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ENHANCING INTROGRESSION OF THE OPAQUE-2 TRAIT INTO ELITE MAIZE LINES USING SIMPLE SEQUENCE REPEATS

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ABSTRACT

To improve on the efficiency of Quality Protein Maize (QPM) breeding in Uganda, the utility of three simple sequence repeats (SSR) markers (phi057, phi112 and umc1066) in selection and introgression of the opaque-2 (o2) gene was investigated. Genomic DNA of six normal and seven (QPM) lines was analysed using a standard PCR protocol. Polymorphisms were detected in the Opaque-2 locus among all the maize lines when using the SSR primers, phi057 and phi112, while no polymorphism was detected with primer umc1066. To facilitate background selection in the heterozygous BC_2 progeny, key phenotypic characters of the recurrent parent (136R) were used as additional markers. The SSR marker phi057 was co-dominant while phi112 was dominant. The polymorphic SSR markers correctly predicted the expression of tryptophan in kernels of all QPM inbreds and five of the six non-QPM inbred lines, However, one non-QPM inbred line (PED49B) had a tryptophan content that is characteristic of the o2 mutation (0.090), suggesting that another genetic system may be responsible for the expression of trytophan in this maize line. Phi057 was employed to monitor the introgression of the o2 allele from CML176 to 136R. Of the 200 BC_1F_1 (136R/CML176//136R) plants genotyped, 104 were found to be heterozygous, producing products corresponding to both alleles (o2 and O2), while 96 produced a single band corresponding to the homozygous dominant (O2O2) condition. The ratio of the two groups in the backcross (BC) population was consistent with the proportion 1:1 accorded by the Chi-square test (X^2=0.16< $X^2_{0.05}$=3.84) for a single gene in a BC population. Therefore, under the conditions of PCR used, SSR markers phi057 and phi112 will constitute the framework for marker assisted introgression of the o2 trait into suitable maize genotypes in Uganda.

Key Words: QPM, tryptophan, Uganda, *Zea mays*

RÉSUMÉ

Améliorer le rendement de Qualité de la Protéine de Maïs (QPM) par reproduction en Ouganda, l'utilité des trois indicateurs simples séquences répétées (SSR) (phi057, phi112 et umc1066) en sélection et introgréssion du gène opaque-2 (o2) était investigué. L'ADN génomique des six lignées normales et sept (QPM) lignées était analysé en utilisant un protocole PCR standard. Les polymorphismes étaient détectés dans le lieu Opaque-2 parmi toutes les lignées de maïs quand on utilisé les premiers SSR, phi057 et phi112, pendant qu'aucun polymorphisme était détecté avec le premier umc1066. Pour faciliter une sélection de fond dans les hétérosygeux progénie BC2, les caractères phénotypes clés du parent récurrent (136R) étaient utilisés comme indicateurs additionnels. L'indicateur SSR phi057 était co-dominant pendant que le phi112 était dominant. Les indicateurs SSR polymorphiques ont correctement prédit l'expression du tryptophane dans les graines de tous les QPM innés et cinq des six QPM des lignées non innées. Cependant, une QPM lignée non innée (PED49B) avait un contenu en tryptophane qui est caractéristique de la mutation o2 (0.090), suggérant qu'un autre système génétique peut être responsable de

l'expression de tryptophane dans cette lignée de maïs. Phi057 était employé pour contrôler l'introgréssion de l'allèle o2 du CML176 à 136R. Des 200 BC1F1 (136R/CML//136R) plantes génotypées, 104 étaient retrouvées être hétérosygeuses, produisant des produits correspondant aux deux allèles (o2 et O2), pendant que 96 ont produit une bande unique correspondant à la condition homosygueuse dominante (O2O2). Le rapport des deux groupes dans la population après croisements avec les parents (BC) était consistent avec la proportion 1 :1 donnée par le test Chi carrée (X^2=0,16<$X^2_{0,05}$=3,84) pour un gène unique dans une population BC. Par conséquent, sous les conditions utilisées de PCR, les indicateurs SSR phi057 et phi112 constitueront le cadre pour l'introgréssion assistée de l'indicateur de trait o2 dans les génotypes adaptés de maïs en Ouganda.

Mots Clés: QPM, tryptophan Ouganda, *Zea mays*

INTRODUCTION

Maize (*Zea mays* L.) is an important food and animal feed worldwide (FAO, 1992). While primarily used as a source of carbohydrates (NRC, 1988), it is also a significant source of essential amino acids required by humans and livestock for healthy nutrition. However, the relative expression and amino acid composition of seed storage proteins in normal maize kernel causes the grain to be of inferior quality for monogastric animals (Ueda and Messing, 1993). Specifically, the zeins, which account for 60% or more of the endosperm proteins in normal maize seeds (Gaziola *et al.*, 1999), are rich in glutamine and hydrophobic amino acids, but very poor in essential amino acids lysine and tryptophan (Azevedo *et al.*, 1997). A diet low in lysine and tryptophan leads to symptoms of protein deficiency, even if plenty of the other essential amino acids are being consumed. In addition, tryptophan is a precursor for endogenous synthesis of niacin, one of the B-vitamins, and maize is also low in available niacin (FAO, 1992) since the latter is chemically bound in a manner that renders it inaccessible unless properly treated.

Based on their solubility, genetic properties, and the apparent molecular masses, zeins have been classified into α- (22 and 19 kDa), the most abundant, β- (14 kDa), γ (27 and 16 kDa) and δ-zein (10 kDa) (Wilson, 1991). The o2 mutation typically reduces the α-zein content by one-half and enhances the synthesis of a number of non-zein proteins that contain relatively higher levels of lysine and tryptophan (Damerval and de Vienne, 1993; Habben *et al.*, 1993; Gaziola *et al.*, 1999). In addition, the free amino acid content in the endosperm of the o2o2 genotypes is also higher than in the normal genotypes (Sodek and Wilson, 1971), with a specific increase especially in lysine and a decrease in glutamic acid, proline and leucine (Sodek and Wilson, 1971). The reduced quantity of leucine, together with more tryptophan, improves the synthesis of niacin. These changes in protein and amino acid composition make o2 maize lines nearly twice as nutritious as normal maize (Briggs, 2000).

Besides regulating the synthesis of zein, the o2 mutation has a wide range of pleiotropic effects on the plant that limit its use (Mario *et al.*, 2003), including (a) unacceptable (soft/opaque) kernel texture, (b) reduced kernel weight, (c) greater vulnerability to ear rot organisms, (d) more infestation by weevils during storage, and (e) slower drying of grain following physiological maturity (Srinivasan *et al.*, 2004). More recently, breeders at the International Maize and Wheat Improvement Center (CIMMYT) combined the o2 mutation with a system of genetic modifiers that restore the vitreous phenotype of maize kernel. The use of modifier genes has allowed the selection of modified *o2* lines that maintain the increased concentrations of lysine and tryptophan in a modified-vitreous endosperm with a good grain yield (Lefevre *et al.*, 2002). The hard endosperm o2 stocks are designated quality protein maize (QPM) to distinguish them from soft o2 strains (Crow and Kermicle, 2002). Although plenty of QPM germplasm is now available from CIMMYT's breeding programme, susceptibility to major foliar diseases remains a major weakness of this germplasm (Pixley, 2001). Most of it is very susceptible to *Turcicum* Leaf Blight (TLB), which, together with Maize Streak Virus (MSV) are considered major diseases of maize in sub-Saharan Africa, including Uganda (NRI, 2005).

To utilise the nutritional potential of QPM in Uganda, efforts are now being made by the

National Maize Program to introgress the o2 mutation and its associated modifiers into elite maize genotypes. However, because the expression of the o2 allele is specific to seeds and recessive, conventional introgression approaches require the inclusion of a selfing progeny test, simultaneously or alternately, to monitor the introgression of the o2 allele within each BC population (Babu *et al.*, 2004a). Standard conventional BC schemes that alternate backcrossing with progeny testing, although extremely reliable, are prohibitively inefficient, involving a higher plant population, enormous time, labour and spatial resources (Dreher *et al.*, 2000; Babu *et al.*, 2004b).

Numerous advantages such as reduced time and population size are known to accrue to the breeder by use of marker-facilitated genotype selection rather than classical phenotypic selection (Tanksley *et al.*, 1988; Paterson *et al.*, 1991). However, molecular markers must be polymorphic and informative across populations for them to be used in a marker assisted breeding programme.

This study was, therefore, undertaken to determine whether marker assisted selection (MAS) can be integrated into the current QPM breeding programme in Uganda. The specific objectives were: (1) to determine SSR polymorphisms for the Opaque-2 locus among selected QPM and normal maize lines; (2) to initiate a backcross scheme to introgress the o2 allele from one QPM line to at least one of the normal inbreds tested in (1); (3) to test the effectiveness of the polymorphic SSR marker(s) in (1) in discriminating between genotypes in BC progeny developed in (2); and (4) identify a phenotypic marker system that could be employed to enhance background selection in the heterozygous BC_2 progeny.

MATERIAL AND METHODS

Plant materials. This study used 7 QPM and 6 non-QPM (normal) inbreds. The QPM lines used were CML 144, CML159, CML173, CML176. CML181 and CML182. Nnalongo (Longe 5), a popular local open pollinated QPM variety, was included as a check. The non-QPM lines used included CML387, CML395, CML444, PED49A, PED49B and 136R. The last three lines were materials developed at Namulonge Agricultural and Animal Research Institute (NAARI) under recurrent selection and were considered well adapted to Ugandan conditions. The rest were inbreds that had been registered and released as CIMMYT Maize Lines (CMLs).

Genomic DNA extraction. Genomic DNA was extracted from maize leaf tissue, collected from 6 week old plants, with a slightly modified form of the cetyltri-methylammonium bromide (CTAB) protocol described by Agbios (1999). Two plants were used to represent each of the 13 genotypes. Briefly, young leaf tissue was ground into powder in liquid nitrogen, and mixed with 1 ml of pre-warmed (65°C) CTAB buffer [100 mM Tris pH 8, 20 mM EDTA, 1.4M NaCl, 2% (w/v) CTAB, 1% (v/v) PVP and 0.2% (v/v) 2-mecarpto ethanol]. The samples were incubated at 65° C for 2 hours and frequently inverted. The mixture was separated by centrifugation, the supernatant extracted with chloroform:isoamyl alcohol (24:1; v/v) and precipitated with cold isopropanol. DNA pellets were washed with 70% ethanol, air-dried, and resuspended in TE (10 mM Tris-HCl pH 8, 1 mM EDTA). The DNA was treated with RNase, quantified, and stored at 4° C.

SSR Polymorphism Assay for the Opaque-2 locus. SSR polymorphism at the Opaque-2 locus was assessed by PCR amplification of genomic DNA with the SSR primer sets Phi57, phi112 and umc1066. The primers were synthesised by MWG Biotech Company. Primer sequences and their repeat motifs are available in the Maize Genomics Data Base (http://www.maizegdb.org/). For primer sets umc1066 and phi057, a consensus PCR mix was established containing 100 ng of template DNA, 0.4 mM of each dNTP (dATP, dGTP, and dCTP, dTTP), 0.25 µM of each of the forward and reverse SSR primer, 2.5 mM $MgCl_2$ (Promega), 0.2 units of Taq DNA polymerase (Promega), and 1X Mg-free buffer (Promega). For phi112, the PCR mix was as above except that 0•2 µM of each of the forward and reverse primer was used. All reactions were performed in a final volume of 15 µl.

Thermocycling was carried out on a 96-well iCycler PCR System (Bio-Rad) which holds 0.2-mL thin-walled PCR tubes. Amplification was

optimised according to the requirement of each SSR marker. For umc1066, the PCR profile consisted of a pre-denaturation step at 95° C for 2 mins, followed by a 'touchdown' thermal cycle. The touchdown cycles consisted of 95°C for 1min, 65–55° C for 1min (with a decrease by 1°C every cycle), and 72° C for 1min 30 seconds. An additional 30 cycles with annealing at 55° C were performed. A final extension step at 72° C for 5 min was included, followed by termination of the cycle at 4° C. For phi057, the following PCR profile was used: a pre-denaturation step at 94° C for 3 min, followed by 35 cycles of a denaturation at 94° C for 1min, annealing at 64° C for 2 mins, and extension at 72° C for 2mins. A final extension step at 72° C for 5 min was included, followed by termination of the cycle at 4° C. For phi112, the PCR profile consisted of a pre-denaturation step at 94° C for 2 mins, followed by 35 cycles of a denaturation at 94° C for 2mins, annealing at 59° C for 1min, and extension at 72° C for 1mins. A final extension step at 72° C for 5 min was included, followed by termination of the cycle at 4° C. PCR products for phi057 and umc1066 were separated in 5% Metaphor agarose gels (1 Metaphor: 1 Seakam agarose) and 1XTBE buffer, while 2% ordinary agarose in 1X TAE buffer was used for phi112. Samples were run into gels at 120 Volts for 3 hours, and visualized under UV by ethidium bromide staining.

To validate the correspondence between SSR marker genotype and plant phenotype, the level of tryptophan in the grain of 12 of the 13 inbred lines was then determined following procedures described by Villegas *et al.* (1984).

Population development for marker assisted backcrossing. Maize inbred 136R was targeted for conversion to the QPM version using CML176 as the o2 donor parent. The two inbred lines were hybridised and the F_1 backcrossed to 136R to form a population, which was to be selected over three BC generations using foreground marker-assisted selection, and background selection based on phenotypic resemblance to the recurrent parent in the BC_2F_1. The F_1 was made using the recurrent parent 136R as the female and CML176 as the pollen parent. The first backcross (BC_1) was made with the F_1 hybrid as the female and 300 BC_1 seed were planted in an ear-to-row deck. A similar population size was developed in BC_2. MAS was applied on a screening population of 200 plants in each BC progeny so as to optimise response to background selection in the selectable (heterozygote) population. An average of five plants per row was selected to derive the screening population.

To facilitate background selection, the phenotypic characters of inbreds 136R and CML176 were studied over two seasons at NAARI. The two genotypes were grown in a randomised complete block design with three replicates. Each plot consisted of one 4 m-row with 0.75 m spacing between plots. Phenotypic characters measured included plant height, ear height, days to 50 % pollen shedding (D50%P), pollen shed duration (PSD), days to 50% silking (D50%S), and tassel colour. The presence of MSV, TLB, and rust diseases was assessed under natural infestation, about 30 days after pollination. MSV severity was measured on a scale of 1-5 as described by Martin *et al.* (1999). TLB disease index was also scored on a scale of 1-5, where 1=no symptoms and 5 = 45-75% of leaf surface diseased. Rust severity was rated on a modified method reported by Groth *et al.* (1992). Plant and ear height measurements were the average of five plants randomly selected in each row. The five plants selected in each plot were tagged and observed daily to determine the progress of tassel development and pollen shed. Ear and plant heights were obtained by measuring the distance from the ground to the primary ear node and the collar of the flag leaf, respectively, after the tassel was fully expanded.

Data analysis. Allele designations and approximate size range for the amplification products for each SSR locus was determined based on the positions of the bands relative to a 100 bp–molecular weight ladder. The banding pattern generated by the three SSR markers was scored either in a codominant manner (1, 2), or as present (1) or absent (0). The segregation of the selected marker locus (in the BC_1 population) was checked for deviations from the expected Mendelian ratio in a backcross population (1:1) by standard Chi square tests. ANOVA was employed to detect significant differences between the QPM and non-QPM inbreds for tryptophan

content, and between CML176 and 136R for various phenotypic parameters. LSD test was used for significant means separation. To better meet the assumptions of ANOVA analysis, tryptophan measurements were transformed whenever necessary by square roots transformation (Kilinganire and Hall, 1993).

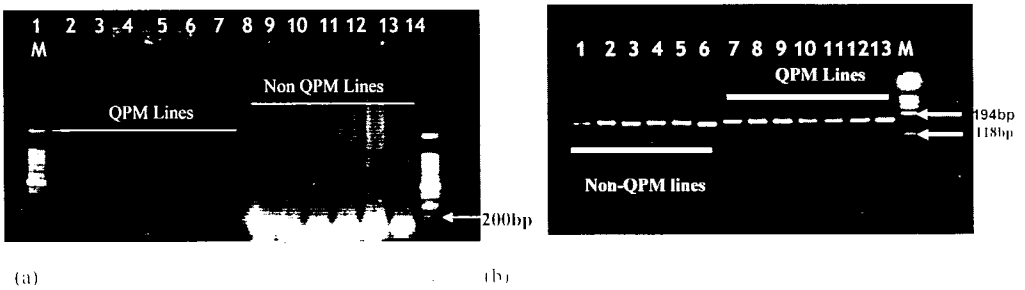
RESULTS AND DISCUSSION

SSR polymorphisms in the Opaque-2 locus. Of the 3 SSR loci analysed, phi057 and phi112 were polymorphic whereas umc1066 was non-polymorphic across the tested materials. SSR marker phi112 exhibited a dominant pattern of polymorphism, amplifying a DNA fragments of between 118 and 194 bp (Fig. 1a) within the normal (O2) allele only. Locus phi057 exhibited codominant polymorphism, with the o2o2 genotypes amplifying a band of larger size (Fig. 1b). The band sizes of products from phi 057 and phi112 were similar to those previously reported (CIMMYT Molecular Genetics Service Laboratory, 2000).

Genotype-phenotype correlation. Variable level of tryptophan expression was observed in both QPM and non-QPM inbreds. Nonetheless, the polymorphic SSR markers phi057 and phi112 correctly predicted the expression of tryptophan in kernels of five of the six non-QPM inbred lines ($P<0.001$, Fig. 2). However, inbred line PED49B had a higher than expected tryptophan content that was characteristic of the o2 mutation (0.090, Fig. 2), although SSR genotyping suggested it to be homozygous dominant at the opaque-2 locus (Figs. 1 and 3).

Foreground selection for opaque-2 in BC_1 generation. Because primer phi057 exhibited codominant polymorphism, it was employed as the standard for o2 analysis in the BC populations. Of the 200 BC_1F_1 plants screened (136R/CML176/ /136R), 104 were found to be heterozygous producing products corresponding to both alleles (o2 and O2), while 96 produced a single band corresponding to the homozygous O2O2 condition. The ratio of the two groups in the backcross population was consistent with the proportion 1:1 accorded by the Chi-square test (Table 2, $X^2=0.16< X^2_{0.05}=3.84$) for a single gene in a BC population.

Trait polymorphisms between CML176 and 136R. Phenotypic analysis demonstrated significant differences between inbreds CML176 and 136R for some of the traits evaluated (Table 1). CML176 was significantly ($P<0.001$) taller



(a) (b)

Figure 1. SSR polymorphism observed between QPM and Non-QPM maize lines using (a) marker phi112 and (b) marker phi057. Lanes 13 in (a) and 4 in (b) = PED49B; M(a)=G210A DNA ladder; M(b)PhiX174 HaeIII ladder.

(165 cm) than 136R (145.4 cm) (P<0.001), but 136R had a higher ear placement. A pronounced difference between 136R and CML176 also occurred in the tassel. The anther colour of 136R was purple and CML176 had a cream shade. The two genotypes did not differ in their timing of pollen shed. However, differences in pollen shed duration (PSD) were significant (P<0.001), with 136R displaying a longer duration of pollen shed (7days) than CML176 (2 days). The two inbreds also differed in the level of resistance to the diseases studied. For example, 136R showed resistance to MSV (score of 1) as opposed to CML176 with a score of 3. In contrast, 136R was

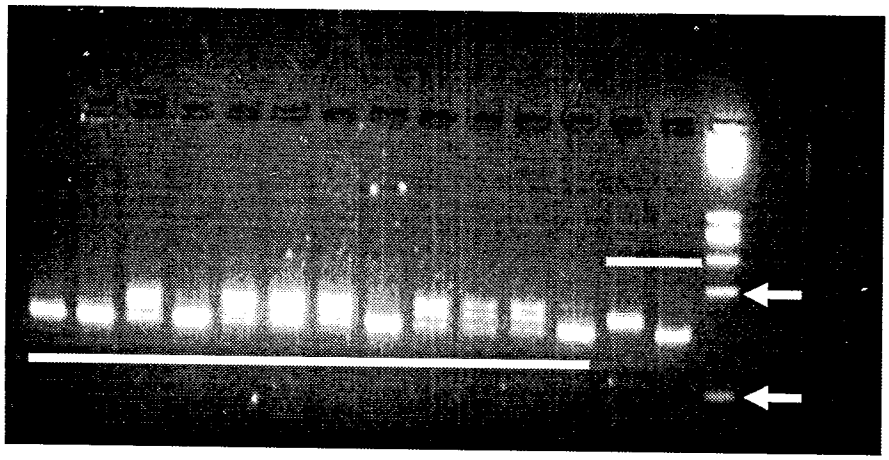


Figure 2. Foreground selection in BC_1F_1 employing phi057: Lanes 1, 2, 4, 8, and 12=O2O2; 3, 5, 6, 7, 9, 10, and 11= o2O2; 13=CML176 (QPM parent); 14=136R (non QPM parent); M=PhiX174 HaeIII marker.

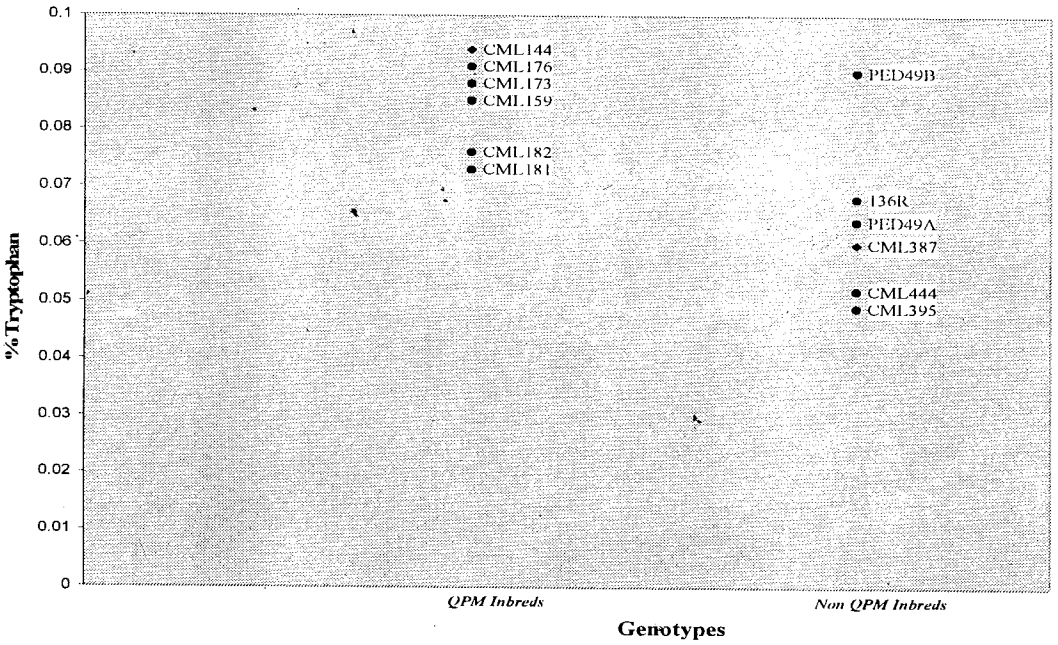


Figure 3. Mean values for trytophan of six QPM and six non QPM inbreds. LSD (0.05) = 0.009315; (ANOVA after square root transformation). *Not significant at (P<0.005).

more susceptible to rust (score 3.0) than CML176 (score 2.0) for CML176. However, differences in TLB rating were insignificant.

Pattern of SSR Polymorphisms and their utility in introgression of o2. A key objective of this study was to identify useable SSR polymorphisms for the Opaque-2 locus and test their effectiveness in marker-assisted introgression of the o2 allele in our QPM breeding programme. Three SSR markers already mapped within the o2 locus were chosen for validation using PCR amplification. Two of the three SSR markers (i.e., phi057 and phi112) could detect polymorphisms between all seven QPM and five of the six normal parents. SSR locus phi057 was codominant, as is typical of most of the SSR markers (Ferreira and Grattapaglia, 1998). However, the marker amplified by the primer phi112, was dominant and in coupling phase with the dominant allele (O2). This is consistent with earlier findings of Tian *et al.* (2003).

The codominant segregation of phi057 successfully discriminated between the two possible genotypes (dominant homozygotes and heterozygotes) at the Opaque-2 locus in the

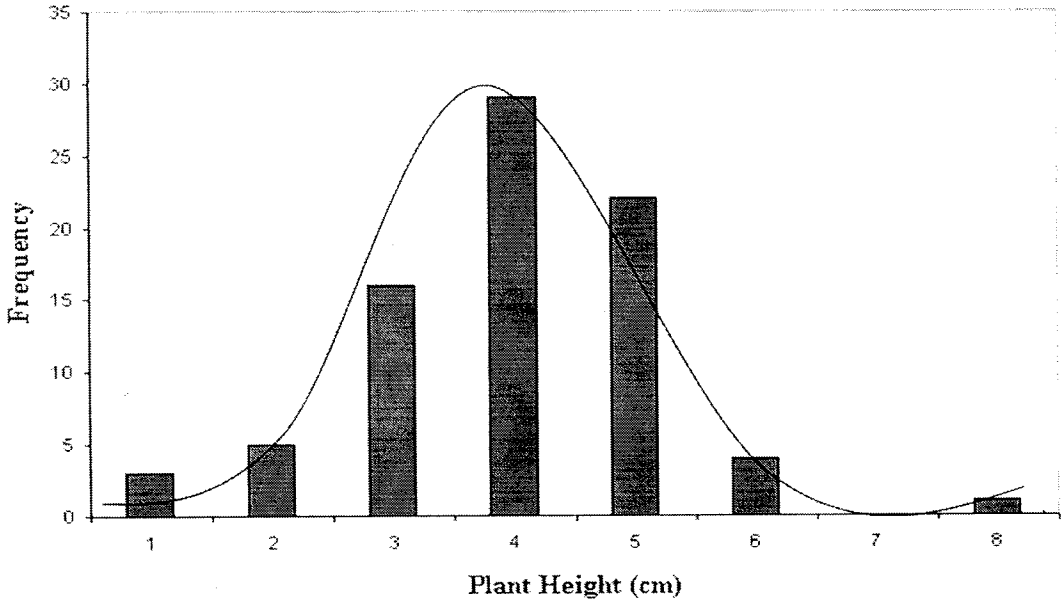


Figure 4. Distribution of plant height in the progeny of 16 BC_2F_2 Families. 1=121-130 cm, 2=131-140 cm, 3=141-150 cm, 4=151 =160 cm, 5=161-170 cm, 6=171-180 cm, 7=181-190 cm, 8=191 -200 cm.

TABLE 1. Phenotypic differences between inbreds 136R and CML176 for selected traits

Entry	Plant height (cm)	Ear height (cm)	D50% P	PSD	Anther pigmentation	D50% S	MSV score	Rust score	TLB score
136R	145.40	56.10	68.00	7.00	Purple	70.33	1.00	3.00	2.00
CML176	165.00	56.00	68.00	2.33	Cream	66.67	3.00	2.00	2.50
LSD (0.005)	2.23	4.13	0.00	1.60	-	1.43*	-	-	-
C.V (%)	0.70	2.10	0.00	15.70	-	0.70	-	-	-

*Not Significant at P<0.005. PSD: Pollen Shed Duration; D50%P: days to 50 % pollen shedding

backcross population, demonstrating its potential utility in national QPM breeding efforts. Since linkage disequilibrium between phi057 and target gene (o2) is complete, individual plants could be scored directly for the gene eliminating the probability of occurrence of false negatives and positives, and consequently, improving the precision of backcrossing. As is the case with all recessive genes, the two conventional selection methods used to introgress the o2 gene rely on the inclusion of a selfing procedure that is done alternately or simultaneously with every backcross step, to differentiate between normal homozygotes and the desired heterozygotes in the backcross generations. Whenever opaque-2 kernels segregating in normal ears of maize can be definitively identified by simple visual examination and/or light table analysis, this would obviate the need to employ MAS. However, the efficacy of visual examination may be limited by the variable translucence that may be encountered in different genetic backgrounds, making some of the o2 phenotypes indistinguishable from their normal counterparts. Therefore, more definitive phenotypic evaluations to differentiate normal and opaque-2 forms would be required including biochemical analysis and zein electrophoretic and chromatographic profiles (Villegas *et al.*, 1992). Since genotypes are identified after the selections are made for the seed and plant characteristics (Kata *et al.*, 1994), a large breeding (BC) population is required. These selection methods are not only expensive, but also delay the breeding process. The lack of capacity to conduct such phenotypic evaluations by our National Maize Programme further complicates the situation. By employing phi057, MAS could offer both cost and time-saving advantage over the most reliable conventional breeding schemes used for QPM line conversion.

TABLE 2. Mean Values for % Trytophan in PED49B and its hybrids

Entry	% Trytophan
PED49B	0.0900
CML181/PED49B	0.0557
CML182/PED49B	0.0567
PED49B/CML173	0.0563
LSD (0.05)	0.00815
C.V (%)	6.7

Because the SSR marker amplified by the primer phi112 was in coupling phase with the normal (O2) allele, the backcross progeny (BC_1) amplified with a similar band profile. Hence, this marker could not distinguish between heterozygous and homozygous plants within the BC_1 population. Its utility in this study was, therefore, limited to the initial parental genotype discrimination. Nevertheless, this marker may also be of use in checking the seed purity during routine field maintenance of QPM inbreds (Babu *et al.*, 2000b).

Pattern of tryptophan expression. As shown in Figure 3, grain tryptophan contents of all the QPM lines tested were superior to five of the six normal inbreds. Inbred line PED49B had a phenotype (tryptophan level) characteristic of the o2 mutation (Fig. 2). Bearing in mind that the opaque-2 mutation conditions high lysine and tryprophan primarily by repressing zein synthesis and stimulating albumins, globulins and glutelin synthesis (Damerval and deVienne, 1993; Habben *et al.*, 1993), it may be envisaged that the high tryptophan level of the "normal genotype" PED49B inbred might also be a consequence of the same phenomenon. Since the genotyping of PED49B with both phi057 and phi112 indicates that it is homozygous dominant at the Opaque -2 locus, we speculated that other genetic system (s) comparable to but outside the O2 locus, may have caused the observed anomaly. In fact, a number of mutations have been identified that affect storage protein synthesis in maize endosperm (Coleman and Larkins, 1998), some of which also condition an opaque kernel phenotype similar to the o2 mutation (Azevedo and Lea, 2003). These include the recessive mutations traditionally classified as "*opaque*" (e.g., *opaque1-15*), the semidominant "*floury*" mutations (e.g., *floury1-3*), and several dominant opaque mutations, *defective endosperm (De)-B30* and *Mucuronate* (Soave and Salamini, 1984).

Although these mutants have a reduced zein content and show altered expression of specific types of zein genes, the degree to which zein synthesis is reduced among them is variable (Hunter *et al.*, 2002). For example, zeins in o1

(Kim *et al.*, 2004), o9, *o11* and *Mc* (Hunter *et al.*, 2002) have been found to be within 80 to 90% of the amount found in normal maize; so zein synthesis in these mutants is barely affected (repressed). The largest reductions in zein protein synthesis have been found to occur in the *o2*, *DeB30* and *fl2* mutants, which have approximately 35 to 55% of the wild-type level of storage proteins (Hunter *et al.*, 2002). Therefore, based on its tryptophan content, we hypothesized that mutant DeB30 or fl2 might also be operative in PED49B. To test this hypothesis, PED49B was crossed with QPM inbreds CML173, CML181 and CML182 and the F_1 progeny analysed for tryptophan content. The results indicated that the level of tryptophan in all three hybrids was significantly less than that in either parent (s) ($P<0.001$, Table 2). We propose that an uncharacterised recessive single gene-two allelic locus may be affecting tryptophan expression in PED49B.

Utility of phenotypic markers in background selection. The differences in phenotype exhibited by 136R were employed as markers to characterise the genetic contribution of 136R to the heterozygous BC_2 progeny developed through use of 136R as the recurrent parent. BC_2F_1 progeny plants displayed the same degree of phenotypic expression of days to 50% pollen shedding, pollen shed duration, tassel pigmentation, MSV, rust and TLB as the recurrent parent. On the other hand, plant height segregated continuously, as is typical of quantitative traits owing to the influence of simultaneous segregation of several genes and environment and considerable heterotic influence of donor height QTLs. However, the probability distribution of plant height appeared negatively skewed (towards the recurrent parent height) (skewness=-0.16), demonstrating the significance of the genotypic contribution of 136R in the BC_2 progeny.

Classically, the expected fraction of genome from the recurrent parent in the *b*th backcross generation is calculated as $1-(^1/_2)^{b+1}$. The theoretical proportion of the recurrent parent genome in the BC_2 progeny would, therefore, be 87.5%. However, this formula ignores "linkage drag" (Brinkman and Frey, 1977), i.e., the persistence of donor genetic material linked to the gene to be introgressed. We hypothesized that the transgressive expression of height in the BC_2 progeny might be due to linkage drag and the theoretical 12.5% donor genome retained in the BC_2. To test the role of linkage drag, we identified 16 BC_2 families in which at least two of the five plants tagged for marker screening were o2 carriers. This allowed us to derive two sets of height means considering the families as random effects-one based on five plants, and the other based on two o2 carriers. Since the two means are correlated, we used a paired t-test on all 16 families together to assess whether, in general, the heterozygote means differed from the mean of all five plants. Results showed no significant difference between the two sets of means ($t_{15}=0.12<t_{15,\ 0.005}=2947$), suggesting that plant height stayed relatively neutral to selection for the o2 allele. Therefore, most variations in height in the BC_2 progeny was considered to result from the contribution of portions of the genome unaffected by marker assisted selection for the o2. Using this information, we envisaged two scenarios for background selection based on height.

The first scenario is based on the premise that the actual donor or recurrent parent genome content (DGC/RPGC) in any BC generation might vary around the mean theoretical value. In BC_1 generation, for example, the expected DGC is 0.50, but in individual BC_1 plants it may vary from 0.25 to 0.75 (Stam, 2003). In the BC_2 generation, the theoretical proportion of the RPG in the BC_2 progeny is estimated to be 87.5%. However, using 88 SSR markers spanning, all the 10 chromosomes of maize genome in background analysis, Babu *et al.* (2004b) found the actual RPG content to vary from 83.7 to 94.8%. On this basis, we hypothesised that o2 carriers with closest height to the recurrent parent had the highest proportion of the recurrent parent genome. Using this criterion, we identified 5 o2 carriers (18%) (mean height 145.4cm±s=2.50) from the selectable population of 32 plants (mean 155.3cm, s= 8.44cm) that we advanced to BC_2F_2. The second scenario was based on the view that results of selecting for height could improve if the proportion of phenotypic variability contributed by genetic variance is high. To assess the utility of this criterion, we subjected the height data to ANOVA and the variance components deduced

considering family effects to be randomly distributed.

Broad-sense heritability was calculated as $H = V_G/V_P$, where V_G is genetic variance [EMS of genotype] and V_P is the phenotypic variance. The expected response to selection was calculated as R= HS; the selection differential (S), is the difference between the mean of the whole population and the mean of the selected group, expressed in standard deviation units (Falconer, 1989). Broad-sense heritability was high for height (62%) and, therefore, a good indicator of genetic merit of selection. Hence, an attempt was made to breed the BC_2F_2 with a mean height corresponding to the RP phenotype from individual plants with height exceeding the RP phenotype. Using this criterion, we identified six o2 carriers from the base population with mean 149.50 cm and standard deviation 1.64 cm. The selection differential was 6.81 cm, and the predicted response to selection was 4.22 cm.

CONCLUSION AND RECOMMENDATIONS

Under the conditions of PCR used, SSR markers phi057 and phi112 are polymorphic in most of the populations used and will, therefore, constitute the framework for marker assisted introgression of the opaque-2 trait into suitable maize genotypes in Uganda, particularly when visual selection is not possible. Optimized phenotypic background selection may be a realistic way to obviate the need for the more expensive marker assisted background selection. However, the opportunity for phenotypic selection might differ between years and locations. In addition, breeding regimes that select on the basis of phenotype alone might require a much larger number of backcrosses and plant populations to enhance the success of selection. The accuracy of our phenotypic selection needs to be validated using molecular markers. The reasons for the high levels of trytophan in PED49B are not yet verified, although other potentially useful genetic systems that repress zein synthesis like the o2 mutation might be responsible. Additional research to elucidate this is needed.

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