[BIO07] Identification of molecular markers for disease resistance genes to *Fusarium oxysporum* f. sp. *cubense* in *Musa acuminata* ssp. *malaccensis* for marker assisted selection (MAS)

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Introduction

Fusarium wilt is a serious soil-borne disease and caused considerable loss to the banana export trade (Rutherford, 1999). Four physiological races of FOC have been reported based on their selective pathogenecity in different banana cultivars. Race 1 had been found virulent on Gros Michel (AAA genotype) while Race 2 on Bluggoe (ABB) and Race 3 on Heliconia. Finally, Race 4 attacking the Cavendish cultivars (AAA) and also virulent on Gros Michel and Bluggoe. Numerous disease control such as soil amendment with Ca or organic matter as well as fumigation with methyl bromide but it only provide temporary solution to this problem. Therefore planting disease resistance bananas is the best approach as it is economical, effective and practical in the long run.

Attempts on developing resistant clones to Fusarium wilt using conventional breeding have limited success due to low reproductive fertility, polyploidy and complete sterility. Wild banana *Musa acuminata* ssp. *malaccensis* showed a high resistance to *Fusarium oxysporum* f. sp. *cubense* race 4. The seed progenies derived from *in vitro* embryo culture had been found to be segregating for resistance and susceptible to FOC race 4. Therefore, they seem to be a great source for the breeding strategies as they are fertile and produce a large number of seeds.

Recent development in molecular marker provided tools for genetic relationship studies among breeding lines (Staub & Serquen, 1996; Saghai *et al*., 1997). Quantitative Trait Loci (QTL) analysis made possible by numerous improved method of molecular marker analysis. Identification of important QTL regions could enhance plant breeding. PCR markers such as RAPDs, microsatellites and AFLPs which were cheaper, safer and more markers per unit of DNA provided framework map around which the polygenes/QTL could be located (Kearsey & Farquhar, 1997). Identification of important QTL regions could enhance plant breeding efficiency by marker-assisted selection (MAS).

Materials and Methods

Development of wild banana seed progenies and F_1 hybrid populations.

Embryo rescue culture of four wild bananas *Musa acuminata* ssp. *malaccensis* from different locations was carried out (Asif *et al.*, 2000). Seeds were extracted and sterilized with 50% Chlorox and 70% ethanol before exposing the embryos. Embryos were transferred on to MS media under sterilized condition and kept in the dark for a week to induce rooting before exposing it into light for shoot elongation. Individual plantlets were subcultured several times to develop clonal population and hardened in the greenhouse for Fusarium screening.

Screening to FOC race 4.

FOC (*Fusarium oxysporum* f. sp. *cubense*) race 4 was isolated from infected corm tissue of Cavendish bananas (AAA) and cultured onto 1% (w/v) PDA (potato dextrose agar) media and maintained at 28°C with 16h light. Conidial suspension was prepared by transferring the mycelia into Armstrong liquid media containing sucrose 20g; $MgSO_4$. $7H_2O$, 400mg; KCl, 1.6g; KH_2PO_4,, 1.1g; $Ca(NO_3)_2$, 5.9g; $FeCl_3$, 0.2 g/ml; $MnSO_4$, 0.2 g/ml; $ZnSO_4$, 0.2 g/ml. Spores suspension was measured into a concentration of 10^6 spores/ml by using haemocytometer.

Screening was carried out using double tray method (Mohamed *et. al.,* 1999) and `Hot Spot' trial and evaluation of disease infection was done

based on LSI and RDI symptoms followed by Brake *et al*, (1995).

RAPD Analysis

DNA was extracted using a modified CTAB method based on the protocol of Doyle and Doyle (1987). RAPD analysis was carried out following William et al, (1990) with modifications by Weising et al, (1995) using 94ºC for 4 minutes for initial denaturing and 45 cycles of [15s 94ºC denaturing, 45s at 36ºC annealing, 90s at 72ºC extension] and a final extension at 72ºC for 4 minutes. Final concentration of the reaction solution was 1.5mM $MgCl_2$, 100µM dNTPs, 100mM PCR buffer, 0.5µM primer, 100ng template DNA and 0.2U/10 l of Taq DNA polymerase (Promega). PCR products were run in 1% (w/v) agarose gel and run at 80V for 2 hours. Amplified DNA markers visualized by Geldock were scored as present (1) and absent (0) bands.

STMS analysis

STMS was performed following Kaemmer *et al*., 1997 using twenty three different primer sets. PCR conditions were 94ºC for 4 minutes for initial denaturing and 35 cycles of [30s 94ºC denaturing, 30s at annealing temperature (depending on primer pair), 30s at 72ºC extension] and a final extension at 72ºC for 10 minutes. Final concentrations in the reaction solution was 1.5mM $MgCl_2$, 100µM dNTPs, 100mM PCR buffer, 0.5µM primer, 50 ng template DNA and 0.2U/10 l of Taq DNA polymerase (Promega).

PCR products were analyzed on by 6% to 8% polyacrylamide gels at 20-25V/cm, 55ºC fo 3 hours. Gels were stained by first fixing in 10% (v/v) ethanol and 5% (v/v) acetic acid for 20 minutes followed by 0.1% (w/v) $AgNO_3$ containing 0.06% formaldehyde for 30 minutes. Signals were developed by soaking gels in 500ml of 3% Na_2CO_3 with 200µl of a 10% (w/v) $Na(S_2O_3)_2$ and 1.5ml of 38% formaldehyde. Gels were rinsed with distilled water and photographed. Homozygous alleles were scored with the presence of fast or slow moving single band while heterozygous alleles showed two bands.

Results

Development of wild banana seed progenies and F_1 hybrid populations.

Four populations of *Musa acuminata* ssp. *malaccensis* wild seed population were developed from samples collected from four different locations. Germination rates of the populations were 66.67% from IPTJ (University of Malaya), 60.0% from GH (Genting Highland), 48.0% from BARI (Johol, Negeri Sembilan) and 49.3% from MIKU (Negeri Sembilan). However, due to labour and time constraints only IPTJ populations were selected for crosses and further analysis.

Germination rates of hybrid populations were 56 plants (26.7%), 89 plants (35.6%), 78 plants (38.0%) and 98 plants (43.75%) respectively had been obtained through embryo rescue technique.

Screening against FOC race 4

Screening was done on IPTJ population in 2 replicates. A total of 137 wild banana plantlets were screened along with 20 Berangan (AAA) plantlets as a controlled. All of 20 control plants were died showing FOC race 4 symptoms. Results of double tray screening showed different response to *Fusarium oxysporum* f. sp. *cubense* race 4 inoculations. Thirty six plants (26.3%) were showing resistance with the scale 0 and 1, sixty one plants (44.5%) tolerance (scale 2-4) while forty (29.2%) was susceptible to FOC race 4 with the scale of 5 and 6.

Results were recorded after a year of screening in the `Hot Spot'. Out of 60 plants tested, 54 plants (90.0%) were resistant to FOC race 4 whereas another 6 plants (10.0%) and all the control plants succumbed to FOC race 4 within 4-5 months. Dissection of rhizome revealed no disease symptoms in resistant wild banana seed progenies tested.

RAPD Analysis

Five primers (Table 1) adapted from Howell *et al*., (1994) were used to screen wild banana seed progenies. 1.5 mM of $MgCl_2$ that produced defined scorable band (Javed *et al*., 2004) was used for amplification.

TABLE 1 Nucleotide sequences of four arbitrary 10-mers primers used for screening.

Primer code	5' Sequence 3'
OPA –3	AGTCAGCCAC
Primer-21	CGCTGTCCTT
Primer-24	GTGCGTATGG
Primer -25	GACAGACAGA
Primer-27	CTCTCCGCCA

All primers showed large amount of polymorphism among the seed progenies of IPTJ populations with the number of scorable RAPD bands varied from 4-8 and the average of six markers per primer. In general, amplified fragments were range from 200-1500bp. Primer-27 showed two bands (350bp and 680bp) observed consistently in most of the plants (Figure 1) with the percentage of 78.5% and 72.3% respectively.

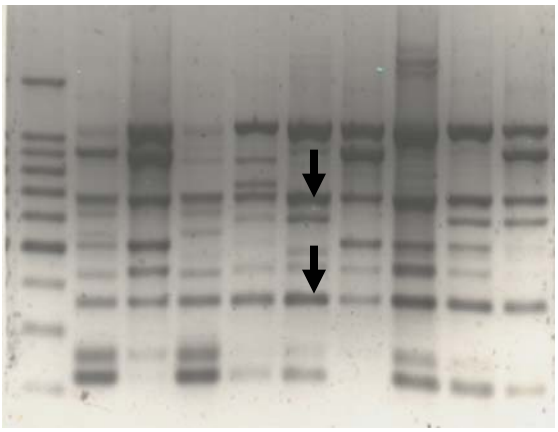

FIGURE 1 Primer-27 produced average of seven major scorable bands with two major bands observed in most of the plants.

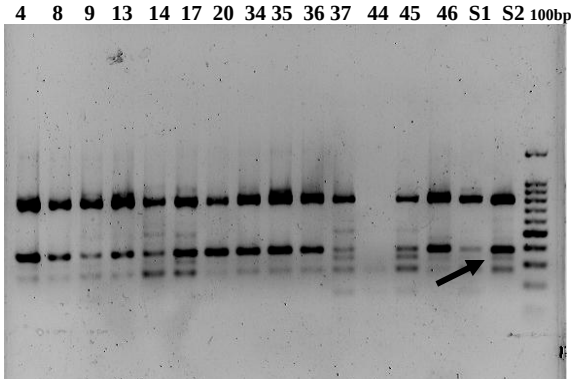

FIGURE 2 Primer-25 showing 250bp bands present in the resistant mother plant but absent in the susceptible

OPA-3 showed three bands (1.0kbp, 350bp and 250bp) shared by most of the plants with the percentage of 67.7%, 64.6% and 73.9% respectively. Primer-21 showed three bands (1.0kbp, 800bp and 250bp) shared by most of the plants with the percentage of 69.2%, 75.4% and 69.2% respectively while primer-24 showed two bands of 300bp and 250bp, which shared by most of the plants with the percentage of 95.4% and 98.5% respectively.

However in RS hybrid populations, Pri-21 showed 3 bands (700bp, 450bp and 350bp) shared by most of the hybrid populations with the percentage of 80.4%, 76.1% and 85.5% respectively. In comparison to its parents, 14 plants shared 250bp bands present in the susceptible parent but absent in resistant parent. Primer-25 showed 3 bands (800bp, 390bp and 280bp) shared by most of the plants with the percentage of 88.4%, 86.1% and 74.4% respectively. 7 plants shared 250bp bands present in the resistant mother plant but absent in the susceptible parent (Figure 2).

TABLE 2 Table showing variation of seven microsatellite loci in *Musa acuminata* ssp. *malaccensis*

Primer Sets	5'-primer sequence-3'	Annealling temperature	λ^2 value
AGMI 2 AGMI25	TTTGATGTCACAATGGTGTTCC TTAAAGGTGGGTTAGCATTAGG	55°C	4.4401^{ns}
AGMI 9 AGMI 93	GATCTGAGGATGGTTCTGTTGGAGTG AACAACTAGGATGGTAATGTGTGGAA	55°C	0.7257^{ns}
AGMI 10 AGMI 103	CCCTTTGCGTGCCCCTAA ACAGAATCGCTAACCCTAATCCTCA	55°C	5.298^{ns}
AGMI 35 AGMI 36	TGACCCACGAGAAAAGAAGC CTCCTCCATAGCCTGACTGC	55°C	49.197*
AGMI 105 AGMI 108	TCCCAACCCCTGCAACCACT ATGACCTGTCGAACATCCTTT	53°C	2.973^{ns}
STMS 1FP STMS 1RP	TGAGGCGGGGAATCGGTA GGCGGGAGACAGATGGAGTT	60°C	12.913*
STMS 13FP STMS 13RP	TTGAAGTGAATCCCAAGTTTG AAAACACATGTCCCCATCTC	50°C	20.202*

* Significantly different at 0.05 level of significance
ns non-significance at 0.01-0.05 levels

STMS Analysis

All STMS primer sets required optimization due to unexpected negative results from all PCR reactions under standard condition. Optimization was carried out by varying the annealing temperature between 55°C to 65°C while maintaining the concentration of other factors ($MgCl_2$, primers, buffer and DNA). Optimum annealing temperatures were selected for further use.

A total of 23 sets of primers were tested to amplify specific products in PCR reactions of wild *Musa acuminata* seed progenies. Of the 23 primers tested, seven (30.4%) amplified products resulting in discrete, repeatable polymorphic bands, twelve (52.2%) amplified unspecific products; even changing the amplification conditions and four (17.4%) did not amplify products in some genotypes. The diversity of alleles in seed progenises of *Musa acuminata* ssp. *malaccensis* using seven sets of primers was studied. Data of the allelic frequencies were tested for genetic equilibrium to Hardy Weinberg by using Chi-square test (Table 2).

Disscussion

Allelic disequilibrium observed in some of the markers could be due to the small sample size had been used. In small population, allele frequencies can be altered by random genetic drift, which refer to random changes in allele frequencies due to sampling error. In other words, allele frequencies may drift from generation to generations as matter of chance. Division of population into sub-populations or groups as observed in case of wild bananas reduces the genetic variability and increases homozygosity. Since mating between similar individuals (inbreeding) takes place in subpopulations, genetic variability within the group decreases with some genes fixed while some are eliminated thus increasing homozygosity (Wright, 1951).

STMS markers proved to be useful because of their highly reproducibility results obtained. However cost of production of these markers is relatively higher. Optimization of PCR condition

is still needed to avoid null alleles. Several attempts were carried out including altering the annealing temperatures, applying touch down program and adjusting the $MgCl_2$ concentration. To ensure that the occurrence of null allele was not a failure of reaction, the assays were repeated several times. Only those giving quality sharp bands were selected for further analysis. More markers are still needed for QTL analysis and mapping in order to facilitate the effectiveness of these markers for MAS.

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