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Full Length Research Paper

Marker assisted introgression of opaque2 gene into herbicide resistant elite maize inbred lines

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Marker assisted selection in combination with conventional breeding can greatly accelerate the introgression of modified opaque2 genotype into herbicide resistant maize. By combining these two approaches, time and costs are greatly minimized. The application of opaque2 allele specific SSR markers was done on materials already undergoing selection in a breeding program for converting herbicide resistant maize lines into quality protein maize (QPM) which is the equivalent of modified opaque2 phenotype. The breeder had selected QPM lines using the light table in the previous cycle and we used leaf samples to extract DNA for analysis of the presence of the opaque2 gene using SSR markers. Two co-dominant SSR markers phi057 and umc1066 and a dominant marker phi112 were used. Umc1216, a modifier marker was also tested in combination with the opaque2 markers with the objective of using the marker to select for modifiers for the opaque2 phenotype. The modified FTA paper technology protocol was applied in field sampling. The results showed 97% of the lines were opaque2 while 3% were non-opaque2. Both methods of conventional breeding using light table and marker assisted selection (MAS) were comparable. However, the application of SSR markers and the FTA technology offers the breeder a fast, time saving, reliable and less labour intensive method of screening QPM maize during the early growing stages instead of having to wait to screen the kernels on the light table after harvesting. Moreover, the routine biochemical analysis for high lysine and tryptophan levels need not be carried out at each backcross since the presence of the opaque2 gene is confirmed with markers.

Key words: SSR markers, quality protein maize (QPM), FTA technology, opaque2 modifiers, marker assisted selection (MAS).

INTRODUCTION

Maize is a staple food for millions of people in poor countries around the world but is deficient in two essential amino acids, lysine and tryptophan. However using the maize mutant opaque2 (o2) discovered in the early 1960s (Nelson, 1969; Mertz et al., 1964) scientists developed high lysine and tryptophan maize with soft, chalky endosperm with increased susceptibility to insect pests and reduced yields. Before long plant breeders recognized genes that improved the opaque2 phenotype resulting in normal kernels of vitreous appearance with high lysine content (Ortega and Bates, 1983). More specifically CIMMYT breeders developed high lysine corn from opaque2 genotypes by selecting for the opaque2 phenotype with normal endosperm texture and increased level of lysine and tryptophan. These modified opaque2 maize were designated “Quality Protein Maize” or QPM (Nelson, 2001; Cordova, 2001; Bjarnason and Vasal, 1992; Gevers and Lake, 1992; Vasal et al., 1980).

The conventional breeding procedures have had success in releasing several QPM hybrids both in Africa and Latin America. The process is however cumbersome with each kernel undergoing selection under the light table. The opaque2 trait is expressed in the recessive state whereby the mutant kernels have a typically starchy endosperm texture and low density. These endosperms when placed on a light table do not transmit light compared with the normal wild-type kernels that are vitreous

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and translucent. This forms a very important step in the selection process for QPM maize. The opaque2 maize was unpopular with breeders because of low yields and susceptibility to ear rot and stored grain pest (Crow and Kermicle, 2002). The ability to overcome the negative effects such as softness is through opaque2 modifiers. The opaque2 modifiers were used by CIMMYT breeders to develop QPM opaque2 varieties with characteristics of normal maize (Prasanna et al., 2001).

Numerous agronomic and processing problems associated with opaque2 in the conversion of elite materials into high yielding commercial cultivars have prevented its acceptance by plant breeders throughout the world (Glover and Mertz, 1987). The maize endosperm is approximately 90% starch and 10% protein. About 70% of the protein is prolamin composed of several proteins known as zeins (Gibbon and Larkins, 2005). The opaque2 mutation increases the lysine content in maize endosperm by decreasing the content of zeins, while the opaque2 modifiers alter the soft texture of the opaque kernels into hard endosperm. Therefore to develop the QPM high lysine maize, breeders had to systematically introgress the modifier genes into opaque2 germplasm to develop normal looking maize with high lysine. This is obviously a very tedious process of maintaining the high lysine homozygous recessive opaque2 locus and converting to increased yield hard kernels using the modifiers.

Molecular markers have been identified that are associated with the opaque2 phenotype and opaque2 modifiers (Lopez et al., 2004; Bantte and Prasanna, 2003). With the rapid advances in genome research and molecular technology, MAS holds promise in enhancing selection efficiency and expediting the process of development of new varieties and hybrids with higher yield potential (Ribaut and Hoisington, 1998). Since the advent of DNA marker assisted selection (MAS) in the 80s, marker technology has dramatically increased the effectiveness of selection in breeding and shortened the development time of varieties (Peleman et al., 2003). MAS offer increased reliability, efficiency, cost and time saving advantages. Also MAS is gaining considerable importance due to the efficiency and precise transfer of genomic regions of interest (foreground selection) and the recovery of the recurrent parent genome (background selection) (Babu et al., 2004). Therefore QPM breeders can utilize MAS tools for conversions of elite lines having other favourable traits such as the herbicide resistance trait into QPM maize. For effective MAS application the breeders will also need economical, easily adaptable protocols for DNA sampling and analysis.

MATERIALS AND METHODS

Maize BC1 materials

QPM maize inbred lines CML154, CML159, CML182, CML176, CML173, CML144, and non-QPM herbicide resistant inbred lines CML202, CML204, were crossed and planted at Kiboko Kenya. All the materials were at the BC1 stage. Twenty eight days after planting the plants were sprayed with imidazole to select for herbicide resistance. After about 1 month all the susceptible plants to the herbicide were dead and only resistant plants survived. Samples were then taken for opaque2 analysis using markers in the surviving plants.

DNA sampling and preparation

Sampling was done in the field on 2-3 month old plants using the modified Whatman FTA paper protocol (Mbogori et al., 2006). Briefly, a young leaf was excised from the plant and wrapped round the FTA paper and put in a polythene bag. A pair of pliers was used to press the leaf sample extract on to the FTA paper. Ethanol (70%) was used to clean pliers in between samples to prevent cross contamination. The FTA card was then hanged on the drying line for 4-5 hours air drying and later stored in air tight containers. The DNA sample was prepared by taking two FTA discs measuring 1.2 mm punched for each parent or BC1 plants and placed in a PCR plate containing 50 µl of FTA wash solution and incubated for ten minutes at room temperature with shaking. Using clean tips, the FTA solution was discarded and replaced with fresh FTA wash solution and incubated for ten minutes at room temperature. The FTA solution was discarded and replaced with 100 µl of double distilled water, and incubated for 5 min with shaking. The double distilled water was discarded and replaced with 50 µl of absolute ethanol, incubated for 5 min at room temperature and discarded. The plate was placed at 55°C for 15 min to dry the FTA discs. Twenty (20 µl) PCR mix containing the dNTPs, primer, and magnesium chloride were added in each well.

PCR primers

Primer sequences (F=forward and R=reverse) used were as follows (www.maizegdb.org):

phi057:
F, 5'-CTCATCAGTGCCGTCGTCCAT-3';
R, 5'-CAGTCGCAAGAAACCGTTGCC-3':

umc1066:
F, 5'-ATGGAGCACGTCATCTCAATGG-3';
R, 5'-AGCAGCAGCAACGTCTATGACACT-3':

phi112:
F, 5'-TGCCCTGCAGGTTCACATTGAGT3';
R, 5'-AGGAGTACGCTTGGATGCTCTTC- 3':

umc1216:
F, 5'-TTGGTTGTTGGCTCCATATTCA-3',
R, 5'-GTTATATGCCCGTGCATTGCTA-3'.

The primers were synthesized and fluorescent labelled by Applied Biosystems Company.

PCR optimization using FTA discs

Primers phi057, umc1066, phi112 for the opaque2 loci and primer umc1216 for modifier gene were used to screen for polymorphism between the parents. Optimization was done for parameters magnesium chloride concentration, effect of10% glycerol, annealing temperatures and number of cycles. PCR optimization was performed for each primer. Twenty (20 µl) PCR mix containing the dNTPs, primer, and MgCl were added in each well. Gradient PCR

was performed using the TC-512 PCR machine using the following profile:

Initial denaturation 2 min	94℃	
Denature	1 min	94℃
Anneal	2 min	60℃ gradient 13
Extension		2 min 72℃
Final extension	2 min	72℃
Number of cycles 40		
Final hold		5℃

An optimum annealing temperature of 60℃ was found suitable for all the primers.

PCR amplification and product analysis

Amplification reaction contained 20 µl of PCR mix (1x Reddymix, 3 mM MgCl, 1.25 U Taq, 0.2 mM dNTPs, 40 pM each primer) and 2 FTA disc. For all the reactions, one drop of mineral oil was added in each PCR well to prevent evaporation. PCR amplification was carried using the profile of one cycle initial denaturation for 2 min at 94℃, and 40 cycles of denaturation for 1 min at 94℃, annealing temperature of 60℃ for 2 min, extension for 2 min at 72℃ and a final extension of 10 min at 72℃. The amplified fragments were separated on 4% metaphor agarose at a ratio of 2:1 metamphor: saekem. The PCR products were also analysed with ABI 3730 sequencer.

Data analysis using ABI 3730 sequencer

Resolution of PCR fragments for SSR markers phi057, phi112, umc1066 and umc1216 on 3% agarose, 4% metaphor agarose gel electrophoresis showed that polymorphism detected was tight and requires polyacrylamide gels for accurate resolution in comparison to agarose. PAGE is a very tedious procedure and also requires special equipments like fume hoods and proper waste disposal due to silver staining. We therefore opted to use ABI 3730 which is automated and considered accurate. We also used ABI 377 but this was also very manual and tedious. In addition, ABI 377 does not have the filters for the new improved fluorescent dyes such as NED, VIC and PET. Briefly, 12 µl of 500LIZ size standard is mixed with 1ml of HiDi formamide. Nine (9) µl of this mix is added to 1µl of PCR samples and sent for automated ABI 3730 analysis. Data is then analysed using genemaper3.7 software. The results were consistent with those obtained from the agarose gels (results not shown).

RESULTS AND DISCUSSION

We used fluorescent labelled primers to identify polymerphism on ABI3730 sequencer. On agarose gels, primer umc1066 and umc1216 gave poor resolution and polymorphism could not be clearly detected. However, Babu et al. (2004) obtained codominant polymorphism and could successfully discriminate between all the three possible genotypes for the *opaque-2* gene viz., dominant homozygotes, heterozygotes and recessive homozygotes with the three markers using agarose gels. Our use of the ABI 3730 also proved very effective in not only identifying polymorphism for the co-dominant markers but also gave accurate peak sizes. The process is automated and we could run both the 96 and 384 well plates. Data analysis and documentation was analysed using Genemaper3.7 software. Phi057, phi112 and umc1066 are also known as opaque endosperm2 and are all located on chromosome 7.01. While umc1216 is also known as 27 kD gamma zein or opaque2 modifier.

Primer Phi 057: This primer showed a very good polymorphism with QPM donor parents, showing a band size at 165 bp and the non-QPM donor lines (CML202, CML204) peak size at 159 bp (Figure 1). The polymorphism was well applicable for discriminating between the QPM and non QPM inbred lines and between homozygous and heterozygous opaque2 progeny (Table 1).

Table 1. Discriminating between the QPM and non QPM inbred lines and between homozygous and heterozygous opaque2 progeny.

Parents/progeny	Phi057-non dominant		Percentage of plants
	Allele 1	**Allele 2**	
CML154 (QPM)	165	165	
CML202 (non QPM)	159	159	
Homozygous O2	159	159	
Heterozygous O2	159	165	3 %
Homozygous o2	165	165	97 %
primer	**Phi112- dominant**		**Percentage of plants**
	Allele 1	**Allele 2**	
CML154 (QPM)	none	none	
CML202 (non QPM)	136	136	
Non QPM O2	136	136	3%
Homozygous o2	none	none	97%

One hundred and twenty samples were sampled from the field for opaque2 gene analysis using fluorescent labelled primers Phi057 co-dominant and Phi112 dominant. The materials had already been screened previously prior to planting using the light table for opaque2 gene. The lines were BC1 of cross CML154 (QPM) x CML202 (non QPM). Primer Phi057 discriminated the heterozygotes O2 from homozygotes O2 and homozygotes o2 lines. Only 3 percent of the plants were found to be heterozygous with primer Phi057. However, the dominant primer Phi112 could not discriminate between the homozygotes O2 and heterozygotes O2. Phi 112 primer amplifies for O2 dominant gene. All lines showing no peaks at the expected size of 134 bp for the non QPM line were scored for QPM. The data was analysed using ABI 3730.

Primer UMC 1066: This primer had very good amplifications with strong bands visible on agarose electrophoresis. However, there was no polymorphism detected between QPM and non-QPM donor (134 bp fragment) both on agarose and fluorescent labeled primers. This primer appears monomorphic under our experimental conditions and requires further investigation (Figure 1).

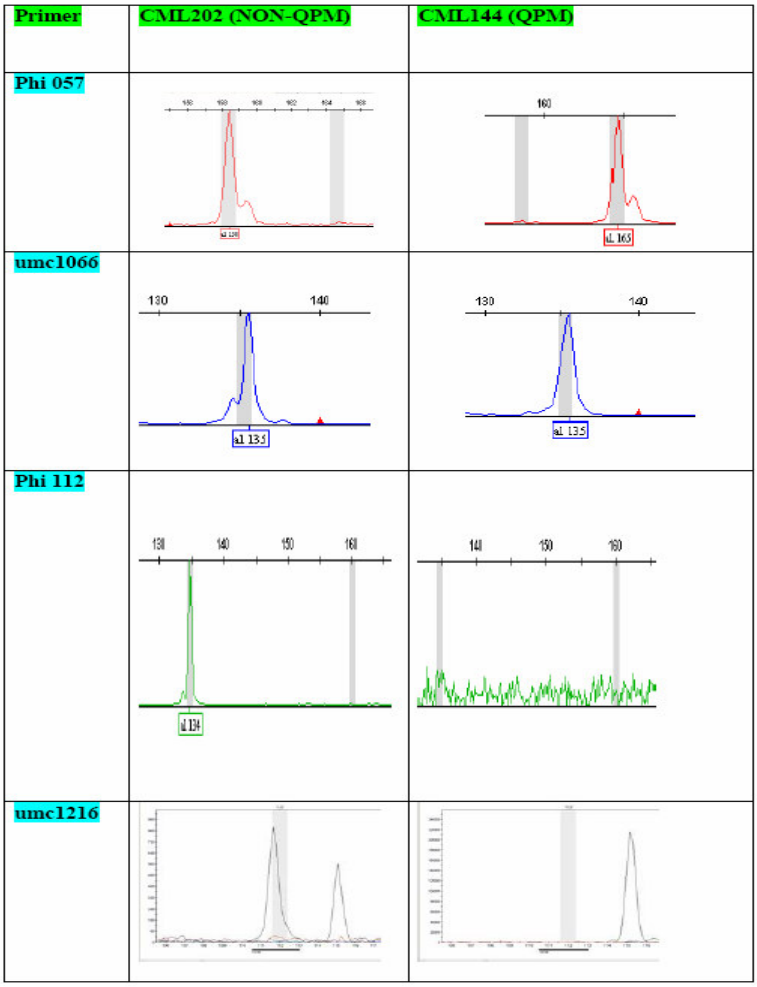

Figure 1. Analysed PCR fragments using fluorescent labelled primers. The figure summarizes the peak sizes as scored by the ABI 3730 sequencer. Inbred line CML202 is the non QPM parent and CML154 is a QPM parent. Phi057 is a co-dominant marker with polymorphism at 159 bp for non QPM and 165 bp for QPM line. Primer umc1066 is codominant marker showing no polymorphism between the QPM and non QPM (135 bp). Phi112 is a dominant marker that shows no amplification for QPM and a fragment size 134 bp for non QPM CML202 and 160 bp for CML204 (not shown). The modifier opaque2 primer umc1216 shows a peak at 112 bp and 115 bp for non QPM and only 115 bp for QPM.

Primer Phi 112: The primer showed a null fragment with all QPM parents and a band size of 136 bp and 160 bp for non-QPM parents CML202 and CML204 respectively (Figure 1). This primer is highly recommended for MAS. However this primer could not be used in discriminating homozygous and heterozygous backcross progeny (Table 1).

Primer UMC 1216: The umc1216 marker is also known as 27-kD gamma zein protein and located on chromosome 7.02. Marker application for modifier gene using primer umc1216 showed two peaks for the non QPM donor at 112 bp and 115 bp while only one peak for the QPM donor at 115 bp (Figure 1). This marker showed reliable discrimination between QPM and non QPM lines. We further scored only for allele 112 bp in non QPM (lines 1-14), QPM (lines 15-24) and the allelic composition of genotypes in a segregating population in crosses randomly selected from a QPM breeder's field (lines 25 to 35) using the genemapper software as shown in Table 2. The allele at 112 bp was detected in only the non QPM lines. This result suggests that the allele at 112 bp is dominant. The observation was consistent with acrylamide gels. Only non QPM lines and one cross of a QPM and a non QPM showed presence of umc1216 opaque2 modifier.

Table 2. Allelic composition of genotypes in a segregating population in crosses randomly selected from a QPM breeder's field.

Normal maize / opaque2 donor	phenotype	Result (bp)
ECAVL2	normal	111.91
SADVLA	normal	111.66
P501SRCO	normal	111.75
P502SRCO	normal	111.76
CML 202	normal	111.96
CML 204	normal	111.80
EM11-133	normal	111.75
EM12-210	normal	111.73
EC 573(R12)C8S3-4	normal	111.90
OSU 23i	normal	111.66
EC 573- (R12) C8S3-93-2	normal	111.64
EC 573-(R12) C8S3-14-1	normal	111.69
CML144	Opaque2 donor	None
CML 150	Opaque2 donor	None
CML 152	Opaque2 donor	None
CML 153	Opaque2 donor	None
CML 154	Opaque2 donor	None
CML 159	Opaque2 donor	None
CML 173	Opaque2 donor	None
CML 175	Opaque2 donor	None
CML 176	Opaque2 donor	None
CML 185	Opaque2 donor	None
(CML 384x CML 176)(F3)4-1-1-2	Cross	111.97
(CML 384x CML 176)(F3)11	Cross	None
(CML 384x CML 176)(F3)98	Cross	None
(CML 384x CML 176)(F3)135	Cross	None
(CML 384x CML 176)(F3)147	Cross	111.71
QPM 1	Cross	None
QPM 2	Cross	None
QPM 3	Cross	None
QPM 4	Cross	None
QPM 5	Cross	None
QPM 6	Cross	None
Water	control	None
Water	control	None

Fourteen non QMP normal maize lines for conversion to QPM and opaque2 donor lines were evaluated. Also included were single cross hybrids between a QPM and normal elite line. Non QPM (lines 1-14), QPM (lines 15-24) and the allelic composition of genotypes in a segregating population in crosses randomly selected from a QPM breeder's field (lines 25 to 35). Modifier marker umc1216 was selected from the maizegdb and we used genemapper software for analysis using fluorescent markers. A peak at 112 bp was present for all non QPM lines and was absent for QPM lines respectively. Only one cross between QPM and non QPM showed the presence of allele 112 bp. This suggests that the allele at 112 bp could be dominant for the modifier opaque2 gene. Opaque2 donor line has the recessive opaque2 allele. Normal maize lines have no opaque2 allele. All the figures in the result column are presented in 2 decimal places.

Therefore the conclusion from these observation is that all the crosses the breeder had planted that were sampled were all of the opaque2 phenotype except cross CML 384 x CML 176) (F3) line 147. These are a reasonable argument considering the fact that the breeder selects only the opaque2 phenotype kernels using the light table rejecting all the modified or normal kernels. There is a great interest by breeders for modifier gene markers. The breeders can select QPM grains efficiently using the light table at a score of 1-5 (Figure 2). Score 1 is fully modified or may be non QPM normal kernels while a score of 5 is chalky, opaque with soft kernels. The breeders always

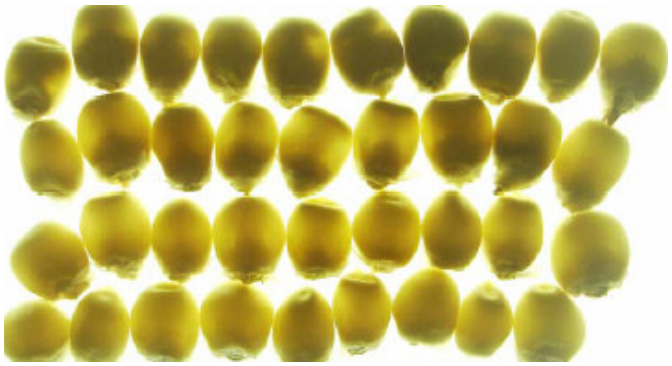

Figure 2. Kernels are arranged according to the degree of opaqueness using the light table. The top row shows opaque kernels with typical opaque2 recessive phenotype usually given a score of 5. The bottom row shows modified opaque2 kernels appearing as normal maize and is given a score of 1. The breeders will usually select the kernels in the middle rows during the early selection cycles.

select a score of 2-3 in the early breeding cycle and make a calculated conclusion that the recessive opaque2 allele is present when they select for the fully modified QPM kernels at later breeding cycles. But the selection has to be repeated through several backcrosses to have complete modification without losing the opaque2 gene. Therefore it became a challenge to find markers that can be used to select for complete modification of opaque2 crosses at early breeding cycles. Precisely, to be able to select lines that carries the opaque2 modifiers for converting the opaque2 phenotype into normal looking maize or QPM with high lysine content. Secondly, use opaque2 markers to detect the opaque2 allele from young leaves in the field. This will eliminate a large population of materials not carrying the opaque2 allele. Even though our results show that the modifier marker could reliably discriminate between the QPM and non QPM lines at the 112 bp allele, further tests for high throughput analysis using a large number of segregating lines is recommended to ascertain its reliability for modifier marker selection without the assistance of the light table.

The opaque2 modifiers create a vitreous kernel by causing a two to three fold increase in the amount of gamma zein protein synthesized in the endosperm (Lopes and Larkins, 1995). The expression of the gamma zein genes may be influenced by the parental background (Burnett and Larkins, 1999). The QPM genotypes contain twice as much 27 kD gamma zeins, which are shown to have a direct relationship with the opaque2 modifier gene dosage (Lopez and Larkins, 1991). Therefore the ability to identify lines with the modifier genes using molecular markers could be an important component of the QPM breeding process.

To the best of our knowledge this is the first report in the use of modifier opaque2 umc1216 marker for MAS. The application of the opaque2 markers and the modifier markers is not only time saving but also economically beneficial to the breeder. We have shown that it is possible to select for fully modified QPM kernels using markers that would otherwise be discarded as non QPM under the light table. Our results confirmed very importantly that markers were reliable for selecting QPM kernels and can be further extended to identifying fully modified QPM kernels. Hence MAS is an important tool for QPM breeders in Africa.

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