Current status on molecular breeding for quality protein maize (QPM) and resistance to downy mildew pathogen in Indonesia

Ruswandi, D.[1], N. Carsono[1], N. Wicaksana[2], M.B. Pabendon[3], M. Azrai[3], F. Kasim[4], M. Rachmadi[1], F. Damayanti[5], and A. Ismail[5]

[1] Assistant Professor. Laboratory of Plant Breeding. Faculty of Agriculture. Padjadjaran University. Bandung 40600. Contact e-mail: ruswandi@hotmail.com

[2] Graduate research assistant. Laboratory of Plant Breeding. Padjadjaran University

[3] Assistant of scientist. Indonesian Cereal Research Institute. Maros

[4] Research scientist. Indonesian Cereal Research Institute

[5] Research Assistant. Laboratory of Plant Breeding. Padjadjaran University

Demand on high quality of maize for domestic consumption is growing every year due to expanding of food and feed industry using maize as its main components. Since domestic maize production could not meet the demand, Indonesia had to import and it is projected to increase. This condition indeed will bother food self sustain in Indonesia for the future. Improvement high yield cultivar possessing high quality protein is the proper solution to multiply maize production in Indonesia.

A basic problem found to improve and to construct high yielding maize cultivar possessing both high lysine and tryptophan and resistant to *P. maydis* is facing the following constraints: (i) poor of gene pool for these traits, (ii) high quality protein of maize possessing high lysine and tryptophan is controlled by a recessive opaque 2 gene and is difficult to select in heterozygous form, (iii) resistant gene to *P. maydis* is complex and is controlled by polygene so that it take much effort to be undertaken. Molecular approach is the best solution to facilitate in creating maize cultivar for these important traits.

Set of experiment has been conducting for the last two years with the ultimate goal to construct an inbred line possessing both high quality of protein and resistant against downy mildew pathogen as parental for synthetic or hybrid cultivar. An integrative approach including field experiment for high yield test, screening under artificial inoculation, and molecular technique using Simple Sequence Repeats (SSR) was conducted to select a high polymorphic pair of parent and to test the hybrids. Results showed that this approach could be effective to improve maize possessing both complex and recessive traits.

Keywords Maize . Q*uality protein maize . Downy mildew resistance . Genetic relationship . Simple sequence repeats.*

Maize (*Zea mays* L.) is the third most important crop in food production in the world, after wheat and rice. Maize is bred and produced for four main purposes: for the fresh food market, for feed of livestock, for cultivation activity, for the maize food processing industry (cornflakes and snacks, for instance) and for non-food industrial uses (e.g. starch and other derivatives). Maize is a rich source of energy, as mentioned that, per 100 g of maize grain contain 1,690 energy (kJ), carbohydrate 83.0 g, lipid 4.9 g, and protein 10.5 g (Leung *et al*., 1972 *cit*. Grubben *et al*., 1996). However, its protein content is lacking from lysine and tryptophan since percentage of such amino acids in the maize

grain less than 4%. FAO recommended standard should be at least 4%. Human body and monogastric animal could not synthesize both amino acids (Vasal, 1999). Currently, there is an increasing demand on high quality of maize for domestic consumptions due to the expanding of food and feed industry using maize as their main component. Consumers and businessmen have realized that quality of maize determines the final product either as food or feed. Unfortunately, in Indonesia, farmers produce low quality of maize especially in its protein content. In order to improve quality protein of maize, some quality protein maize (QPM) lines from CIMMYT, Mexico, have been introduced in Indonesia. Interesting researchs have been undertaking to introduce this QPM into Indonesian maize lines.

Other drawback in domestic maize production is Java downy mildew attack caused by *Peronosclerospora maydis* in Lampung, South Sulawesi, East Java, Central Java, etc. Development of partial resistance to *P. maydis* entails a great effort by plant breeders and plant pathologists. Additive genes with dominance and epistatic effect regulate the resistance to *P. philipinensis* (Ruswandi *et al.*, 2003). According to these reasons, it is actually needed to construct high-yielding cultivar of maize possessing both high quality of protein and resistance to *P. maydis*.

This paper will describe current status of research activities in quality protein maize and downy mildew pathogen case study in Indonesia

Molecular Markers

A basic task in any breeding program is to identify a large number of genotypes that can pass on their desirable characteristics to the progeny. Conventional breeders are forced to rely on phenotypic evaluation, which does not indicate underlying information present in plant genome (Dreher *et al.*, 2000). Environmental factors often influence the phenotypic appearance, and in addition, genetic interaction can obscure the presence or absence of specific alleles, making it difficult to identify plants that really seek. Many approaches have been used by breeders in the past for conducting such activities, in terms of selecting genotypes possessing specific traits, such as, morphological evaluation, isozymes and storage proteins analysis like patatin in potato, glutenin in wheat, glutelin in rice, etc. However, as revealed by many researchers, molecular marker offers an effective technique for evaluation and selection of desired genotypes (Law *et al.*, 1998). Molecular markers are molecules that could be used to trace a desired gene(s) in examined genotype (Ovesna *et al.*, 2002), they can inform breeders about the presence the desirable alleles in the genotype (Dreher *et al.*, 2000). The main advantages in using molecular markers are, that markers segregate as single genes and they are not affected by environment.

Currently molecular marker techniques used for genome mapping and for tagging different traits are: restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA

(RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphisms (SNP), and microsatellites. All molecular markers offer great advantages as well as limitations. A rational consideration should be taken into account for selecting those markers. For reason of cost and simplicity, single sequence repeat (SSR) markers are widely applied in maize breeding and genomic project. Microsatellites are sequences that consist of a direct repeat of di-, tri-, or tetra-nucleotides such as $(TG)_n$, $(TA)_n$, $(CAC)_n$ where *n* can vary from 8 to 30. Microsatellites are very abundant in plant genome and often highly polymorphic. They are estimated to occur at least once in 10^5 bp, which means that maize genome size is 2.5 x 10^9 bp (Hughes, 1996), and probably as many as 25000 microsatellites or may be more are present. Generally, the larger the number of repeats (n) the larger the number of alles (Genomics, 2001). Microsatellites are co-dominant markers.

PCR amplification protocols used for microsatellites employ either unlabelled primers pairs or primer pairs with one of the primers being radioactive-labeled or fluoro-labeled. Electrophoresis of unlabelled PCR products can be carried out on vertical polyacrylamide gels or horizontal agarose gels (Ovesna et al., 2002) or using automated system.

Microsatellites are commonly identified through (1) screening of small-insert or microsatellite-enriched genomic libraries by hybridization with oligonucleotide primers followed by sequencing, and/or (2) searching DNA sequence databases. Database searching is only suitable for development of SSR markers in plant species well represented in public databases. As result, maize is one of the better-characterized plant species with 79 996 entries available in the GenBank database (status January 2001). Moreover, as more plant expressed sequence tag (EST) and genome-sequencing projects become established, public databases will contain an abundance of sequence data that may be exploited for SSR development. The maize research community now has the most detailed and comprehensive SSR marker set of any plant species. This can be found in MaizeDB at URL www.agron.missouri.edu (Sharopova *et al*., 2002).

Quality Protein Maize

Quality protein maize phenotype (QPM) is controlled in a part by a mutant allele of a gene called *opaque2* (Dreher *et al*., 2000). The recessive genotype (o2o2) has endosperm with an opaque (chalky) appearance, reduced level of 22 kDa zeins, enhanced level of two essential amino acids, lysine and tryptophan. The structure of the *Opaque2* gene (dominant) is typical of plant gene having six exons. This gene codes for a transcription factor, a protein that binds to the upstream region of other genes and is required for initiation of transcriptions of those genes (White and Hoogenboom, 2003). Over time, the QPM limitation i.e. having soft endosperm, has been improved by modifier genes action that restored the desired hard endosperm phenotype in materials containing the

recessive *opaque2* mutation (Dreher *et al*., 2000). The agronomically acceptable, nutritionally enhanced materials later came to be known as Quality Protein Maize (QPM).

The great advantage of QPM: (i) increase quality of grain for human diet, which in turn could reduce malnutrition and mortality, (ii) reduce the cost production of feed since it provides well balanced feed and reduce import soybean flour as source of protein.

SSR-based molecular marker analysis for QPM breeding begins with the extraction of a DNA sample from the plant to be studied. Primers that flank a region containing SSRs within the *opaque2* gene are then used to amplify the sample using the well-known polymerase chain reaction (PCR) method. When the number of repeated sequences between the flanking primers differs in the normal *opaque2* and mutant *opaque2* alleles, the amplified DNA fragments vary in length. By electrophoretically separating the amplified DNA fragments on gels and analyzing them, researchers can determine if the plant possesses two copies of the normal allele, two copies of the desirable mutant *opaque2* allele, or one copy of each allele.

In the context of QPM line conversion, MAS (Molecular Assisted Selection) offers the possibility of overcoming the two main constraints faced by conventional breeders. First, since marker analysis can be done using DNA samples extracted from leaf tissue of very young plants, it allows QPM plants to be identified early in the breeding cycle. This allows the breeder to discard plants that do not contain mutant *opaque2* alleles prior to pollination, reducing the size of the breeding population and saving both time and money. Second, with molecular markers, breeders can distinguish between homozygous recessive plants that carry two copies of the mutant *opaque2* allele (these will express the QPM trait) and heterozygous plants that carry only one copy (these will not express the trait, because the mutant *opaque2* allele is recessive). Armed with this knowledge, breeders do not have to go through the laborious and costly process described at the end of the previous section (Dreher *et al*., 2000)

Downy Mildew Pathogen

Downy mildew caused by *Perenosclerospora maydis* is among the most important diseases affecting maize production in Indonesia. The pathogen invades through the stomata, shoots apex and stem and thereafter, infects the succeeding growing parts of the plant (Dalmacio and Exconde, 1969). Advincula (1975) detected the organism in the flower, kernel, rachilla, peducle and stem of systemically infected maize. The organism was also observed to infect the seeds by invading the parenchymatous tissues, and develop in the vascular strand of the plant to the ovary wall of the developing caryopsis. The hypha then establishes in the pericarp layer leaving the endosperm and the embryo uninvaded. Two types of symptoms appear in the second or third leaf in the form of

chlorotic stripes as early as 12 days after planting. The systemic symptoms appear in the first true leaf in the form of complete chlorosis or chlorotic stripes 9 days after planting.

Improvement of resistance against the downy mildew pathogen required a great attempt by plant breeders and pathologist. For one, the resistance is governed by additive genes with dominant and epistatic effect (Ruswandi et al., 2003) which make selection for the trait take a longer time to complete. Another constraint is that selection should be done under severe epiphytotic condition in which potential superior genotypes possessing high yield may loss (Kaneko and Aday, 1980).

Molecular marker assisted selection could be applied to improve resistance against downy mildew pathogen. Molecular markers linked to quantitative trait loci (QTL) associated with downy mildew resistance traits could allow selection for resistance to different pathogens, i.e. populations in a single location even in the absence of the pathogen. QTL map for resistance gene against the pathogen of downy mildew have been developing extensively nowadays by collaborations research conducted by Asian Maize Biotechnology Network (Ruswandi, *et al.,* 2002a). Based on analysis using A x B (CML 139 x Ki 3) recombinant inbred lines (RIL), six QTL confer resistance against *P. philippinensis* were identified (AMBIONET, 2001). These QTL were distributed in chromosome 1, chromosome 2, chromosome 5, and chromosome 10. In line to this QTL map, Ruswandi et al. (2002b) indicated QTL on chromosome 1, 5, 6, and 8 for disease incidence. Furthermore, they hypothesized that the gene controlling resistance to three different downy mildew pathogen, namely: *P. philippinensis*, *P. sorghi*, and *P. maydis* that were located on chromosome 1 could have similar conserved gene. Azrai et al (2002) investigated fourteen RFLP markers, which were associated with six chromosomes for Java downy mildew resistance gene in tested material of hotspots in Bogor.

Research program and major achievements for introgression opaque 2 gene into dmr maize lines

Currently, a collaborative research program between Laboratory of Plant Breeding, Faculty of Agriculture, Padjadjaran University, Bandung with Indonesian Cereal Research Institute has been conducted since the year of 2002 and it was continued to develop maize cultivars possessing both high quality protein and resistant to downy mildew pathogen. Seven QPM genotypes and four DMR genotypes were introduced for this purpose. The seven introduced QPM lines were CML 161, CML 162, CML 163, CML 164, CML 165, CML 171, and CML 172. The four introduced DMR lines were Ki-3, MR 10, Nei 9008, and P-345.

The main activities under this collaborative research in the first year from 2002 to 2003 were mentioned as follows: (a) seed propagation of introduced QPM and DMR lines for developing F_1 hybrids; (b) hybridization of DMR X QPM to initially introgress opaque-2 gene from QPM line into

DMR line; (c) assessment of polymorphic DNA level using SSR markers; and (d) evaluation of single cross DMR x QPM.

Six introduced QPM and four introduced DMR lines can successfully be propagated in Bogor, West Java. Generally, all introduced maize lines phenotypically performed well so that they can yield enough seeds. Only CML 165 phenotypically performed poor so that its seed can not be produced.

Hybridization of DMR x QPM was successfully done based on line x tester mating pattern where DMR lines was used as recurrent parent lines and QPM lines were used as donor tester. Those DMR lines were Ki-3, MR 10, Nei 9008, and P-345; meanwhile the QPM testers were CML 161, CML 164, and CML 172. Those single cross hybrids to be formed, namely: Ki 3 x CML 161, Ki 3 x CML 164, Ki 3 x CML 172, Nei 9008 x CML 161, Nei 9008 x CML 164, Nei 9008 x CML 172, P 345 x CML 161, P 345 x CML 164, P 345 x CML 172, MR 10 x CML 161, MR 10 x CML 164, and MR 10 x CML 172.

Set of experiment was conducted at the Lab. of molecular biology, Biotechnology Research Institute for Food Crop Bogor to finger-print downy mildew resistance and quality protein of maize lines to be used as parental lines in the breeding program. Five of downy mildew resistant (DMR) inbred lines and six of quality protein maize (QPM) inbred lines were used in the study. Molecular marker characterization using eleven simple sequence repeats (SSR) were done to profile these inbred lines. There are three groups of parental lines based on Jackard coefficient similarity at 0.3 (Figure 1). These groups include CML 161, Ki 3, MR 10, and CML 164 for group 1. CML 165, CML 172, Nei 9008, AMATLCOHS, and P 345 for group 2. CML 162 and CML 162 for group 3.

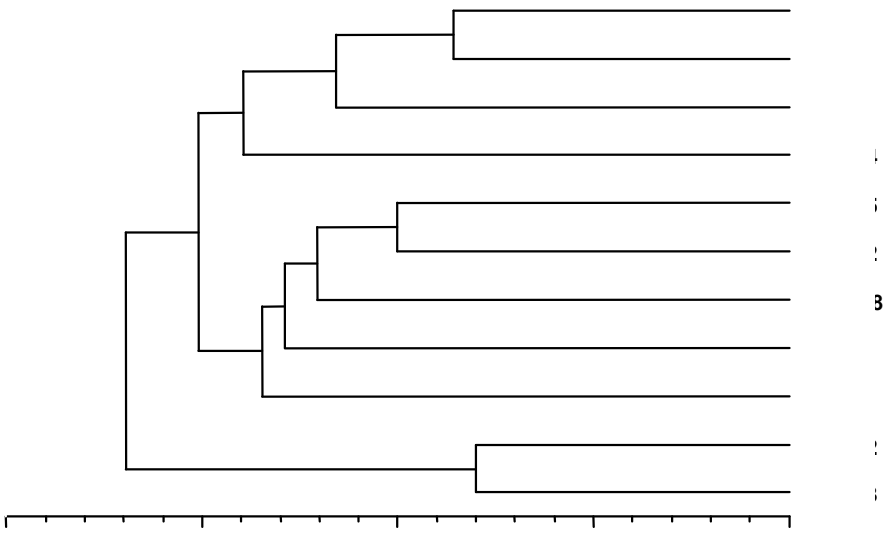

Figure 1. Genetic similarity of DMR and QPM lines based on Jackard's coefficient similarity at 0.3

Conclusion

Breeding for improving quality of maize and resistance to downy mildew pathogen have been conducted and for two coming years it will be accomplished.

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