., 1996) and software available in public domains. Just as the nucleic acid and protein sequence databases depend on input from many groups, public microarray databases will similarly stimulate a level of analysis that is not possible with narrowly defined data sets. Patenting and trade marking should not be allowed to affect technology development, distribution and access (Duyk 2002). Co-operation seems to be a more likely option and has solved similar problems in the microelectronics industry before (Bassett et al. 1999).

CONCLUSIONS AND FUTURE DIRECTIONS

DNA microarray technology has a great number of potential applications that could be used in the improvement of forest trees. Geneticists have predominantly improved forest trees based on phenotypic characteristics. Due to the substantial time between seed germination and sexual maturity, trees have not benefited greatly from traditional breeding approaches. The availability of automated, highly

efficient, fast and productive technologies must clearly enhance the opportunities for, and the rate of tree improvement. This can be achieved by using DNA microarray technology. DNA microarrays allow genome-wide and high-throughput analysis for the identification of trait-linked markers available to the plant breeders in future, and therefore improve the efficiency of breeding programs (Borevitz et al. 2003). This will not only make loci and gene-specific markers available to the plant breeders, but will also increase our understanding of tree genomes for breeding purposes.

Despite remarkable progress, DNA microarrays are still in their early stages of development and this presents many challenges. One of the most challenging aspects relating to microarray technology in the next decade will deal with the flow of data generated (Bassett et al. 1999). Efforts to normalise and analyse data, as well as to centralise the information in public repository databases, are clearly needed. In addition, more effective software tools will be needed for the efficient handling of hybridisation-based DNA fingerprints or to extract binary scores from hybridisation data. The large amounts of data generated in microarray analyses often go beyond the research interests of the individual scientists that have produced them. Therefore, data should be shared by all researchers to ensure maximum impact of research. In future, researchers will most likely be required to submit their microarray data to a public depository before publication.

Although there are difficulties associated with data analysis and management of microarray results, useful applications are already emerging and others will undoubtedly follow. Innovative efforts, coupling fundamental biological and chemical sciences with technological advances in the field of micro-fabrication should lead to even more powerful devices that will accelerate the realisation of large-scale genetic testing (Wang, 2000). In the short term, we expect that DNA microarray technology will continue to be evaluated as a marker technology. DNA microarray technology and structural genomic tools, such as sequencing, will help to improve trees. Sequencing of plant genomes will allow for the saturation of existing forest tree maps. This will make loci and gene specific markers available to plant breeders in future.

In the long term, we expect that the ability to exploit and manipulate the genomes of forest trees will be possible. This is because of advances in the output of large-scale sequencing efforts and achievable advances in array technology (Lockhart et al., 2000). The combined use of microarray technology, sequence information, computational tools, integrated knowledge databases, biology, chemistry, physics, mathematics and genetics will increase the understanding of the mechanisms of disease. Locating, identifying and cataloguing genotypic differences will be the first steps in relating genetic variation to phenotypic variation in both normal and diseased states. However, the full potential of this technology as a marker system will depend on the number of laboratories that will use and evaluate the potential of microarray technology in theoretical and applied forest tree breeding. For

microarray technology to meet its full potential, it will need to become an integral part of the daily activities of the molecular biology laboratories (Mc Dowell et al. 2003).

The potential applications of DNA microarrays as a method for genetic marker analysis in forest tree breeding has been described in this review. It is likely that DNA marker technology will evolve rapidly in the coming years. Instruments for preparing microarrays and more effective software programs for analysing data from microarray experiments are continuously being improved. We thus expect that DNA microarray analysis as a method for genetic marker analysis will greatly assist forestry breeding programs.

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