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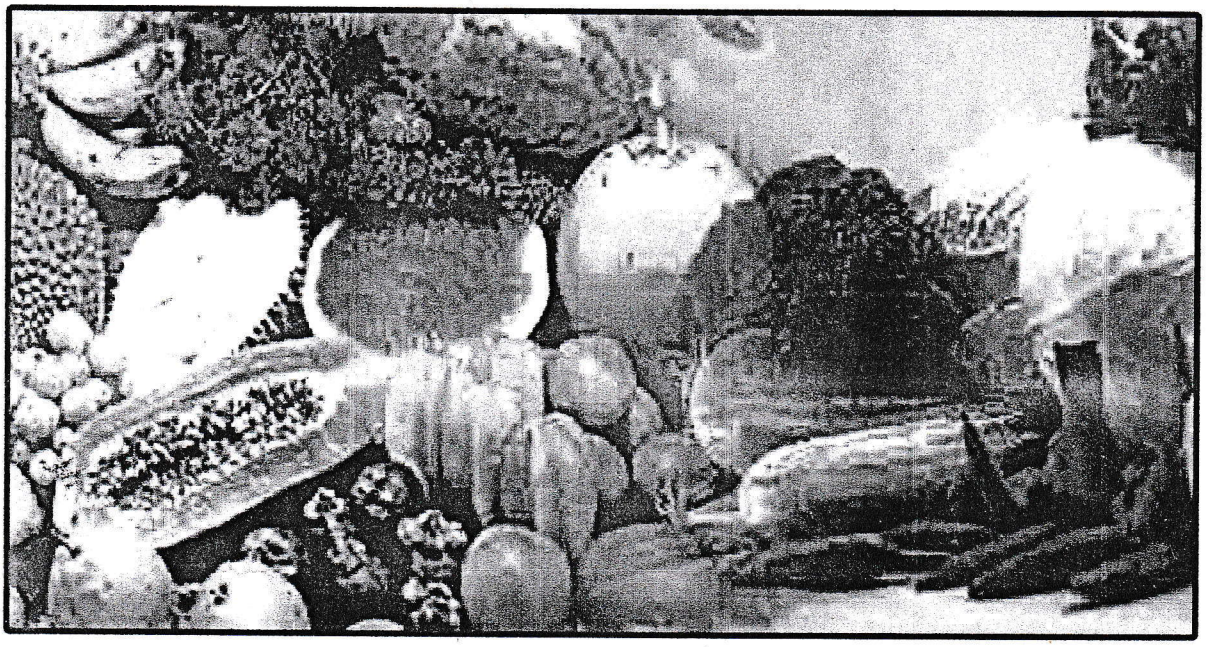

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INTROGRESSION OF CMV TOLERANCE GENES TO HYBRID PARENTS OF HOT PEPPER: EMPLOYING MORPHOLOGICAL AND RAPD MARKER TO IDENTIFY RECURRENT PARENT CHARACTERISTICS IN BC2 POPULATION

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ABSTRACT

Superior and CMV tolerant hybrid cultivar can only be developed by crossing a pair of high heterobeltiosis parents and both of which are CMV tolerance parents. Gene introgression have to be accomplished if the tolerance is not exist in the parents. This research was objected to employ DNA markers on identifying reccurent parent characteristics in BC2 generations to cut down the backcrossing cycle in CMV tolerance gene introgression. This research employed five BC2 populations generated from crosses of hybrid parents PBC1354 and PBC378 with tolerant parents of C1024, C1042 and C1043. The BC2 populations were selected for their CMV tolerance before and were characterized morphologically and molecularly. The results showed that genotype A24, A25, A29 of BC2A ([378/ [378/(378/1042)]-11]); B12, B2, B4 of BC2B ([378/[378/(378/C1024)]-6]), C16, C33, C4 of BC2C ([378/[378/(378/C1043)]-13]) population were tolerance individuals and resemble to recurrent hybrid parent PBC378. While individuals D11, D33, D38 of BC2D ([1354/[1354/(1354/C1043)]-18]); and individuals E12, E20, E31 of BC2E ([1354/[1354/(1354/C1024)]-4]) population were CMV tolerance and resemble to recurrent hybrid parent PBC1354. Employing of both morphological and RAPD marker were efective to identify the recurrent parent characteristics in early backcross generation.

Keywords : Capsicum, CMV tolerance, gene introgression, RAPD

INTRODUCTION

Hot pepper (*Capsicum annuum* L.) is one most important vegetable crop in Indonesia. However, the national production has not yet been able to fulfill the increasing demand annually. For instance, in the year of 2006, Indonesian government had to import hot pepper product as much as 11,885.5 ton (BPS, 2007). The main constraint of national hot pepper production are genetically low production potential of vareties planted by famers and CMV infection in the field. Among 45 different viruses detected from hot pepper fields, CMV is the most widespread and has potential to cause heavily yield losses (Duriat, 1996). Although quantitative yield loss due to CMV is not well documented, this virus tends to cause total yield loss in the field. Infection during early growth stage decreased 81.4% and 82.3% of total number of fruit and fruit weight per plant respectively (Sari *et al.*, 1997). In Korea, a field survey on paprika producing region showed that CMV is the most dominant virus in the field, followed by PepMoV, PMMoV, and TSWV (Mun *et al.*, 2008; Ryu *et al.*, 2009).

The most efficient and promising control measure against CMV and to increase pepper production is cultivation of high yielding hybrid cultivars tolerance to CMV. Such hybrids can merely be developed by crosses of pairs of parents with high heterobeltiosis potential and both of which tolerance to CMV. Introgression of tolerance into hybrid parents is compulsory if the trait does not exist in both parents. The conventional method to do gene introgression is a backcross breeding.

The final objective of the backcross selection is to find individuals identical to the recurrent parent with an additional characteristic, CMV tolerance. To identify the recurrent parental characteristics in a segregating backcross population is the most important and difficult step during the backcross selection. The identification process have to be carried out accurately, otherwise this will lead to an ineffective selection. Therefore, efforts to employ as many traits as possible will be helpful to do the selection in an early backcross population.

Two groups among the traits are morphology and DNA markers. Morphological traits such as plant height, number of dichotomous branch, leaf length, leaf width, fruit length, fruit diameter, total number of fruit, and total fruit weight per plant are commonly used in morphological characterization of hot pepper (IPGR, AVRDC and CATIE, 1995). DNA markers have been used to assist selection in breeding program on biotic stress (Klein-Lankhorst *et al.*, 1991; Wechter *et al.*, 1995; Saidi dan Warade, 2008). DNA markers were employed to assist introgression of high protein content into a superior wheat cultivars by mean of backcross breeding (Davies *et al.*, 2006).

The objective of this study was to employ morphological traits and RAPD markers to identify recurrent parental characteristics in early backcross populations during introgression of CMV tolerance gene into hybrid parents by means of backcross breeding.

MATERIALS AND METHODS

The study was conducted in the greenhouse of Dept. of Agronomy, Faculty of Agriculture, Univ. Bengkulu, and RAPD analysis was carried out at RGCI Laboratory of Bogor Agricultural University in the period of June to October 2009.

Development of MAS of Recurrent Parent

A MA of recurrent parent is a set of DNA markers used to identify the similarity of BC individuals to their recurrent parent. Genetic materials used in this study were the recurrent parents PBC378 and PBC1354. Chemicals used were DNA isolation and amplification kit REDExtract-N-Amp Plant PCR Kits XNAP (Sigma-Aldrich USA), 58 random primers of OPERONS, and electroforesis chemicals. Principal apparatus employed were Eppendorf micro pippete (0.5-2,5 µl, 10-100 µl, 100-1000 µl), eppendorf tips, tubes, waterbath, high sonic centrifuge Sorvall RC-55 Dupont, DNA drier, DNA Thermal Cycler PE Gene Amp PCR system 2400, electroforesis set, UV translumintor and digital camera.

Some leaf disks 0.5 cm in diameter were placed in a 2 ml plastics tube containing 100 µl of XNAP SIGMA KIT extraction solution and incubated at 95°C for 10 minutes. Dilution solution of 100 µl was then added in to the tube and shaked gently to maximize the extraction process. The solution was then removed into new tubes and added with 200 µl of aqua bidestilata and 100 µl CIA (cloroform:isoamyl alcohol, 24:1), and centrifuged at 10000 rpm, 4°C, for 10 minutes to separate extracted DNA from other leaf tissues. The liquid phase was then pippeted carefully and put into new tubes, and the DNA was precipitated with 1 ml of 95% alcohol, and incubated at 4°C for at least 30 minutes. Presipitant of DNA genom was centrifuged at 10,000 rpm, 4°C, for 10 minutes to separate DNA pellets from their solution. DNA pellets were then dried in a vacum drier. The final step, the DNA pellets were then dilluted with 100 µl sterilized ion free water.

DNA amplification was performed with random primers able to amplify hot pepper DNA genom following the RAPD tehnique of William *et al.* (1990). The RAPD markers were generated through polymerase chain reaction (PCR) using PE 2400 gene Amp-DNA thermal cycler. Electrophoresis was run on agarose gel (0.8 w/v) in TAE buffer and gel staining was done by shoaking the gel in etidium bromide solution (0.5 mg/l) for 20 seconds. DNA bands were visualized by UV transluminator and documented by Nicon D1000 digital camera.

Selection of BC2 Population

DNA amplification products of the recurrent parents were than used as marker assisted selection. DNA fingerprint of recurrent parents along with their morphological trait were then used to identify similarities of BC2 individuals to their recurrent parents. Genetic materials selected in this study were 5 populations of BC2 generated from a cross between parental hybrids and CMV

tolerance donor, namely BC2A= PBC378/[PBC378/ (PBC378/C1024)]-11, BC2B= PBC378/[PBC378/(PBC378/C1042)]-6, BC2C= PBC378/ [PBC378/(PBC378/C1043)]-13; BC2D= PBC1354/[PBC1354/(PBC1354/C1043)]-18; dan BC2E= PBC1354/[PBC1354/(PBC1354/C1024)]-4. DNA genom of five most CMV tolerance individuals of each BC2 population were isolated and amplified by random primers which previously were used to characterize their recurrent parents. Those individuals were also characterized morphologically. Based on morphological traits and RAPD markers they were analyzed their similarity to their recurrent parents by *Cluster Analysis*. Three most identical individuals to their recurrent parent were selected to generate further BC generation.

RESULTS AND DISCUSSION

Development of MAS of Recurrent Parent

Primer selection was done to determine primers used to characterize recurrent parents. Amplification of DNA genome with 58 random primer of OPERONS OPA, OPC, OPE, OPF, OPH and OPM showed that most of them were able to amplify DNA genome of hot pepper. However, less than 10 primers produced more than 4 bands, and the best 5 primers were then used in further study. They were OPE20 (AACGGTGACC), OPE7(AGATGCAGCC), OPE15 (ACGCACAACC), OPH5 (AGTCGTCCCC), and OPH13 (GACGCCACAC) (Figure 1).

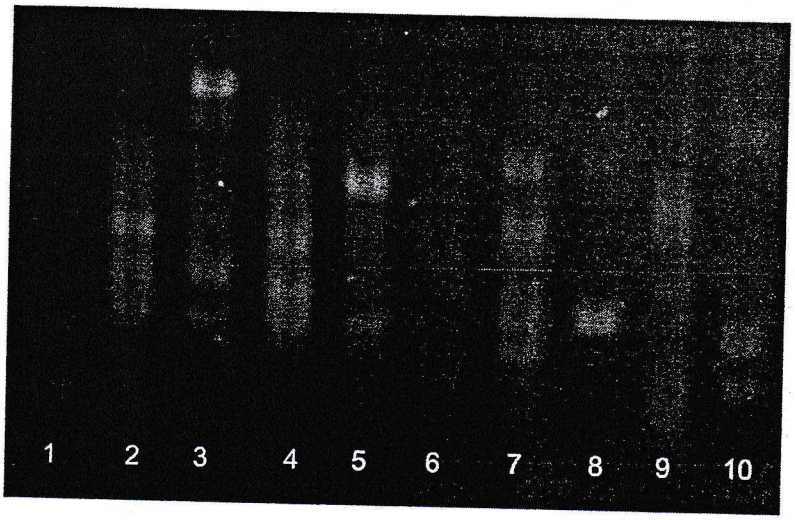

Figure 1. Example of visualisation during primer selection on recurrent parent PBC378: 1=OPE9, 2= OPE15, 3=OPE20, 4=OPH13, 5=OPH14, 6=OPH8, 7=OPE7, 8=OPE8, 9=OPH19, 10=OPH12

Based on the amplification of those five primers, DNA fingerprints of PBC1354 were characterized by the occurence of band patterns at 1150, 650, and 300 bp when amplified with OPE20; at 1050, 800, 600, 500, 400, and 250 bp with OPE7, at 800, 700, 600, dan 350 bp with OPE15, at 700, 550, dan 300 bp with OPH5 and at 800 dan 600 bp with OPE13. DNA fingerprints of PBC378 were characterized by band patterns at 1150, 650, 600 and 300 bp when amplified with OPE20; at 800 and 500 bp with OPE7; at 800 dan 700 bp with OPE15, at 700, 500, dan 350 bp with OPH5; and at 800, 650 dan 400 bp OPH13 (Table 1).

Table 1. Characterics of recurrent parents based on band pattern of DNA

Band pattern	PBC1354	PBC378
OPE20-1150	+	+
OPE20-650	+	+
OPE20-600	-	+
OPE20-550	+	-
OPE20-350	-	+
OPE20-300	+	-
OPE7-1050	+	-
OPE7-800	+	+
OPE7-600	+	-
OPE7-500	+	+
OPE7-400	+	-
OPE7-250	+	-
OPE15-800	+	+
OPE15-700	+	+
OPE15-600	+	-
OPE15-350	+	-
OPH5-700	+	+
OPH5-550	+	-
OPH5-500	-	+
OPH5-350	-	+
OPH5-300	+	-
OPH13-800	+	+
OPH13-650	-	+
OPH13-600	+	-
OPH13-400	-	+

+ presence, - absence

Fingerprints of both recurrent parents had dissimilarity level of 46% meaning that both of them were genetically not closely related. The similarity level of 54% was due to both of them belong to the same species, *Capsicum annuum.*

Selection of BC2 Population

Morphologically, based on plant height, total number of dycotomous branch, leaf length and width, total number of fruit, total fruit weight, fruit length and diameter, each of the BC2 population was segregated. This indicated that the recurrent and donor parents for each of BC population were morfologically different. The range of calculated coeficient variability on those variable were variably within each of selected BC2 individuals. The range coeficient variability of BC2A, BC2B, BC2C, BC2D, and BC2E were 9.47-34.67, 5.61-57.69, 6.91-36.39, 7.51-51.64, and 8.58-33.71 in precentage, respectively.

Similarly, the DNA amplification product (Figure 2) also showed variability between individuals within each BC2 population which indicated that between recurent and donor parent were genetically different. The calculated coeficient variability based on RAPD marker ranging higher than those of morphological traits. The range coeficient variability of BC2A, BC2B, BC2C, BC2D, and BC2E were 76.25-161.02, 67.27-147.96, 95.93-195.00, 76.25-226.78 and 80.90-147.96 in precentage, respectively. The segregation in each of BC2 population also indicated that eventhough

they were previously selected for their CMV tolerance, there were still segregation among the selected indiduals.

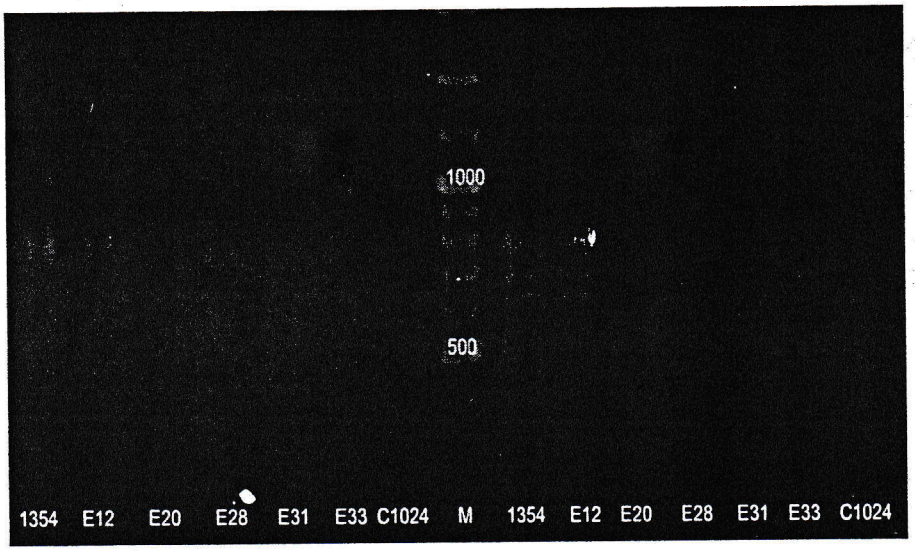

Figure 2. Example of DNA band pattern form RAPD analysis with primer OPE5 and OPE15

Employing all variable measured, selection was performed based on similarity level to the recurrent parents. On BC2A population, all selected individuals had similarity level of 97.57% to their recurrent parent PBC378. Therefore, each of the individuals previously selected for CMV tolerance could be used similarly to generate further BC ger.eration. On BC2B population, B12 was most identical individual to PBC378 with similarity level almost 100%, and followed by B2 and B4 with similarity level of 99%. So that those three individuals were best for further generation. The last alternative individuals were B37 and B49 with similarity level of 97.08 to their recurrent parent. Individuals of BC2C population almost identical among them with similarity level were more than 99%. All of them formed one group which was 98.25% similar to PBC378. Therefore, each of them was suitable to develop further BC generation. Genotype D11 and D33 of BC2D were most identical to their recurrent parent PBC1354 with their similarity level about 98% and each of them, therefore, was suitable to develop further generation. The next alternative was either D3 or D38 which the similarity level to their recurrent parent was about 93%. Genotype E12, E31, and E20 of BC2E population were most identical to their recurrent parent, PBC1354 with similarity level of about 99%. Therefore, they were the best genotype to be used to develop further generation. The rest genotype, E28 and E33, merely 96.27% similar to their recurrent parent.

Comparing all BC2 population in this study, it was noticebly that there was different variation pattern between genotypes within each BC2 population although all of them were the selected genotype for CMV tolerance. Some genotypes were highly identical to their recurrent parent and the others were less similar to their recurrent. There seem to be depending on combination of the donor and recurrent parent used in the breeding program.

The results indicated that employing both morphological trait and RAPD marker were sucessfull to identify genotypes highly identical (more than 99% similarity level) to their recurrent parent in the BC2 generation. This indicated that the use of morphological in combination to molecular trait was promising to shorten the backcross breeding program in introgressing CMV tolerance gene to a hybrid parent.

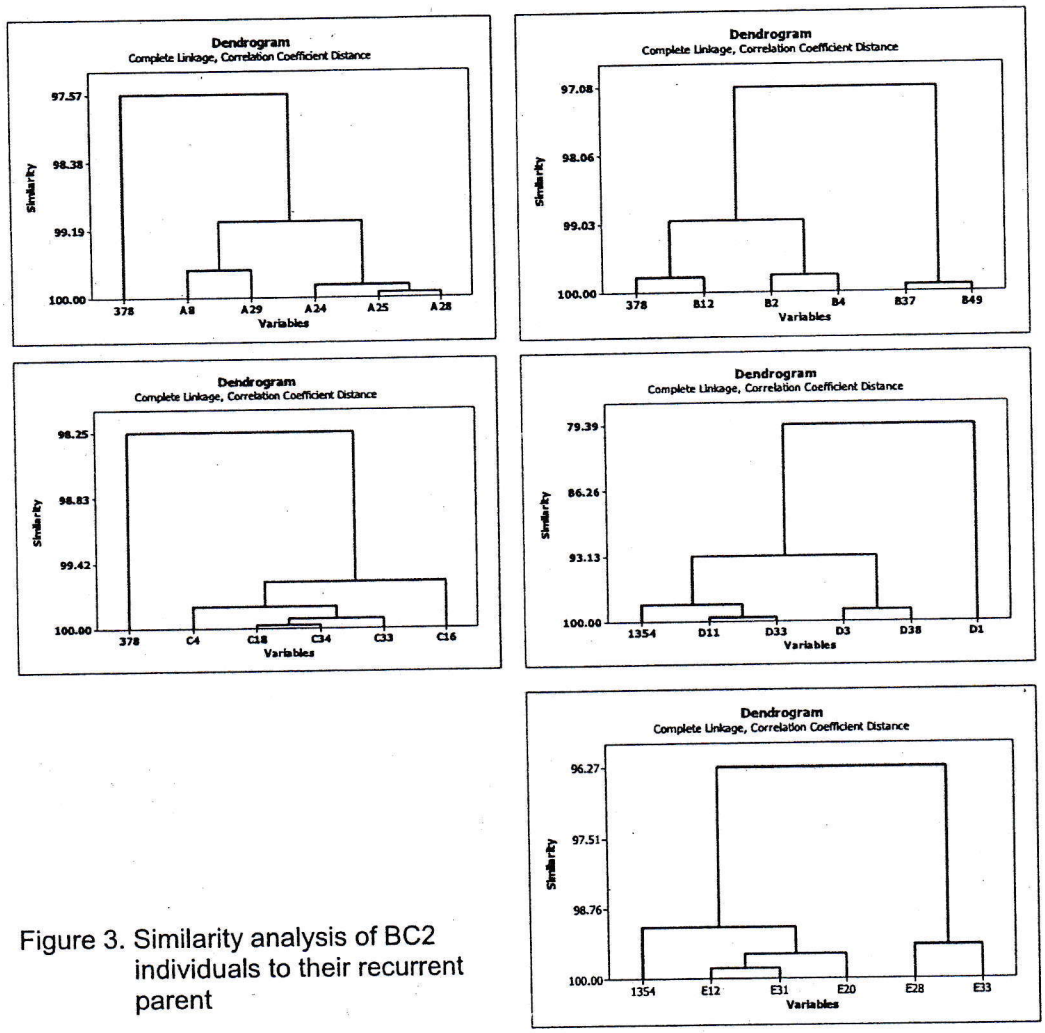

Figure 3. Similarity analysis of BC2 individuals to their recurrent parent

CONCLUSSION

Genotype A24, A25, A29 of BC2A ([378/ [378/(378/1042)]-11]); B12, B2, B4 of BC2B ([378/[378/(378/C1024)]-6]), C16, C33, C4 of BC2C ([378/[378/(378/C1043)]-13]) population were tolerance individuals and resemble to recurrent hybrid parent PBC378. While individuals D11, D33, D38 of BC2D ([1354/[1354/(1354/C1043)]-18]); and individuals E12, E20, E31 of BC2E ([1354/[1354/(1354/C1024)]-4]) population were CMV tolerance and resemble to recurrent hybrid parent PBC1354. Employing of both morphological and RAPD marker were efective to identify the recurrent parent characteristics in early backcross generation.

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