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Sequence-Tagged Site of Defense-Related Genes for Resistant/ Susceptible Eucalypt Selection to *Cryptosporiopsis eucalypti*

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ABSTRACT

Leaf spot and shoot blight caused by the fungal pathogen, *Cryptosporiopsis eucalypti*, is the most severe disease for eucalypt plantation in Thailand. The objective of this study was to create a set of DNA markers for resistant clone detection. Twelve clones of eucalypt were selected and screened for disease resistance using intact seedling inoculation and detached leaf techniques in the greenhouse and laboratory. Six clones, SF01, SF06, SF18, SF36, SF94 and SF98, were classified as resistance while the other six, SF03, SF07, SF14, SF16, SF70 and SF86, were susceptible clones. The sequence-tagged-site (STS) marker of pathogenesis-related (PR) genes, plant disease resistance (R) genes, and defense-related genes were analyzed in both DNAs of resistant and susceptible groups using polymerase chain reaction (PCR). Amplified DNA fragments were separated by agarose gel and polyacrylamide gel electrophoresis. The results showed that six markers (Ce1, Ce2, Ce3, Ce4, Ce5 and Ce6) from thirty five primer pairs could be used for susceptible and resistant detection of eucalypt.

Key words: *Eucalyptus*, *Cryptosporiopsis eucalypti*, leaf spot, DNA marker

INTRODUCTION

Clonal selection in eucalypt plantation is based on growth rate alone and no disease resistance screening has been developed for field implementation. In some areas, superior clones were grown but they were damaged from pathogens. In Southeast Asia, the most damaging diseases are caused by leaf and shoot blight pathogens, especially *Cylindrocladium quinqueseptatum*, *Phaeophleospora destructans* and *Cryptosporiopsis eucalypti* (Old and Mohammed, 2003). *Eucalyptus camadulensis* is the most popular eucalypt species in Thailand, but plantation productivity has been decreased by *C. eucalypti* infection (Old *et al.*, 2002). The plantations seriously infected were observed in Eastern and Western part of Thailand. Clonal selection for disease resistance is necessary to control the widespread of this disease. However, field assessment for this disease is difficult because its development depends on environmental conditions in each year. Furthermore, techniques for screening by greenhouse and laboratory inoculation and DNA-based approaches are quicker and easier, especially DNA-based approaches which have no effect from environmental factors. This study aimed at

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screening eucalypt clones infected with *C. eucalypti* in the greenhouse and laboratory then a set of DNA marker was developed to detect the resistant and susceptible clones before the field growth.

MATERIALS AND METHODS

Plant and fungal materials

Twelve clones of eucalypt (SF01, SF03, SF06, SF07, SF14, SF16, SF18, SF36, SF70, SF86, SF94 and SF98) were gave from Siam Forestry Co., Ltd. Six-to eight-month of seedlings and leaves were used for fungal screening. Three isolates of *C. eucalypti* were collected from plantations at Ratchaburi, Kanchanaburi and Chachoengsao provinces and coded as R001, K001 and C001, respectively.

Fungal inoculation

Fungal isolates were grown on potato dextrose agar (PDA) for 2 weeks at 25°C in the dark. Conidia were harvested by washing the plates with sterilized water. The conidial concentration in the suspension was measured using a haemacytometer and adjusted to 5×10^5 conidia/ml.

Intact seedling inoculation experiment was performed by 3x8 factorial experiment in completely randomized design (CRD) with three replications. Treatments consisted of three isolates of *C. eucalypti* and eight clones of eucalypt. Detached leaf inoculation was performed by CRD with four replications. Seedlings and mature leaves of each eucalypt clone were inoculated with conidial suspension. The control plants were sprayed with sterilized water. All inoculated and control plants were incubated in a high humid chamber.

The plants were evaluated after 8-11 days of inoculation determined from the leaf area and disease leaf area. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were conducted using SAS statistical software. Disease reactions were classified as resistance (R), moderately resistance (MR), moderately susceptible (MS), susceptible (S) based on the percentage of disease.

Primer design

Sequence of PR-genes, R genes, and defense-related genes were searched from GenBank database. Multiple-sequence alignment of these genes was analyzed using ClustalW (http://www.ebi.ac.uk/clustalw/). The conserved sequences were used for primer designing by Primer3 program (http://www-genome.wi.mit.edu) and primers synthesis was done by KU-Vector service unit.

PCR amplification and electrophoresis

The genomic DNAs were extracted from eucalypt leaves using the method described by Agrawal *et al*. (1992). STS primers were used for PCR amplification. PCR reaction was performed in a 20-μl volume containing 100 ng of genomic DNA, 0.2 mM dNTPs, 2 μl of PCR buffer (include 20 mM of $MgCl_2$), 5 ρmol forward and reverse primers and 0.5 U *YEA* DNA Polymerase. Amplification was programmed for 3 min at 94° C for initial denaturation and 40 cycles consisting of 0.5 min at 94°C, 1 min at 45°C, and 1 min at 72°C, followed by a final 5 min extension at 72°C. Amplified DNA fragments were separated on 1% agarose gel and 6% polyacrylamide gel electrophoresis (PAGE), and stained with ethidium bromide and silver nitrate, respectively. Dendrograms of DNA banding patterns were constructed using NTSYSpc, version 2.01e. The presence and absence of DNA bands were changed to binary number as 1 and 0. A similarity matrix was generated by SIMQUAL program, and cluster analysis was performed with the unweighted pair group arithmetic mean method (UPGMA). Polymorphic bands between resistant and susceptible groups were selected as markers.

Cloning and sequencing

DNA fragments from resistant group separated on agarose gel were excised and purified using QIAquick gel extraction kit (QIAGEN) following the manufacturer's instructions. DNA fragments detected on polyacrylamide gel were excised and reamplified by PCR. All expected bands were cloned into the pGEM-T vector (Promega) using the manufacturer's instructions and transformed into JM109 *E. coli* competent cells by heat shock transformation (Sambrook *et al*., 1989). Blue-white colony selection and PCR amplification were used to detect positive clones. Plasmids were extracted and purified using QIAprep spin miniprep kit (QIAGEN) following the manufacturer's instructions and sequenced by Bioservice Unit. All sequences were compiled and analyzed by BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

RESULTS

Screening of resistant clone

The average percentages of disease from intact seedling and detached leaf inoculation are shown in Table 1. There were highly significant differences at P > 0.0001 in the percentage of disease among clones. Disease reactions were classified into four levels according to disease percentage. The resistance of intact seedling inoculation experiment was classified as R = ≤ 1%, MR = 1.1-2%, MS = 2.1-3% and S = >3% while the resistance of detached leaf inoculation experiment was classified as R = 1-20%, MR = 21-40%, MS = 41-60% and S = 61-100%. Disease reactions of clones are shown in Table1.

DNA markers were developed from both resistant and susceptible groups. MR was combined into R group and MS was combined into S group. Resistant group was composed of SF01, SF06, SF18, SF36, SF94 and SF98 and susceptible group was composed of SF03, SF07, SF14, SF16, SF70 and SF86.

Molecular marker for clone selection

Among 35 designed primer pairs, 12 were from PR-genes, 4 were from resistance gene, and 19 were from defense-related genes (Table 2).

Table 1 Average percentage of disease and disease reaction of eucalypt clones after inoculation with isolates of C. eucalypti.

Clone	Intact seedling inoculation		Detached leaf inoculation		Disease reaction
	Average percentage of disease (%)	Disease reaction	Average percentage of disease (%)	Disease reaction	
SF07	2.87a1	MS	86.50a1	S	S
SF70	2.41a	MS	83.50a	S	S
SF16	2.04a	MS	67.34b	S	S
SF03	2.03a	MS	49.67c	MS	S
SF14	2.49a	MS	40.50c	MS	S
SF86	2.44a		-	-	S
SF18	-	-	22.50d	MR	R
SF01	-	-	10.50de	R	R
SF94	-	-	6.34de	R	R
SF98	-	-	5.25de	R	R
SF36	0.63b	MS	2.00e	R	R
SF06	0.39b	RR	1.92e	R	R
	C.V. (%) = 64.12		C.V. (%) = 32.72		

[1/] Means within a column followed by the same letter are not significantly different (P=0.05) by DMRT

From primer pair screening, 4 pairs could not generate any amplification product, 4 could only generate monomorphic band, and 27 could generate at least one polymorphic band. The estimation of similarity was based on 103 polymorphic bands which were calculated and used for dendrogram construction. Similarity coefficient showed that the genetic similarity of

Table 2 Thirty five primer pairs designed from sequence of pathogenesis-related genes, plant disease resistance genes and defense-related genes.

Primer	Gene	GenBank Accession Number
PR1	antifungal	D90196
PR2	fl-1,3-glucanase	AF435089
PR5	thaumatin-like protein	X89008
PR8	chitinase type III	AF241266
PR9	lignin forming peroxidase	NM101321
PR10	ribonuclease-like	X61365
PR12	defensin	X91916
PR14	lipid-transfer protein	AY395741
PR15	oxalate oxidase	X91957
PR16	oxalate oxidase-like	U75206
Chi	chitinase	X16938
Rcht2	chitinase (Rcht2) gene	L40336
RPS2	RPS2	U12860
Pib	Pib	AB013448
Xa1	XA1	AB002266
Xa21	receptor kinase-like protein gene	U37133
I-SOD	iron-superoxide dismutase	AB014056
CBP	calcium-binding protein	AB021259
APX1	ascorbate peroxidase	AB050724
SalT	salT	AF001395
C/Z-SOD	copper/zinc-superoxide dismutase	D00999
APX2	ascorbate peroxidase	D45423
CatB	CatB gene (catalase)	D64013
OsMADS45	MADS box protein	U31994
ATPase	Ca^{2+}-ATPase	U82966
Rab21	water-stress inducible protein	Y00842
Waxy	waxy gene	X64108
ATN	metal transporter	Q9SAH8
Fe	yellow stripe1	AF186234
Fe1	yellow stripe1	AF186234
Fe2	yellow stripe1	AF186234
IRT	Zrt-Irt related protein	NM179215
MY1	maize yellow stripe1	-
ZIP1	zinc transporter	AF033535
ZIP4	metal transporter	NM100972

the 12 eucalypt clones ranging from 23-100%. At 35% similarity level, 12 clones were clustered into two distinct groups. Six resistant clones were clustered into cluster A and the other six susceptible clones were clustered into cluster B. This result was similar to the grouping of disease reaction. Six primer pairs could amplify DNA fragments and using as markers to identify resistant or susceptible eucalypt clone. The Ce1 and Ce5 markers were amplified by PR5 and the Ce2, Ce3, Ce4 and Ce6 markers were amplified by RPS2, Pib, Xa1 and ATN, respectively. The Ce1, Ce2 and Ce3 marker could identify resistant clone. Ce1 marker (Figure 1) showed 220 bp but Ce2 marker gave 750 bp on polyacrylamide gel while Ce3 marker had 420 bp on agarose gel. On the other hand, Ce4 (Figure 2), Ce5 and Ce6 markers gave 340 bp, 420 bp and 510 bp, respectively, on agarose gel for identification of susceptible clones.

The Ce1, Ce2, Ce3 and Ce4 markers were compared to see their sequence similarity using BlastN programs. The results showed that they were not homology to any defense response genes. However, Ce2 marker showed 82% and 78% homology in two regions with the sequence in the GenBank accession number NM_128386 encoded cyclic nucleotide-regulated ion channel of *Arabidopsis thaliana.*

The nucleotide sequence of Ce4 marker in the susceptible group resembled the sequence in resistant group but contained 60 base pairs shorter than the resistant group, except SF36 which had different nucleotide sequence from the others.

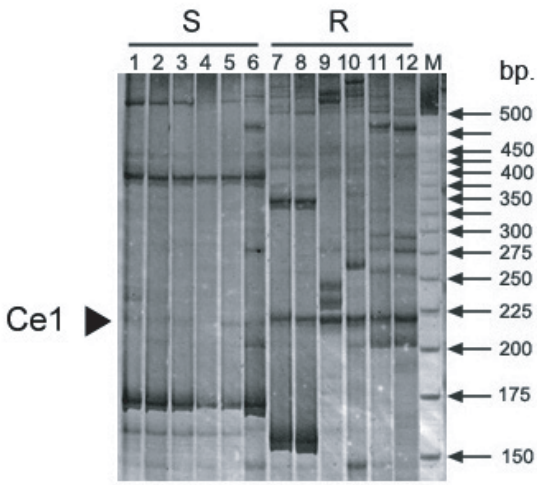

Figure 1 Polymorphic bands of susceptible and resistant clones amplified by the PR5 primer pair on 6% polyacrylamide gel. M : 25 bp. DNA ladder, lane 1-12 : SF03, SF07, SF14, SF16, SF70, SF86, SF18, SF01, SF06, SF36, SF94, and SF98.

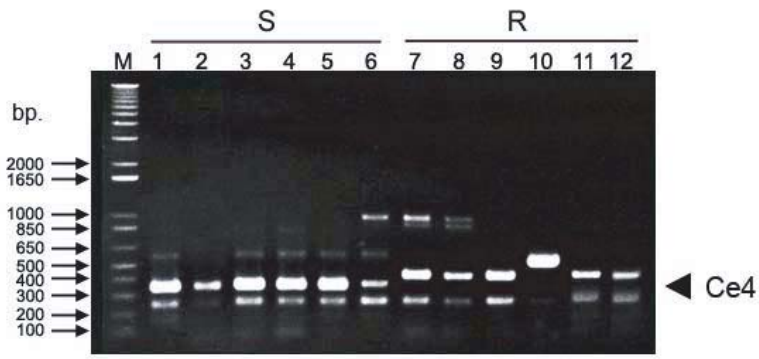

Figure 2 Polymorphic bands of susceptible and resistant clones amplified by the Xa1 primer pair on 1% agarose gel. M : 1 kb. DNA ladder, lane 1-12 : SF03, SF07, SF14, SF16, SF70, SF86, SF18, SF01, SF06, SF36, SF94, and SF98.

DISCUSSION

Detached leaf inoculation method was more efficient to screen the resistant and susceptible eucalypt clones than using intact seedling inoculation method. Seedling inoculation method gave rather low average disease percentage which probably due to instability and high level of incubation temperature (24-33°C). This was similar to the work done by Sankaran *et al*. (1995) having this fungus inoculated on seedling in a high humid chamber at 32±2°C which gave no development of leaf spot symptoms, even for a month long of inoculation. Old *et al*. (2002) also reported that growth and sporulation of this fungus at 32°C reduced when compared to that at 24°C. The suitable temperature for symptom development should be limited to the range of 23-30°C.

From the field evaluation of leaf spot, shoot blight and canker caused by *C. eucalypti* showed that CT76 had low disease severity, while CT37 had high disease severity (Doungnamol, 2004). This was similar to the severity caused by SF06 and SF07, thereby, SF06 or CT76 was classified as resistance while SF07 or CT 37 was classified as susceptible.

In this study, PAGE was more effective to detect polymorphism than using agarose gel electrophoresis. These observations corresponded to Chen *et al*. (1998) showing that numerous PCR-amplified products could be detected by high-resolution electrophoresis, which greatly increased the power of detecting polymorphism. Furthermore, their study indicated that the resistance-gene analogous (RGA) markers could serve as candidate genes for cellular recognition and informative markers for inferring genetic relationships among germplasms. In this study, the conserved sequence of R genes was also used to design primers. DNA fragment derived from PCR amplification by these primer pairs was speculated to be RGA. So, markers Ce2, Ce3 and Ce4 might be RGA markers and they might be candidate genes for cellular recognition. However, nucleotide sequences of these markers were not similar to the other sequences of R gene and RGA. In contrast to this study, Cordero and Skinner (2002) reported that degenerate oligonucleotide primers designed from conserved regions of the nucleotide binding site (NBS) were used to amplify the NBS regions from genomic DNA of alfalfa (*Medicago sativa* L.). These sequences of RGA were highly homologous to NBS regions of other species. Noir *et al.* (2001) reported that RGA could be isolated by PCR with degenerate primers designed from two conserved motifs in the NBS regions of R genes of various plants. Moreover, nine distinct classes of RGA of the NBS-like type, representing a highly diverse sample, were isolated from *Coffea arabica* and *C. canephora* species.

In mutational analysis of *Arabidopsis dnd1* (defense, no death) gene encodes a mutated cyclic nucleotide-gated ion channel. The *dnd* mutants have high salicylic acid levels but do not produce hypersensitive response (HR) to avirulent *Pseudomonas syringae* pathogens (Yu *et al.*, 1998; Clough *et al*., 2000). Although lacking of the HR, *dnd* mutants could give high levels of PR-gene expression and limit pathogen growth. In this study, the sequence of Ce2 marker for cyclic nucleotide-regulated ion channel of *Arabidopsis thaliana* was found in two regions which was due to the short sequence of this marker.

CONCLUSION

Six markers can be used for selection of eucalypt resistant clones. Markers for resistant clone identification were composed of Ce1, Ce2, and Ce3 marker while markers for susceptible clone identification were composed of Ce4, Ce5, and Ce6 marker. To obtain the best results for clonal selection, all markers from both groups should be used. If the core markers have to selected, Ce1 and Ce4 would be the most suitable

ones. For more efficient markers, all of them should be tested in several populations of the cross between susceptible and resistant clones.

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