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CHAPTER THREE

IDENTIFICATION OF MICROARRAY MARKERS ASSOCIATED WITH *CHRYSOPORTHE AUSTROAFRICANA* TOLERANCE IN *EUCALYPTUS* USING DIVERSITY ARRAY TECHNOLOGY (DART) AND BULK SEGREGANT ANALYSIS

ABSTRACT 91

INTRODUCTION 92

MATERIALS AND METHODS 95

PLANT MATERIAL AND DNA EXTRACTION 95
CANKER ASSESSMENT 95
MICROARRAY ANALYSIS 96
SEQUENCING OF DArT MARKERS 99
CAPS MARKER DESIGN AND ANALYSIS 99

RESULTS 101

CANKER ASSESSMENT 101
IDENTIFICATION OF DArT MARKERS ASSOCIATED WITH TOLERANCE 101
SEQUENCING OF MICROARRAY MARKERS ASSOCIATED WITH TOLERANCE 102
CAPS MARKER DEVELOPMENT 102

DISCUSSION 104

REFERENCES 107

ABSTRACT

Eucalyptus spp. are important plantation trees in South Africa and other parts of the world. The canker fungus *Chrysoporthe austroafricana*, previously known as *Cryphonectria cubensis in South Africa*, has resulted in serious damage, particularly to clonal plantations. Selection of disease tolerant *Eucalytpus* planting stock is thus a priority of the South African forestry industry. The aim of this study was to develop DNA-based molecular markers for Chrysoporthe tolerance in *Eucalyptus grandis* using a bulk segregant analysis (BSA) approach and Diversity Array Technology (DArT). A genotyping microarray had previously been prepared by arraying a partial library of genomic restriction fragments of 24 *Eucalyptus grandis* trees onto a glass slide. The multi-locus marker genotypes of tolerant and susceptible bulks, as well as individual genotypes of the progeny, were determined using a 384-feature prototype genotyping array. BSA revealed a total of 109 scorable, polymorphic loci, of which nine appeared to be associated with tolerance or susceptibility. Two DArT markers were converted to cleaved amplified polymorphic sequence (CAPS) markers, which discriminate susceptible and tolerant individuals. This study shows that DArT-based genotyping in combination with BSA provides a powerful approach for the detection of markers associated with disease tolerance in forest trees, and it should be equally applicable in other crop species.

INTRODUCTION

Chrysoporthe cubensis, previously known as *Cryphonectria cubensis* (Gryzenhout et al. 2004), is one of the most important pathogens of plantation-grown *Eucalyptus* spp. (Hodges et al. 1979, Hodges 1980, Florence et al. 1986, Wingfield et al. 1989). The fungus causes a serious stem canker disease and it is widely accepted to have influenced changes to vegetative propagation of *Eucalyptus* spp. for plantation development (Wingfield et al. 2003) in areas where climatic conditions favor the development of the disease (Hodges et al. 1979, Alfenas et al. 1982, Sharma et al. 1985b, Florence et al. 1986).

A fungus very similar to and originally thought to be the same as *Chr. cubensis* (Gryzenhout et al. 2004) causes a serious stem canker disease of *Eucalytpus* in South Africa (Gibson 1981, Hodges et al. 1986, Micales et al. 1987, Wingfield et al. 1989, Roux et al. 1999, 2003). Specimens previously treated as *Cryphonectria cubensis* from South Africa could, however, be distinguished morphologically from those representing *Chrysoporthe cubensis* and have thus been described as *Chrysoporthe austroafricana* (Gryzenhout et al. 2004). The discovery of *Chrysoporthe austroafricana* and the associated canker disease on *Eucalyptus* in South Africa (Wingfield et al. 1989) has caused concern regarding the impact that the disease could have on the success of *Eucalyptus* plantations in this country.

A substantial part of the forestry industry in South Africa depends on the wide-scale planting of *Eucalyptus* clones. The result is large areas of genetically uniform stands that are seriously threatened by pests and diseases (Wingfield 1990). Thus, it has become important for forestry companies both in South Africa and elsewhere in the world to implement an effective disease management strategy for these pathogens (Wingfield 2003).

Deployment of *Eucalyptus* planting stock, tolerant to canker caused by *Chr. austroafricana,* is considered the only feasible means of controlling the disease in affected areas (van Zyl 1990, Wingfield 2003, van Heerden et al. 2003). Trials to test disease tolerance of clonal hybrids using traditional breeding methods are time-consuming and costly. In addition, efficient selection of tolerant clones is possible only after trees are more than one year old (van der Westhuizen et al. 1992). Rapid, yet reliable, screening methods for disease susceptibility or tolerance are thus needed to identify and deploy tolerant genotypes.

Polymerase chain reaction (PCR)-based marker systems have been available for more than a decade. Among these, RAPD (Williams et al. 1990), AFLP (Vos et al. 1995) and SSR (Weber and May 1989) have been used extensively for fingerprinting, gene-tagging and mapping (Brondani et al. 2002, Myburg et al. 2003). These marker systems are technically accessible and rapidly provide polymorphic markers with universal reagents and assay protocols. However, they require gel electrophoresis for marker genotyping. Common limitations of gel electrophoresis are the limited number of detectable polymorphisms (up to 100 on the highest resolution acrylamide gels), and

difficulties in the precise correlation of bands with allelic variants resolved on different gels (Jaccoud et al. 2001).

Recently, methods have been developed for genotyping very large numbers of marker loci in parallel based on microarray hybridization of genomic DNA fragments. Jaccoud et al. (2001) reported the development of Diversity Array Technology (DArT^{TM}). This technique relies on the detection of DNA fragments in a complex mixture of selectively amplified restriction fragments and was recently used to develop the first genetic maps in a plant species (barley) based on microarray markers (Wenzl et al. 2004). Borevitz et al. (2003) reported the use of oligonucleotide arrays for repeatable and informative detection of allelic variation in the *Arabidopsis* genome. Since no oligonucleotide arrays are available for *Eucalyptus*, DArT is the only microarray-based genotyping method currently applicable in these species. This technique provides a tool for the parallel analysis of thousands of restriction fragment-based marker loci in the *Eucalyptus* genome, and we have used it for genome-wide fingerprinting of closely related *Eucalyptus* trees (Lezar et al. 2004).

In this study, DArT was used as a tool to identify markers associated with tolerance to *Chr. austroafricana* in *Eucalyptus*. Putative tolerance-associated markers were identified by bulk segregant analysis (BSA; Michelmore et al. 1991) and converted to cleaved amplified polymorphic sequence (CAPS, Konieczny et al. 1993) markers for further characterization in segregating *Eucalyptus* populations.

MATERIALS AND METHODS

Plant material and DNA extraction

A full-sib pedigree of *Eucalyptus grandis* was used to identify the DArT markers. This pedigree consisted of progeny from a controlled cross of the susceptible *E. grandis* clone ZG14 (Mondi Business Paper South Africa) and a single tolerant *E. grandis* tree TAG-S (Mondi). Genomic DNA of twenty-two clonally propagated F_1 progeny and the two parents were used for the generation of a genomic representation of the whole full-sib family (Lezar et al. 2004). Genomic DNA was extracted from young leaves as described by Murray and Thompson (1980). A DNA sample was also obtained from TAG5 (Mondi), a putative sibling relative of TAG-S, because tree TAG-S was lost during the early stages of this study.

Canker assessment

A total of 210 F_1 progeny (two clonal ramets per F_1 individual) and the two parental genotypes (ramets of clones ZG14 and TAG5) were planted in a commercial trial near Kwambonambi, Kwazulu-Natal, South Africa in 1997. The clonal replicates were planted in a randomized block design and maintained using standard silvicultural procedures. All parent and progeny trees in the trial were inoculated with one isolate (CMW 2113) of *Chr. austroafricana.* This isolate was previously shown to be highly virulent in a population of isolates (van Heerden et

al. 2001). The inoculum was prepared by culturing the fungus on 2% Malt Extract Agar (MEA; 20 g Malt extract/ 1000 ml water) plates. For inoculations, a cambial disc was removed from the main stems at a height of approximately 140 cm using a 20-mm diameter cork borer. A disc taken from the actively growing margin of a culture of *Chr. austroafricana* was placed in each wound with the mycelium facing towards the inside. To reduce desiccation, wounds were sealed with masking tape. Lesion lengths were measured 11 weeks after inoculation. The data collected from this trial were used to identify 11 disease tolerant and 10 susceptible F_1 progeny (Figure 1) that had the most extreme phenotypes and exhibited consistent phenotypes in three consecutive years of disease assessment. Genomic DNA was obtained from disease-free clonal ramets of these F_1 progeny and used for microarray analysis.

Microarray analysis

A 384-probe prototype DArT microarray was constructed by arraying genomic restriction fragments derived from total genomic DNA of 22 F_1 progeny and two parents onto glass slides (Jaccoud et al. 2001, Lezar et al. 2004). The DArT fragments were prepared by digesting a mixture of genomic DNA of all 24 trees with the restriction enzyme *Pst*I followed by ligation of enzyme-specific adapters to the restriction fragments. The genome complexity was reduced by selective amplification using adaptor primers ending in a single selective nucleotide (Lezar

et al. 2004). Eight replicates of the 384 DArT fragments were arrayed on each slide.

For BSA, two bulk genome representations were constructed by mixing equal amounts of genomic DNA of the 10 susceptible and 11 tolerant F_1 genotypes (from the phenotypic extremes, Figure 1), respectively, followed by restriction digestion and reduction of genome complexity as described above. A multi-locus DArT genotype was generated for each bulk genome representation by hybridizing differentially labeled (Cy3 or Cy5) genome representations of the two bulks to the 384-probe DArT genotyping array. Probe DNA preparation, labeling and hybridization were carried out following the published DArT procedure (Jaccoud et al. 2001) with modifications described by Lezar et al. (2004). One technical replicate of each hybridization was performed, each replication consisting of a reverse labeling experiment. In addition, the whole experiment was repeated with fresh DNA samples (i.e. four microarray slides were used in total). DArT genotypes were generated for the individual F_1 progeny included in the bulks and for the parents in a recent DArT fingerprinting study (Lezar et al. 2004). Array Vision 6.0 software (Imaging Research Inc., Molecular Dynamics, USA) was used to quantify the signal intensities of the array features.

For BSA, DArT fragments that differentiated the DNA pools derived from the tolerant and susceptible F_1 progeny were identified. Signal intensity ratios between the susceptible and tolerant bulks were obtained by dividing the normalized average signal intensity (across four reverse labeling replicates and eight spot replicates) of the tolerant bulk by that of the susceptible bulk. The $\log_2$

fold change and significance of the difference in bulk means were computed in SAS (version 8.2, Cary, NC, USA) for each of the 384 DArT features as described elsewhere (Okinaka et al. 2002). A Bonferroni multiple testing threshold (0.05/384 = 0.00013) was used to obtain an experiment-wise significance threshold of α = 0.05.

For analysis of the individual F_1 progeny and parents, the signal intensities of the parents and F_1 individuals were background corrected and normalized across slides by regression on the spot intensity data for parent ZG14 (Lezar et al. 2004). The normalized signal intensity values were used for identification of polymorphisms among F_1 individuals (Lezar et al. 2004).

DArT markers that differed significantly between the two bulks were evaluated in each of the 21 F_1 progeny constituting the bulks and in parent ZG14 using the single-dye (Cy3) experiments reported earlier (Lezar et al. 2004). Polymorphic DArT fragments were identified in Microsoft Excel based on the bimodal distribution of their intensity values across slides, consistent with their segregation as dominant PCR-based testcross (Aa:aa = 1:1) or intercross (A:aa = 3:1) markers. Each polymorphic DArT marker was scored for the presence (AA or Aa) or absence (aa) of the fragment in the genome representations of F_1 progeny and parent ZG14. The observed segregation ratios in the F_1 progeny were used to select a subset of markers with significant signal intensity differences between the two bulks and that were exclusively present in tolerant or susceptible F_1 progeny.

Sequencing of DArT markers

Nine DArT fragments that were putatively associated with tolerance were sequenced and subjected to similarity searches to reveal the possible identity of each tolerance marker. The cloned DNA fragments were recovered from *Escherichia coli* TOP10F' host cells harboring the genomic library. Recombinant *E. coli* clones were grown overnight at 37°C in LB medium containing 50 µg/ml ampicillin. A 1.0 µL aliquot of this solution was used for PCR with the M13 forward (-20) and M13 reverse primers (Invitrogen) as described by Lezar et al. (2004). Amplified fragments were then column-purified (QIAquick PCR purification Kit, QIAGEN GmbH) for sequencing on an ABI 3100 DNA sequencer (BigDye v3.2, Applied Biosystems). All fragment sequences were subjected to similarity searches using BLASTN and BLASTX.

CAPS marker design and analysis

For all nine sequenced DArT fragments, genome walking was carried out in the forward direction (across the 3'end of the cloned fragment) and the reverse direction (across the 5'end of the fragment) in two susceptible and two resistant individuals as described by Siebert et al. (1995). Each successfully cloned genome walking product was evaluated for the presence of a *Pst*I restriction site plus approximately 200 nucleotides beyond the restriction site. Eight pairs of CAPS

primers (Table 1) were designed using the Primer Designer 4 package (Version 4.2, Scientific and Educational Software, Cary, NC). The CAPS fragments were amplified in 20 µl containing 0.4 µM of each forward and reverse primer, 0.25 mM of each dNTP, 1X reaction buffer (Roche Diagnostics GmbH), 0.8 U *Taq* polymerase (Roche), and 6 ng template DNA. The PCR amplification consisted of 10 cycles of denaturation at 94°C for 30 sec, primer annealing at 63°C (reduced by 1°C per cycle) for 30 sec, and primer extension at 72°C for 1 min; 25 cycles of denaturation at 94°C for 20 sec, primer annealing at 53°C for 30 sec, and primer extension at 72°C for 1 min; with an initial denaturation of 94°C for 2 min, and a final extension of 68°C for 15 min. Following amplification, 12 µl of the reaction mix were transferred to a new tube and digested with 5 U of *Pst*I. Digested amplification products were resolved in 2.5% agarose gels. The marker phenotype of each CAPS marker was determined in parent ZG14, the 21 F_1 progeny used for BSA and in the two bulks. The CAPS markers were further tested in an additional 21 susceptible and 20 tolerant F_1 progeny of the same cross that also exhibited reliable disease phenotypes.

RESULTS

Canker assessment

After 11 weeks, inoculations on the parental and F_1 trees resulted in stem lesions of variable length. The F_1 progeny differed significantly in lesion length producing a phenotypic distribution with non-overlapping extremes (Figure 1). This result was expected since the parent trees were known to differ in tolerance. Only the most susceptible and most tolerant trees with consistent data over three consecutive years were selected for further analysis using microarrays.

Identification of DArT markers associated with tolerance

Sixty-two of the 384 array features showed some amount of differentiation between the contrasting bulks. Out of the 62 polymorphic features identified, 23 (6.0%) had significantly higher signal intensities in the tolerant bulk, while 39 (10.2%) had significantly higher intensities in the susceptible bulk at the Bonferroni-corrected threshold. Putative DArT markers differentiating the two bulks were evaluated in the 21 individuals included in the bulks (data available in Lezar et al. 2004, plus four additional individuals).

The CLUSTER and TreeView software (Eisen et al. 1998) allowed visualization of the relationships of the bulk and individual hybridization profiles (Figure 2). Two unique sub-clusters were identified that were associated with

canker tolerance. Sub-cluster I contained five DArT markers that were absent in all tolerant and present in all susceptible individuals (Figure 2), while sub-cluster II contained four markers that were absent in all susceptible and present in all tolerant *Eucalyptus* individuals. Therefore, only 9 (2.3% of 384) of the 62 polymorphic features were present in all individuals of one bulk and absent in all individuals of the other. They were consequently targeted for CAPS marker development (Table 2).

Sequencing of microarray markers associated with tolerance

Similarity searches based on the sequenced DArT fragments revealed that four fragments (78, 167, 189 and 341) were not homologous to any sequences in public databases. However, one fragment (4) showed weak similarity to *Arabidopsis* disease tolerance protein RPP8. Fragment 39 showed similarity to *Lotus japonicus* genomic DNA, while fragments 73 and 312 showed similarity to an *Arabidopsis thaliana* CTP-synthase-like protein and *Solanum tuberosum* mRNA for putative external rotenone-insensitive NADH dehydrogenase respectively. All E-values showed weak similarities.

CAPS marker development

Sequence data obtained from the cloned genome walking fragments revealed that for eight of the nine DArT markers, there were base-pair or indel polymorphisms

among susceptible and tolerant F_1 progeny within the *Pst*I restriction sites at the 3' end of the original DArT fragments. One fragment had a polymorphism in the 5'*Pst*I restriction site. Specific primers were designed to amplify DNA fragments containing the *Pst*I restriction site plus at least 200 nucleotides beyond the site (Table 1). We were able only to amplify eight of the nine CAPS fragments (results not shown). Two of the eight primer sets were successfully used in CAPS marker assays. Primers F178 and R178 amplified a monomorphic band of 600 bp in all parents an F_1 progeny, as expected from the sequence data. After restriction digestion of the fragment with *Pst*I, it was possible to differentiate between susceptible and tolerant individuals (Figure 3 A). A 600 bp undigested band was present in all susceptible F_1 individuals, whereas fragments of 500 bp and 100 bp were observed in all tolerant F_1 individuals. Three susceptible individuals (45, 47, 50) were heterozygous for the CAPS marker. Amplification of DNA with primers F312 and R312 resulted in a monomorphic band of 700 bp. Subsequent restriction digestion with *Pst*I produced a 600 bp and 100 bp band in the 10 susceptible individuals and a 700 bp undigested band in the 11 disease tolerant individuals (Figure 3B).

Primers for DArT markers 352, 4, 189, 73, 167, 39 were also tested in susceptible and tolerant individuals, but the PCR products were found not to be useful for CAPS analysis. The primers 4, 39 did not identify single loci, but produced multiple bands. To overcome this problem, annealing temperatures were increased. However, this resulted in loss of amplification. Primers 352, 189, 167,

73 amplified monomorphic bands, but showed no polymorphisms between individuals after restriction digestion when run on agarose gels (results not shown).

DISCUSSION

In this study, bulk segregant analysis (BSA, Michelmore and Paran, 1991) and DArT enabled us to identify restriction polymorphisms associated with tolerance to stem canker caused by *Chrysoporthe austroafricana* in a segregating full-sib family of *E. grandis* trees. A DArT genotyping microarray was previously prepared by selective PCR amplification of short restriction fragments derived from total genomic DNA of 24 *Eucalyptus grandis* trees onto a glass slide (Lezar et al. 2004). The multi-locus marker genotypes of tolerant and susceptible bulks, as well as individual genotypes of the progeny, were determined using a 384-feature prototype DArT genotyping array. Direct comparison of signal intensities revealed that of a total of 109 scorable, polymorphic loci, nine appeared to be associated with tolerance or susceptibility.

The combination of BSA and DArT is an efficient approach to rapidly identify genomic markers linked to traits of interest. Up to 10 000 DArT fragments can be printed on a single array, which allows very high genome coverage for gene-tagging in segregating families, or even in natural populations. The observed number of putatively associated markers detected with BSA (nine) corresponded

well with that predicted for one or two major tolerance genes. If we assume that linkage can be detected within a third of a chromosome, then 11.6 (384/33) of the markers could be expected to be linked to a single tolerance locus. Of the 384 DArT loci assayed, nine (2.3%) were found to have very different signal intensities in the resistant and susceptible bulks.

Several features of DArT make it attractive for genome-wide fingerprinting of *Eucalyptus* clones for the identification of trait-linked markers. This technique, like AFLP, allows genomic fingerprinting of organisms such as *Eucalyptus* with no prior DNA sequence information (Jaccoud et al. 2001, Wenzl et al. 2004). More importantly, the DNA for hybridization is prepared by amplification of short restriction fragments derived from throughout the genome using generic adapter primers. This eliminates the need for single PCR amplifications with different primers followed by restriction digestion as is used in CAPS (Paran et al. 1993) or PCR-RFLP approaches (Williamson et al. 1994), and allows a very high multiplexing ratio. The technology is, however, fairly expensive. This limits its usefulness for routine marker analysis of large numbers of trees. However, the fact that all DArT fragments are cloned before printing, allows the very quick conversion of DArT markers to simple, co-dominant PCR-RFLP based markers. PCR-RFLP based markers are less expensive to use for routine screening and marker-assisted breeding and more informative than DArT markers at the single marker level.

In this study, BSA revealed a total of 109 scorable, polymorphic loci, of which nine appeared to be associated with tolerance or susceptibility. We were able to successfully convert two of the nine DArT polymorphisms to co-dominant CAPS markers. These co-dominant markers provide a valuable tool for the accurate assessment of the genotype at the linked locus in mostly heterozygous forest trees. The two CAPS markers co-segregated perfectly with the DArT markers from which they were derived. However, failure of the enzyme *Pst*I to digest the amplified fragment would result in the incorrect scoring of genotypes. The activity of the enzyme should, therefore, be monitored by including known resistant and susceptible controls in marker assays. The remaining seven of the putatively associated DArT markers were not successfully converted to CAPS markers. We were not able to amplify one of the CAPS fragments, while the primers designed for two of these markers did not identify single loci. The other four CAPS markers that amplified the same size fragment from all individuals failed to digest with *Pst*I. We are investigating the design of the primers and identity of the PCR fragments.

The power of DArT fingerprinting combined with BSA lies in its ability to track the segregation of specific genomic regions inherited from the two parents in outcrossed pedigrees. Borevitz et al. (2003) reported the use of oligonucleotide arrays to demarcate recombination events along chromosomes of recombinant inbred lines of *Arabidopsis*. Unfortunately, genetic map information is not available for the cross of *E. grandis* parents used in this study and internal sequences of probes could not be used to link polymorphisms to a genome

sequence. Nonetheless, we found that the clustering of the DArT markers according to similar hybridization patterns across individuals suggested the presence of major linkage groups. Clustering of DArT markers allowed us to identify two unique subclusters that corresponded to Chrysoporthe canker tolerance or susceptibility.

The approach used in this study to identify microarray markers associated with tolerance to stem canker in *Eucalyptus* caused by *Chr. austroafricana*, should be applicable to other traits and other plant species. The long-term objectives of the research presented in this study is to analyze linkage relationships in a larger set of F_1 progeny and to associate the two markers identified with tolerance in *Eucalyptus grandis* breeding populations at a genome-wide level.

REFERENCES

Alfenas AC, Hubbes M, Couto L (1982) Effect of phenolic compounds from *Eucalyptus* on the mycelial growth and conidial germination of *Cryphonectria cubensis*. Canadian J Bot 60: 2535-2541.

Alfenas AC, Jeng R, Hubbes M (1983) Virulence of *Cryphonectria cubensis* on *Eucalyptus* species differing in resistance. Eur J Forest Path 13: 179-205.

Brondani RPV, Brondani C, Grattapaglia D (2002) Towards a genus-wide reference linkage map for *Eucalyptus* based exclusively on highly informative microsatellite markers. Mol Genet Genomics 267: 338-347.

Florence EJM, Sharma JK, Mohanan C (1986) Stem canker disease of *Eucalytpus* caused by *Cryphonectria cubensis* in Kerala. Kerala Forest Research Institute Scientific Paper 66: 384-387.

Gibson IAS (1981) A canker disease of *Eucalyptus* new to Africa. FAO, Forest Genetics Resources Info 10: 23-24.

Gryzenhout M, Myburg H, van der Merwe NA, Wingfield BD, Wingfield MJ (2004). *Crysoporthe*, a new genus to accommodate *Cryphonectria cubensis*. *Studies in Mycol* 50: 119-142.

Hodges CS, Geary TF, Cordell CE (1979) The occurrence of *Diaporthe cubensis* on *Eucalyptus* in Florida, Hawaii and Puerto Rico. Plant Disease Rep 63: 216-220.

Hodges CS, Alfenas AC, Ferreira FA (1986) The conspecificity of *Chrysoporthe cubensis* and *Endothia eugeniae*. Mycologia 78: 343-350.

Konieczny A, Ausubel F (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR based markers. Plant J 4:403–410.

Lezar S, Myburg AA, Berger DK, Wingfield MJ, Wingfield BD (2004) Development and assessment of microarray-based DNA fingerprinting in *Eucalyptus grandis*. Theor Appl Genet 109: 1329-1336.

Micales JA, Stipes RJ, Bonde MR (1987) On the conspecificity of *Endothia eugeniae* and *Cryphonectria cubensis*. Mycologia 79: 700-720.

Michelmore RW, Paran L, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc Acad. Sci USA 88: 9828-9832.

Myburg AA, Griffin AR, Sederoff RR, Whetten RW (2003) comparative genetic linkage maps of *Eucalyptus grandis*, *Eucalyptus globulus* and their F_1 hybrid based on a double pseudo-backcross mapping approach. Theor Appl Genet 107: 1028-1042.

Okinaka Y, Yang C-H, Perna NT, Keen NT (2002) Microarray profiling of *Erwinia chrysanthemi* 3937 genes that are regulated during plant infection. Mol. Plant-Microbe Interact 15: 619-629.

Old KM, Murray DIL, Kile GA, Simpson J, Malafant K (1986) The pathology of fungi isolated from eucalypt cankers in south-eastern Australia. Australian Forest Res 16: 21 – 36.

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85: 985-993.

Roux J, Wingfield MJ, Coutinho TA, Bouillett JP, Leigh P (1999) Diseases of plantation *Eucalyptus* in the Republic of Congo. SA J Science 96: 454-456.

Roux J, Myburg H, Wingfield BD, Wingfield MJ (2003) Biological and phylogenetic analyses suggest that two *Cryphonectria* spp. cause cankers of *Eucalyptus* in Africa. Plant Disease 87: 1329-1332.

Sharma JK, Mohanan C, Florence EJM (1985a) Disease survey in nurseries and plantations of forest tree species grown in Kerala. Research Report 36, Kerala Forest Research Institute, India.

Sharma JK, Mahanan C, Florence EJM (1985b) Occurrence of *Cryphonectria canker* of *Eucalyptus* in Kerala, India. Annals Appl Biol 106: 265-276.

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA. (1995) An improved method for walking in uncloned genomic DNA. Nucleic Acids Res 23:1087–1088.

Van der Westhuizen IP, Wingfield MJ, Kemp GHJ, Swart WJ (1992) Comparative susceptibility of *Eucalyptus grandis* clones and hybrids to *Cryphonectria cubensis*. In: Proceedings of the 30th Congress of the South African Society for Plant Pathology, Cintsa, East London, 23 – 26 January, South Africa.

Van Heerden SW, Wingfield MJ (2001) Genetic diversity of *Cryphonectria cubensis* isolates in South Africa. Mycol Res 105: 94–99.

Van Heerden SW, Wingfield MJ (2002) Effect of environment on the response of *Eucalyptus clones* to inoculation with *Cryphonectria cubensis*. Forest Pathol 32: 395 – 402.

Van Zyl LM, Wingfield MJ (1999) Wound response of *Eucalyptus* clones after inoculation with *Cryphonectria cubensis*. Eur J Forest Pathol 29: 161 – 167.

Vos P, Hogers R, Blecker M, Reyans M, van de Lee T, Hornes M, Fritjers A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414.

Wenzl P, Carling J, Kudrna D, Jaccoud D, Hyttner E, Kleinhofs A, Kilian A (2004) Diversity Array Technology (DarT) for whole-genome profiling of barley. Proc Natl Acad Sci USA 101(26): 9915-9920.

Williamson VM, Ho J-Y, Wu FF, Miller N, Kaloshian I (1994) A PCR-based marker tightly linked to the nematode resistance gene, *Mi*, in tomato. Theor Appl Genet 87: 757-763.

Wingfield MJ, Swart WJ, Abear BJ (1989) First record of Cryphonectria canker of *Eucalyptus* in South Africa. Phytophylactica 21: 311-313.

Wingfield MJ (1990) Current status and future prospects of forest pathology in South Africa. S A J Science 86: 60-62.

Wingfield MJ (2003) Daniel McAlpine Memorial Lecture. Increasing threat of diseases to exotic plantation forests in the Southern Hemisphere: lessons from Cryphonectria canker. Australasian Plant Path 23: 133-139.

Table 1. List of primers developed for evaluation of CAPS markers

Spot Number	Primer Name[a]	Sequence 5' 3'
312	**F312**	CATGGTGGGTGTATAGT
312	**R312**	GGTTCCGAAGCTTGAGTAG
178	**F178**	CCAGCTGCTGATGGAT
178	**R178**	TTACCTCTCTCGGCATCT
352	**F352**	TGGGCTCTTCTTCTCC
352	**R352**	CGGCGGAAGGGTAAAA
4	**F4**	CTGCAGTGGGGAAAG
4	**R4**	CTAAGACGCGTTGCACGA
73	**F73**	CAGTGATGCCAGTGTA
73	**R73**	ATCCGCCGGAAGGGTCAAA
189	**F189**	TGCAGTACGGAGGCTTGT
189	**R189**	CCGAGGATCTTGAATCTC
167	**F167**	TCCAGTGCAGTTGCCAACA
167	**R167**	GAGCAGTAACCGTTGAC
39	**F39**	CTGCCTGATGCAGACA
39	**R39**	TACTGTCGGACGGGCAGAT

[a] The forward primers (F) were used for genome walking. The reverse primers (R) were designed based on the genome walking products.

Table 2. Putative markers for tolerance to *Eucalyptus* stem canker caused by *Chr. austroafricana*, based on signal intensity ratios and segregation patterns in F_1 progeny

MARKER IDENTIFICATION SPOT NO	Ratio (Tolerant v. Susceptible)	*P*-value	Individual Data	
			Tolerant	Susceptible
4	2.402	0.00007	11	10
39	2.257	0.00001	11	10
73	2.033	0.00005	11	10
78	0.451	0.00001	10	11
167	0.380	0.00000	10	11
189	0.422	0.00000	10	11
312	0.472	0.00000	10	11
341	3.757	0.00000	11	10
352	4.955	0.00000	11	10

Figure 1. Mean lesion length (mm) of 61 F_1 progeny with consistent disease phenotypes after artificial inoculation with *Chrysoporthe austroafricana* for 3 consecutive years (1997-1999). The results are based on one replicate per tree and three annual measurements. Standard deviations are indicated by error bars. Additional trees genotyped with CAPS markers are indicated by *.

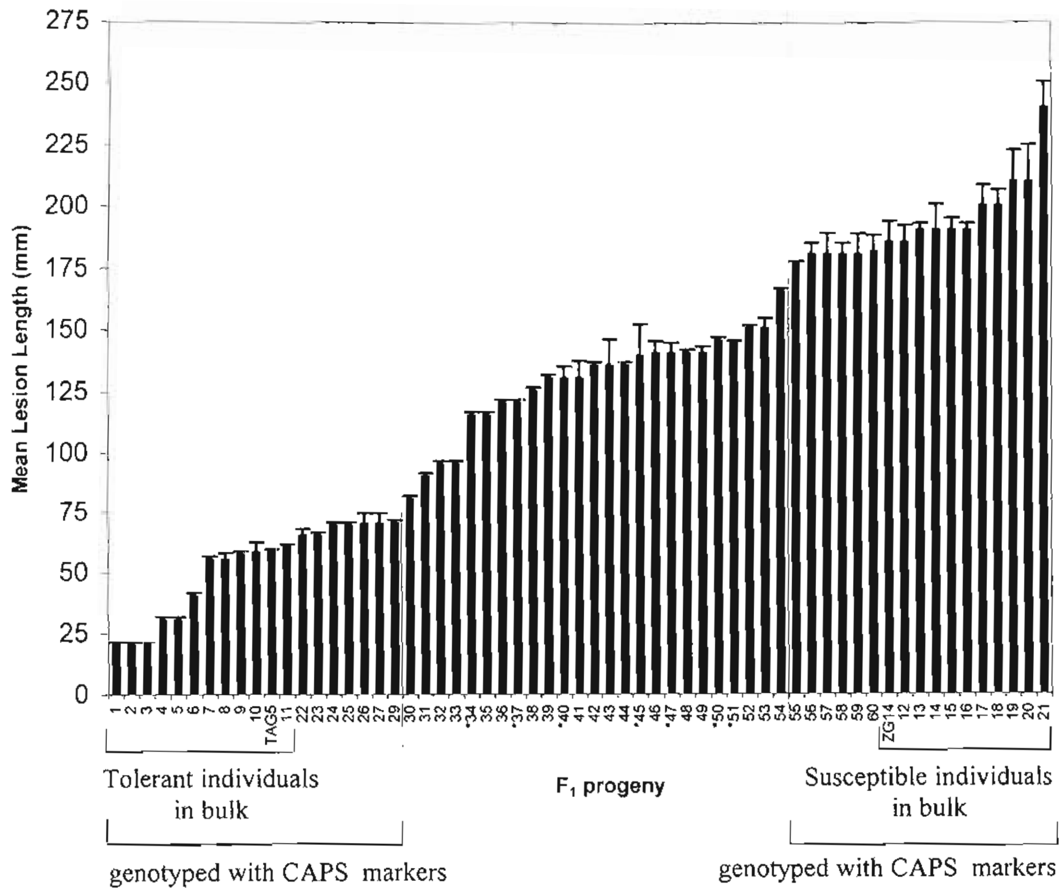
Mean Lesion Length (mm)
275
250
225
200
175
150
125
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 TAG5 11 22 23 24 25 26 27 29 30 31 32 33 •34 35 36 •37 38 39 •40 41 42 43 44 •45 46 •47 48 49 •50 •51 52 53 54 55 56 57 58 59 60 ZG14 12 13 14 15 16 17 18 19 20 21
Tolerant individuals
in bulk
F1 progeny
Susceptible individuals
in bulk
genotyped with CAPS markers
genotyped with CAPS markers

Figure 2. TreeView (Eisen et al. 1998) representation of clustering of hybridization profiles among 21 *Eucalyptus* individuals based on microarray analysis with the 384-probe array. Columns represent hybridization profiles of individuals (or replicates) and rows represent the mean log intensities for labeled DNA/DNA hybridisations across the parent ZG14 and the individuals of the full-sib progeny. *Green* bars and *red* bars indicate high and low mean log intensity values and *black* bars indicate intermediate values. The numbers I and II represent sub-clusters identified associated with canker tolerance. Sub-cluster I contains DArT markers that were absent in all tolerant and present in all susceptible individuals, while sub-cluster II contained markers that were absent in all susceptible and present in all tolerant *Eucalyptus* individuals.

Tolerant Bulk
TAG5
1
2
3
4
5
6
7
8
9
10
11
SUSCEPTIBLE BULK
ZG14
12
13
14
15
16
17
18
19
20
21

II

I

Figure 3. Electrophoretic pattern of PCR-amplified DNA products digested with *Pst*I enzyme among F_1 derivatives of *Eucalyptus grandis*. (A) DNA fragments amplified with primers F78 and R78. Trees TAG5 – 40: F_1 derivatives of parent ZG14 and TAG-S are all tolerant, whereas trees 45 – ZG14 are susceptible genotypes. (B) DNA fragments amplified with primers F312 and R312. Trees TAG5 – 40 are tolerant genotypes and lanes 45 – ZG14 are susceptible genotypes. F_1 progeny with intermediate disease phenotypes after artificial inoculation with *Chrysoporthe austroafricana* are indicated by *.

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