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Development of SCAR Markers for Rapid Identification of Resistance to *Phytophthora* in Durian using Inter Simple Sequence Repeat Markers

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ABSTRACT: Eighty inter simple sequence repeat (ISSR) primers were applied to detect polymorphisms among eight durian clones. From eighty ISSR primers used, only six primers UBC824, UBC860, UBC886, UBC888, UBC889 and UBC891 generated distinct polymorphic fragments. Out of the total of 42 bands produced from six primers, eight were polymorphic. Only 5 fragments successfully cloned to convert into sequence characterized amplified regions (SCAR) markers. Based on the sequences of the cloned ISSR fragments, six ISSR-SCAR primer sets were produced. Three SCAR markers designed from this study were examined using genomic DNA extracted from 4 accessions of *Durio lowianus* and *Durio kutejensis* and 6 accessions of hybrids and clones of *Durio zibethinus*. Resistance to *Phytophthora* infection is an important objective for cultivar improvement programmes of Durian. Molecular markers linked to such key traits can be used to screen resistant individuals for future breeding. ISSR-SCAR marker 886-2 was identified to separate the resistant and susceptible *Durio* species towards *Phytophthora* infections.

Keywords: Durio; Canker; Resistance; Susceptible; Disease and Sequencing.

INTRODUCTION: Durian is one of the important fruit in Malaysia. The country is the world's second largest producer and exporter of durian after Thailand (Vanijajiva, 2012). There are several problems in maintaining durian orchard. One of the most important problems in durian cultivation is the susceptibility to stem canker caused by *Phytophthora palmivora* (Muryati *et al*., 2009). The disease of *Phytophthora* is one of the most serious problems attacking durian plants occurring everywhere in cultivated area including Malaysian Agricultural Research and Development Institute (MARDI) genebank, which represent the world largest collections of durian.

This disease can attack any stage of the plant from seedlings, matured and old trees. The fungus can attack the trunk, twigs, branches, leaves, flowers, fruit and the underground parts of the stem and roots. Various cultivation methods and chemicals have been applied and proven to be somewhat effective (Muryati *et al*., 2009). However, their effectiveness is only for short period of time. Using resistant genetic resources is considered to be the most effective approach against this disease for long-term cultivation (Mohd Shamsudin *et al*., 2000). However, information on the genetic backgrounds of durian genetic resources is still lacking and there is a need to evaluate and utilize the genetic diversity of durian in breeding for resistant varieties.

Breeding of durian for resistance to *Phytophthora* has become an important objective of durian crop improvement program. Conventionally, durian plants were selected based on the visible or measurable traits called the phenotype. Phenotypic reaction to infection of *Phytophthora palmivora* has been previously conducted for MARDI germplasm. Wounded barks of durian trees were inoculated with *Phytophthora palmivora* isolates to observe the reaction (Nik Masdek, 2008). Unfortunately, this conventional method can be difficult due to the influence by the environmental factors and costly. Identification of the disease resistance trait based on molecular markers is a practical method to be approached. Identified region of DNA that showed resistance to *Phytophthora* would be used as a marker to track and develop the trait. These markers will help researchers especially breeders to make selection for resistant plant at the stage of seedlings. The objectives of this study were to develop ISSR-SCAR markers of durian and to identify the potential ISSR-SCAR markers associated with *Phytophthora* resistance in *Durio* species.

MATERIALS AND METHODS:

PCR amplification with ISSR primers: Approximately 80 ISSR primers were tested on 8 durian clones. The reaction volume was 20µl containing 20ng genomic DNA, 1X PCR buffer, (various=1/2) mM MgCl2, 0.2mM dNTPs, 0.6µM primer and 1U Taq polymerase (vivantis). The thermal cycler conditions were 2 min at 94°C; followed by 40 cycles of 94°C for 1 min, annealing temp (various °C) for 1 min, and 72°C for 2min; followed by a final elongation at 72°C for 8 min. A total of 8 distinct polymorphic fragments were selected from 6 ISSR Primers (Table 1).

Cloning of polymorphic fragments and designing of SCAR primers: These fragments were excised and purified using QIA quick Gel Extraction kit (QIAGEN). Then, these fragments were cloned using TOPO TA Cloning Kit for sequencing (Invitrogen). DNA fragments were ligated to vector pCR-TOPO. Then, these ligated products were transformed chemically into *E. coli* competent cells (One Shot TOP10) through heat shock process (42°C for 1 min). The transformed cells were added with SOC medium and incubated at 37°C and lastly spread onto selective LB agar with X-gal and 100µg/ml Ampicillin. The agar plates were incubated 37°C overnight. Single colonies were selected and cultured in 10µl selective LB broth (100µg/ml Ampicillin) at 37°C for 1hour. After that, colony PCR was carried out to identify clones carrying the desired DNA fragment. The right clones were incubated in 8ml selective LB broth (100µg/ml Ampicillin) at 37°C overnight. Plasmids were extracted using QIAprep Spin Miniprep Kit (QIAGEN). The extracted plasmids were digested with enzyme EcoRI (37°C, 5hours) to confirm the presence of the desired insert within the plasmid. The right clones (in plasmid form) were then sent for sequencing. From those fragments, only 5 fragments were successfully cloned and sent for sequencing. Based on these sequences, ISSR-SCAR primers had been designed using primer premier 5 software.

Detection of SCAR markers associate to Phytophthora resistance: DNA samples of 2 Durio species, *Durio lowianus* and *Durio kutejensis*, 3 hybrid of clones (MDUR78, MDUR79, MDUR88) and 1 clone (D24) of *Durio zibethinus* were collected at MARDI field genebank in Serdang, Selangor. Samples have been earlier identified for their phenotypic reaction to *Phytophthora palmivora* infection (Table 2). Only plants that showed extreme reaction of resistant and susceptible to *Phytophthora* infection were used in this experiment (Table 2). Sample of young leaves from each species were extracted using i-genomic Plant DNA Mini Kit from iNtRON Biotechnology. DNAs obtained were quantified by electrophoresis using 1 % agarose gel and 100bp DNA ladder. DNAs concentrations were quantified using spectrophotometer.

A total of three SCAR primers that have been converted from ISSR polymorphisms were used in this experiment which are ISSR-SCAR 888, ISSR-SCAR 886-1 and ISSR-SCAR 886-2 (Table 3). DNA amplification was performed in a 20µl reaction volume containing 20ng genomic DNA, 1x PCR Buffer, 1 or 2 mM $MgCl_2$, 0.2mM dNTPs, 0.6µM primer, and 1U Taq Polymerase. Amplification was performed in a thermo-cycler. The PCR assay was performed in 41 cycles: 1 cycle of 2 minutes at 94ºC, followed by 40 cycles of 1 minute at 94ºC, 1 minute at annealing temperature (various ºC), 2 minutes at 72ºC and finishing with an extension step of 8 minutes at 72ºC. The amplification products were separated in 1.4% agarose gel. The gel was stained with Syber Safe and allele size was estimated by comparing with 100bp DNA ladder. DNA fragments were photographed using Gel documentation system and stored as digital pictures.

RESULTS:

Development of SCAR markers: From eighty ISSR primers used, only six primers UBC824, UBC860, UBC886, UBC888, UBC889 and UBC891 generated distinct polymorphic fragments. From these six primers, number of amplicons per primers varied from 4 (UBC860) to 12 (UBC889) and the amplicon sizes varied from 750bp to 1400bp (Table 1). Out of the total of 42 bands, 8 bands (18.6%) were polymorphic with an average of 1.3 polymorphic bands per primer (Table 1). Figure 1 showed the polymorphic banding profile generated using six primers UBC824, UBC860, UBC886, UBC888, UBC889 and UBC891. From those eight polymorphic fragments, only 5 fragments successfully cloned to convert into sequence characterized amplified regions (SCAR) markers. Based on the sequences of the cloned ISSR fragments, six ISSR-SCAR primer sets were produced (Table 3).

Detection of SCAR markers associate to Phytophthora resistance: Three SCAR markers designed for this study were examined using genomic DNA extracted from 4 accessions of *Durio lowianus* and *Durio kutejensis* and 6 accessions of hybrids and clones of *Durio zibethinus*. All of the primers yielded clear banding profiles with the size of amplicon varied from 1 to 3 and the amplicon size varied from 200bp to 1250bp (Figure 2, 3 & 4). All of the bands, which are 5 in total, were polymorphic (Figure 2, 3 & 4). From these 3 SCAR primers, Primer 886-2 separated the

resistance and susceptible *Durio* species (Figure 4). Five known resistant individual amplified the SCAR markers 886-2. They were 2 accesions of *Durio lowianus*, 1 accession of *Durio kutejensis* and 2 hybrids of *Durio zibethinus* MDUR78 and MDUR79.

Table 1: List of Inter Simple Sequence Repeat (ISSR) primers showing genetic polymorphisms among eight durian clones.

ISSR primer (UBC)	Primer sequence*	PCR condition [$MgCl_2$] & Annealing Temp.	Fragment size	Total Bands	No. of polymorphic bands
824	$(TC)_8G$	2mM;45°C	1.4kb	6	1
860	$(TG)_8RA$	2mM;45°C	800bp	4	1
886	$VDV(CT)_7$	2mM;45°C	1-800bp 2-780bp	7	2
888	$BDB(CA)_7$	1mM;40°C	850bp	8	1
889	$DBD(AC)_7$	1mM;45°C	1-950bp 2-1.0kb	12	2
891	$HVH(TG)_7$	1mM;45°C	750bp	6	1

**Single letter abbreviations for mixed-base positions: Y = (C, T), R = (A, G), B = (non A), D = (non C), V = (non T), H = (non G).*

Table 2: The plant materials collected from MARDI field gene bank, Serdang and their reactions to *phytophthora.*

No.	Samples Name	Reactions to *Phytophtora*	Samples ID
1.	*Durio lowianus* (Wild species)	Highly Resistant (Nik Masdek, 2008; Mohd Shamsudin et al., 2000)	D1
2.	*Durio lowianus* (Wild species)	Resistant (Nik Masdek, 2008)	D2
3.	*Durio kutejensis* (Wild species)	Resistant (Nik Masdek, 2008)	D3
4.	*Durio kutejensis* (Wild species)	Susceptible (Nik Masdek, 2008)	D4
5.	*Durio zibethinus* (Hybrid : MDUR88 – D24x D10)	Intermediate (Zainab & Abidin, 2008; Perak Agriculture Department, 2013)	D5
6.	*Durio zibethinus* (Hybrid : MDUR88-D24x D10)	Intermediate (Zainab & Abidin, 2008; Perak Agriculture Department, 2013)	D6
7.	*Durio zibethinus* (Hybrid : MDUR79-D24x D10)	Resistant (Zainab & Abidin, 2008; Perak Agriculture Department, 2013)	D7
8.	*Durio zibethinus* (Hybrid : MDUR78 – D10xD24)	Resistant (Zainab & Abidin, 2008; Perak Agriculture Department, 2013)	D8
9.	*Durio zibethinus* (Clone : D24)	Very susceptible (Tai, 1971)	D9
10.	*Durio zibethinus* (Clone : D24)	Very susceptible (Tai, 1971)	D10

Table 3: Characteristics of the SCAR loci derived from ISSR markers selected from previous study.

No.	Oligo Name	Primer sequence (5'-3')	No. Bases	Tm value	GC (%)
1.	886-1.F	CCC TTG TAT TTT TTC CTA TC	20	45.0	35.0
	886-1.R	CTC TCT CAT GCT TCT CCC AAC	21	52.7	52.4
2.	888.F	CAC ACA GCA GCA ACA CAA C	17	49.9	52.6
	888.R	GTT GGT GGT GGA GGC AGT C	18	54.7	63.2
3.	889-2.F1	GGG AAC ACG GCA AGG GGA GT	19	61.7	65.0
	889-2.R1	GCT ACG GCG GAG GGC AAA GT	19	62.1	65.0
4.	886-2.F	CTC TCT TAT CCA GGG GG	20	45.8	58.8
	886-2.R	CTT TGC TGT CAT CTG GGT	20	47.3	50.0
5.	891.F	GTG TGT GAT GGA CAG TGA A	19	45.5	47.4
	891.R	CTT TTG CTG TTT CAC ATT CA	19	48.4	35.0
6.	889-2.F2	CAC ACA AGA ATT TAG ATG G	19	41.9	36.8
	889-2.R2	CAC ACA CAC ATA TAT ACA T	20	33.3	31.6

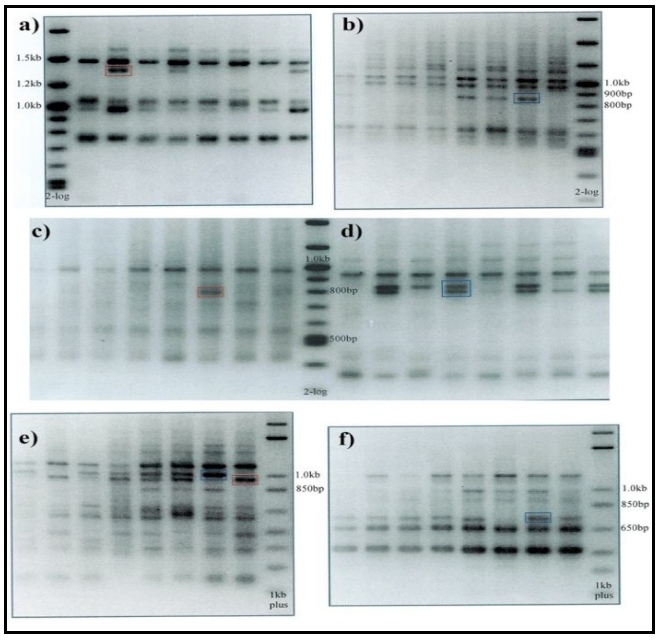

Figure 1: Polymorphic banding patterns of eight durian clones generated using ISSR primers. a) UBC824 b) UBC888 c) UBC860 d) UBC886 e) UBC889 and f) UBC891. Abbreviations ; red box , Failed to clone; blue box, Successfully cloned.

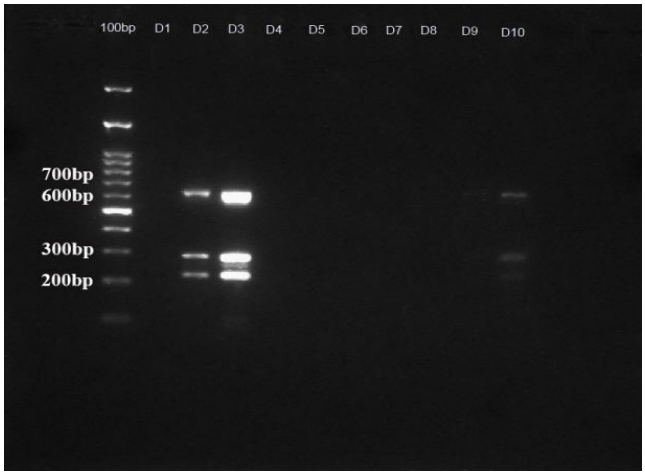

Figure 2: Polymorphic banding patterns of *Durio* species generated using ISSR-SCAR primers 886-1.

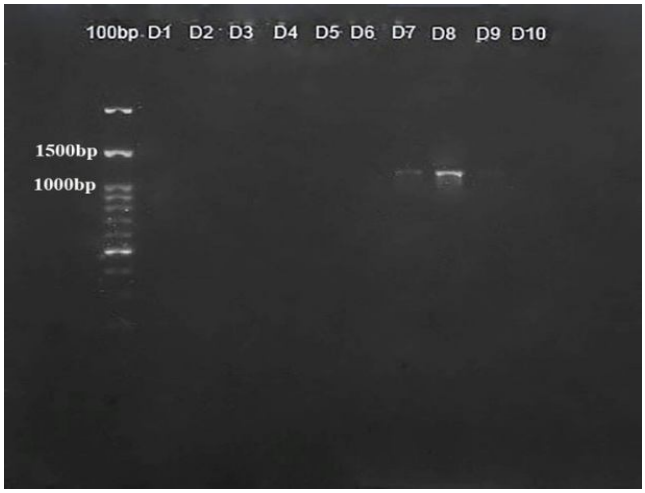

Figure 3: Polymorphic banding patterns of *Durio* species generated using ISSR-SCAR primers 888.

Figure 4: Polymorphic banding patterns of *Durio* sp. generated using ISSR-SCAR primers 886-2.

DISCUSSION: The SCAR markers have some advantages over ISSR markers to distinguish *Durio* plants. Due to ISSR dominant nature, the amplification of multiple loci and their sensitivity to reaction conditions restricted their further use. SCARs are more independent to reaction conditions and are usually codominant markers that amplify a single locus, making them robust and more reproducible assay than could be obtained with short primers used for ISSR analysis (Bautista *et al.*, 2002). Since SCAR markers can identify a single or a few bands instead of a complex pattern, SCAR markers are more straightforward to be analyzed than other molecular techniques such as RAPD, ISSR, SSR and AFLP (Luqua *et al.*, 2013). The SCAR marker technique has been successfully applied in many crops for the marking of resistance genes and marker-assisted selection (Evans and James, 2003; Ardiel et al., 2002; Jei-Wan et al., 2011; Dong Hyeon et al., 2013). In this study, clear and bright, amplified DNA bands from ISSR markers were selected for SCAR marker development. In SCAR, pairs of 17-21bp oligonucleotide primers specific to the sequence of polymorphic bands can be used to amplify the characterized regions from genomic DNA under stringent conditions, which makes this marker more specific and dependable as compared to ISSR marker.

Durio lowianus (D1) reaction towards *Phytophthora palmivora* was the highest resistance among all the durian clones and species used in this study. It can be the best benchmark for choosing the desirable primers which is resistant to *Phytophthora*. MDUR78 and MDUR 79 hybrid were also both resistant to *Phytophthora* infections and also claimed by Zainab & Abidin (2008) and Perak Agriculture Department (2013) to be resistant towards *Phytophthora*. *Durio zibethinus* D24 clone was known to be susceptible to *Phytophthora* disease. The samples were taken from trees that were severely infected by *Phytophthora palmivora*. The study shown that SCAR primer 886-2 detected the *Durio lowianus* (D1 & D2), *Durio kutejensis* (D3), MDUR78 (D8) and MDUR 79 (D7) hybrid only. The D24 clones (D9 & D10) were not amplified with the primer 886-2. This preliminary work to detect *Phytophthora* resistance, has shown that SCAR primer 886-2 can be used to screen for durian trees or seedlings which have the resistance towards *Phytophthora* infections for future breeding or as rootstocks.

Arguably, this markers need to be further tested on the F1 progenies of the resistance and susceptible clones before it can be used. Further study on marker assisted selection (MAS) need to be done. Seggregation of the full progeny for *Phytophthora* resistance must be tested with this marker. SCAR markers by directly sequencing from ISSR products were useful for the MAS and suitable for further applications such as cultivar identification and mapping (Busconi *et al.*, 2006).

CONCLUSION: Six ISSR-SCAR primer sets were produced from three successfully cloned polymorphic ISSR primers which are ISSR-SCAR 886-1, ISSR-SCAR 888, ISSR-SCAR 886-2, ISSR-SCAR 889-2.1, ISSR-SCAR 889-2.2 and ISSR-SCAR 891. ISSR-SCAR marker 886-2 was identified to separate the resistant and susceptible *Durio* species towards *Phytophthora* infections.

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