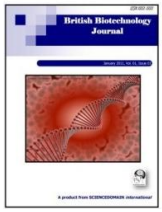

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Characterization of New Quality Protein Maize (QPM) Varieties from a Breeding Program: Analysis of Amino Acid Profiles and Development of a Variety-Diagnostic Molecular Marker

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Authors' contributions

This work was carried out in collaboration between all authors. Author KKN designed the study, compiled the data and wrote the final copy of the manuscript. Author GD performed the molecular analysis, assisted with the writing of the manuscript. Author KM monitored the variety development and field evaluation, and reviewed the manuscript. Author PM monitored the molecular genetic activities and reviewed the manuscript. Author GT assisted with molecular analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims of the Study: This study was to determine the amino acid profile of two newly developed quality protein maize varieties and to develop variety-diagnostic molecular markers.
Methodology: Two new maize varieties, named MUDISHI 1 and MUDISHI 3 have been developed by breeders and farmers using the participatory breeding approach. Total protein and amino acid profiles of the two new lines were compared to their respective parental population and a locally

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released genetically improved normal maize variety. Maize accessions from the DR-Congo breeding program were analyzed using ISSR primers. Variety-diagnostic markers were identified and characterized.

Results: Protein analysis data revealed that MUDISHI 1 and MUDISHI 3 are QPM varieties that are distinct from their original population, Longe 5 QPM from NARI- Unganda and DMR-ESR-W-QPM from the International Institute for Tropical Agriculture (ITTA, Ibadan), respectively. Lysine content in MUDISHI 1, and MUDISHI 3 were 3.5 g and 3.6 g of lysine / 100 g, respectively, which represents a significant increase of 20% and 23% over the genetically improved normal maize variety (Salongo 2) that is currently released. There was a significant increase of 25% of tryptophan and 33% of methionine in MUDISHI 3 compared to its parental variety while the amount of lysine was similar for the two varieties. There were 10% and 15% decrease of lysine and tryptophan, respectively in MUDISHI 1 compared to its original parent Longe 5 QPM. Genomic DNA was extracted from different maize varieties. One ISSR diagnostic-marker of 480 bp that was identified was unique to the QPM variety MUDISHI 3. This sequence was converted to a sequence characterized amplified region (SCAR) marker using a pair of designed primers. This SCAR sequence was not specific to MUDISHI 3 as it was present in all the varieties tested.

Conclusion: The newly developed varieties are typical QPM lines. The development of an ISSR diagnostic marker indicates that it is possible to develop a molecular breeding program involving QPM and normal varieties.

Keywords: Quality Protein Maize (QPM); amino acid profile; molecular markers; variety-diagnostic – marker; DR-Congo; MUDISHI 1 and MUDISHI 3.

1. INTRODUCTION

Maize is a major cereal crop for both livestock and human globally [1]. Several millions of people particularly in developing countries derive their protein and daily calorie requirements from maize [2]. It accounts for up to 15 to 56% of total daily calories in diets of people in 25 developing countries [1]. In these countries particularly African and Latin American, animal protein is scarce, expensive and unavailable to vast majority of the society [3]. Normal maize varieties are deficient in two essential amino acids, lysine and tryptophan required for human nutrition [3,4]. Lysine content in normal maize is 2% which is less than half the amount recommended for human nutrition [5]. Maize contains other amino acid, but low levels of lysine and tryptophan dilutes the contribution of other essential amino acid in maize grains [1]. Due to the poor protein quality in normal maize, there is a high prevalence of malnutrition in countries that rely solely on normal maize as sole source of daily nutrients are faced with high cases of malnutrition [1,6,7]. A genetic approach was taken to address this problem [8]. Researchers at Purdue university (USA) discovered a mutation in maize designated *opaque 2* (*O2*) [7,8]. This mutation doubled the amount of lysine and tryptophan in maize grain compared to the normal variety. They later discovered a pleiotropic effect in *O2* mutants such as soft endosperm, which made them more susceptible to pest attack and kernel damage. This was undesirable for use especially by farmers in developing countries were consumers were familiar with hard kennel normal maize varieties. Efforts to improve the poor grain quality of *O2* maize mutant, led to the development of quality protein maize (QPM) varieties by the international maize and wheat improvement center (CIMMYT) in Mexico in the 1990's [8].

Quality protein maize (QPM) germplasm was developed by the discovery of *opaque 2* (*o2*) endosperm modifier genes [1,9,10]. Two of these modifier genes have been identified. One locus mapped near centromere of chromosome 7 and the second near telomere on the long arm of chromosome 7 [11]. *O2* gene modifiers alter the phenotype of soft *O2* mutant endosperm to vitreous endosperm as well as maintain the double lysine and tryptophan content present in *O2* mutant maize verities. Through back crossing and recurrent selection, breeders at CIMMYT developed a large number of elite QPM varieties for distribution [9,12].

The plant and grain of QPM is similar in appearance and are difficult to distinguish from normal maize. Although similar phenotypically to normal maize, Nutritionally, QPM grains contains approximately 55% and 30% more tryptophan and lysine respectively compared to normal maize varieties [13]. The protein quality of QPM is 90% the nutritional value of skim milk [1].

Several countries in Africa, Latin America along with China have incorporated QPM in their Agricultural development plan [14]. However because of similar phenotypic appearance between normal and quality protein maize, a more reliable method of identifying quality protein maize has to be developed for breeding purpose.

Globally, many Asian, African, and Latin American countries are part of the network facilitated by CIMMYT, for the improvement of QPM in developing countries [1,14]. These breeding programs aimed at developing new QPM varieties that are adapted to specific environments and regional needs. Two new QPM varieties named MUDISHI 1 and MUDISHI 3 have been developed in the DR-Congo maize breeding program. These varieties are adapted to different agro-ecological regions.

The main objective of the present study was to determine the amino acid profile of MUDISHI 1 and MUDISHI 3 and to develop variety-diagnostic molecular markers that could be used to specifically track this accession in a maize breeding program.

2. MATERIALS AND METHODS

2.1 Genetic Materials

The QPM accessions used in the present study are described in Table 1. They include, GPS-5, Salongo – 2, DMR-ESR- W; DMR-ESR-W-QPM, Locale-2, AK9331-DMR-ESR-Y, MUS-1, Locale – 1, QPM LONGE-5, ECAQVE-3, ECAQVE-4, ECAQVE-6, QPM-SRSYNTH, SUSUMA, MUDISHI-1 AND MUDISHI-3. The source and year of introduction of each maize variety used in this are listed in Table 1.

2.2 Development of MUDISHI 1 and MUDISHI 3

MUDISHI 1 and MUDISHI 3 are open-pollinated quality protein varieties developed by the National Institute for Agronomic Study and Research, (INERA – DR-Congo) and Laurentian University, Sudbury, Ontario, (Canada). They were developed by breeders and farmers using the participatory breeding approach. The original variety used to develop MUDISHI 1 was QPM Longe 5 and DMR-ESR-W – QPM for MUDISHI 3. Longe 5 QPM was from NARI – Uganda while DMR –ESR-W QPM was obtained from the International Institute for Tropical Agriculture (IITA) in Ibadan, Nigeria.

MUDISHI 1 and MUDISHI 3 are the results of open pollinations of their parental lines with several QPM and normal maize varieties that were grown in the same location for few seasons. The QPM accessions include QPM Longe 5, ECAVE – 3, ECAVE-4, ECAVE-6, QPM – SR-Synth, and Susuma and the normal maize involved are DMR –ESR-W, AK9331-DMR-ESR-Y, Salongo 2, MUS 1, GPS 5, and Locale 1. The open pollinated plants were grown and progenies were selected in isolation for different agronomic characteristics for several cycles in different environments. The main selection criteria include, spike size, resistance to mildew and maize streak virus, grain yield and nutritional quality (lysine, tryptophan and other amino acid contents), and organoleptic characteristics. Plant selection and variety evaluation were performed using participatory variety selection (PVS) approach with local farmers led by breeders. The new varieties are adapted to agro-ecological conditions of Southern, Central, and Western DR-Congo.

2.3 Protein and Amino Acid Analysis

Amino acid analyses were conducted at the University of Missouri Agricultural Experiment Station Chemical Laboratories (ESCL). Total amino acid profiles were determined for the newly developed QPM varieties, MUDISHI 1 and MUDISHI 3 along with their respective parental lines (QPM Longe 5 and DMR – ES – W- QPM). One locally released and genetically improved normal maize variety (Salongo 2) was also analyzed as reference. All the analyses were conducted in triplicates. The grain amino acid concentration was evaluated using AOC standard method (Method 982.30 E (a, b, c), AOAC [15]. Crude protein was determined by combination analysis (Method 990.03, [15] using the formula crude protein = N x 6.25. ANOVA (two-way) was used to identify significant variation for each amino acid and crude protein. The least significant differences were determined to compare means.

2.4 Molecular Analysis

2.4.1 DNA extraction

Total genomic DNA from maize seedlings were extracted using the cyltrimethylammonium bromide (CTAB) protocol described by Nkongolo [16] and Nkongolo et al. [17] with some modifications. The modifications included the addition of polyvinylpyrrolidone (PVP) and beta-

mercaptoethanol to the CTAB extraction buffer. DNA extracted purity was determined using spectrophotometer (Varian Cary 100 UV-VIS spectrometer).

2.4.2 Amplification of ISSR and RAPD primers

A total of 24 ISSR and 46 RAPD primers synthesized by Invitrogen were used for DNA amplification. PCR analysis was carried out following the procedure described by Mehes et al. [18] and Vaillancourt et al. [19]. Each PCR reaction was performed using a total of 25 µl volume containing 11.4 µl double distilled water, 10 mM tris-HCl pH 8.3 at 25ºC taq buffer, 2 mM $MgSO_4$, and 0.5 µM of each dNTP (Applied Biosystems, Foster City, CA), 0.5 µM primer, 5ng/µl genomic DNA and 0.625 U of taq DNA polymerase (Applied Biosystems, Foster City, CA). For each primer, double distilled water was used as a negative control. Also a drop of mineral oil was added to each reaction to prevent evaporation. The samples were amplified in a thermal cycler (Perkin Elmer, Foster City, CA). The cycles performed were as follows: an initial denaturation at 95ºC for 5 minutes, followed by a 2 minute incubation at 85ºC at which point the taq polymerase was added; 42 cycles of 30 minutes at 95ºC, 90 seconds at 55ºC and 30 seconds at 72ºC; a final extension for 7 minutes at 72ºC was followed by subsequent incubation at 4ºC.

To the PCR product, 5 µl of loading buffer was added to make a total of 30 µl. About half of this volume was loaded in a 2% agarose gel stained with 1 µl ethidium bromide. Running buffers used were 0.5X tris borate EDTA or 1X tris acetate EDTA buffer. These products were run against a 1 kb plus DNA ladder for approximately 150 minutes at 64 volts. The agarose gels were visualized and documented by using the Bio-Rad ChemiDox XRS system and analyzed with the discovery series quantity 1 D Analysis software.

2.4.3 Cloning and sequencing

A variety-diagnostic band was identified at 480 bp in maize variety MUDISHI 3 by amplifying maize genomic DNA with ISSR primer HB 15 (Fig. 1). This band was cloned and sequenced as described by Vaillancourt et al. [19] with the following modifications: Unique diagnostic band was run in low melt 3% agarose gel. It was excised and gel plugs were dissolved with 1X tris-EDTA (TE) buffer. DNA extractions from gel plugs were achieved through several chloroform and phenol DNA extractions described by Vaillancourt et al. [19]. After sequencing, a primer pair was designed and synthesized to target the insert region by using Life technology software (OligoPerfect^{TM} Designer). The primer pair was used to amplify normal and quality protein maize DNA to verify the specificity of the SCAR markers.

3. RESULTS

3.1 Agronomic Characteristics

MUDISHI 1 and MUDISHI 3, two new maize varieties with white grain color were released in 2012 and they are adapted to all the maize growing regions in western, central, and southern DR-Congo. Days to maturity for these varieties in these areas average 115 and 100, for MUDISHI 1 and MUDISHI 3, respectively. The yield in farmer's field without fertilizers is 1 T to 1.5 T for MUDISHI 1 and 0.8 to 1 T/ ha for MUDISHI 3. This yield can reach up to 6 T / ha for MUDISHI 1 and 3 T to 4 T for MUDISHI 3 at research field station under mineral fertilization. MUDISHI 1 is susceptible to down mildew and maize streak virus. MUDIDHI 3 is highly resistant to downy mildew and to lodging and resistant to maize streak virus.

3.2 Amino Acid Profiles

The overall amino acid composition of the maize varieties and the levels of statistical significance obtained from analysis of variance are shown in Table 2. Lysine content in MUDISHI 1 and MUDISHI 3 were 3.6 g and 3.5 g of lysine / 100 g, respectively, which represents a significant increase of 23% and 20%, over the genetically improved normal maize variety (Salongo 2) that is currently released. There was a significant increase of 25% of tryptophan in MUDISHI 3 compared to its parental variety (DRM-ESR-W–QPM) while the amount of lysine was similar for the two varieties. But, there were 10% and 15% decrease of lysine and tryptophan, respectively in MUDISHI 1 compared to its original parent Longe 5 QPM.

The other potentially limiting amino acids are threonine, isoleucine and methionine. Threonine and isoleucine levels were relatively similar in MUDISHI 1, MUDISHI 3, DMR-ESR-W QPM, Longe 5 QPM, and Salongo 2. A significant increase of 20% and 33% of methionine in MUSHISHI 3 over Salongo 2 and the original parent (DMR-ESR-W-QPM), respectively was observed. Likewise there were 34% more methionine in MUDISHI 1 compared to its

parental line, Longe 5 QPM. The levels of leucine and glutamic acid were the same in MUDISHI 3 compared to its original parental DMR-ESR-W-QPM. But the values for these elements were slightly lower in MUDISHI 1, Longe 5 QPM, and Salongo 2. The crude protein content were 9.1%, 8.9%, 9.5%, 8.8%, and 9.6% in MUDISHI 1, MUDISHI 3, DMR-ES-W-QPM, Longe 5 QPM, and Salongo 2, respectively (Table 2).

Table 1. List of maize accessions used in this study

Varieties	Origin	Year of introduction	Type
GPS-5	INEAC-Gandajika-DR-Congo	-	NORMAL
SALONGO-2**	INERA- Gandajika-DR-Congo	1976	NORMAL
DMR-ESR-W**	IITA-Ibadan	1994	NORMAL
LOCALE-2	Farmers- Gandajika-DR-Congo	-	NORMAL
AK9331-DMR-ESR-Y	IITA-Ibadan	1994	NORMAL
MUS-1**	INERA	1996	NORMAL
LOCALE-1	Farmers- Gandajika-DR-Congo	-	NORMAL
DMR-ESR-W-QPM	IITA –Ibadan	1994	QPM
QPM-LONGE 5***	NARI-Uganda	2008	QPM
ECAQVE-3	CIMMYT-Kenya	2008	QPM
ECAQVE-4	CIMMYT-Kenya	2008	QPM
ECAQVE-6	CIMMYT-Kenya	2008	QPM
QPM-SR-SYNTH***	CIMMYT-Kenya	2008	QPM
SUSUMA	CIMMYT-Kenya	2008	QPM
MUDISHI 1	INERA-DR-Congo/Laurentian University	2012	QPM
MUDISHI 3	INERA-DR-Congo/Laurentian University	2012	QPM

***Selected improved normal maize varieties, ***Selected Elite Quality protein maize varieties, CIMMYT: International Maize and Wheat Improvement Center, INERA: National Institute of Agronomic Research and Studies (DR-Congo), INEAC: National Institute of Agronomic Studies (Belgium, Congo), IITA: International Institute of Tropical Agriculture and NARI: Namulonge Agriculture Research Institute*

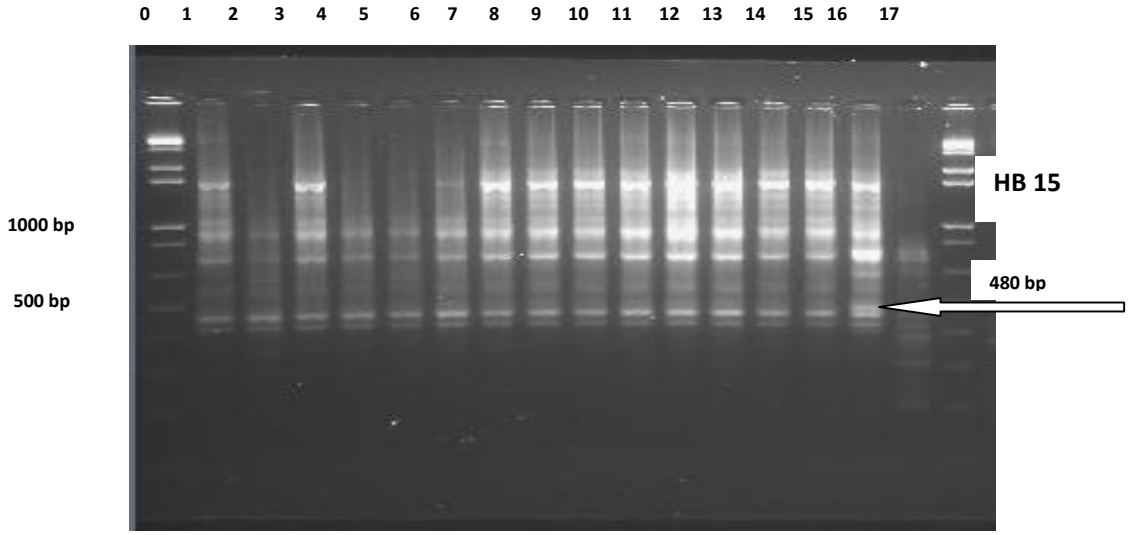

Fig. 1. ISSR amplification of maize DNA samples with the HB 15 primer-generating a 480 diagnostic marker

*Lanes 0 and 17 contain 1-Kb plus ladder; Lanes 1 to 15 contain GPS-5, SALONGO-2**, ECAQVE-6, AK9331-DMRESR-Y, MUS-1**, LOCALE-1, QPM-LONGE5***, ECAQVE-3, ECAQVE-4, DMR-ESR-W**, LOCALE-2, QPM-SRSYNTH***, SUSUMA, MUDISHI 1 and MUDISHI 3; Lane 16, blank. Arrow on the right indicates variety-diagnostic marker at 480 bp*

Table 2. Total protein and essential amino acid content in quality protein maize (QPM) and normal maize varieties from the DR-Congo breeding program

Essential AA w/w (%)*	Corn varieties					LSD
	Mudishi 1	Longe 5 qpm	Mudishi 3	Dmr-esr-w-qpm	Salongo 2	
Taurine	0.11 (1.2)	0.12 (1.4) (1.36)	0.11 (1.2)	0.12 (1.3)	0.03 (0.3)	0.05
Hydroxyproline	0.04 (0.44)	0.04 (0.5)	0.02 (0.3)	0.04 (0.4)	0.03 (0.3)	0.05
Aspartic acid	0.63 (6.9)	0.63 (7.2)	0.56 (6.3)	0.64 (6.8)	0.60 (6.3)	0.58
Threonine	0.33 (3.6)	0.32 (3.7)	0.31 (3.5)	0.35 (3.7)	0.34 (3.6)	0.30
Serine	0.40 (4.4)	0.39 (4. 5)	0.41 (4.6)	0.42 (4.4)	0.44 (4.6)	0.44
Glutamic acid	1.62 (17.8)	1.51 (17.2)	1.63 (18.2)	1.74 (18.4)	1.89 (19.7)	1.00
Proline	0.85 (9.3)	0.87 (9.9)	0.82 (9.4)	0.86 (9.1)	0.86 (9.0)	0.70
Lanthionine	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	-
Glycine	0.38 (4.2)	0.39 (4.5)	0.38 (4.3)	0.40 (4.2)	0.35 (3.7)	0.45
Alanine	0.63 (6.9)	0.57 (6.5)	0.65 (7.3)	0.68 (7.2)	0.75 (7.8)	0.71
Cysteine	0.24 (2.6)	0.23 (2.6)	0.22 (2.5)	0.22 (2.3)	0.20 (2.1)	0.40
Valine	0.46 (5.1)	0.47 (3.4)	0.44 (5.0)	0.48 (5.1)	0.47 (4.9)	0.45
Methionine	0.21 (2.3)	0.15 (1.7)	0.20 (2.3)	0.16 (1.7)	0.18 (1.9)	0.10
Isoleucine	0.32 (3.5)	0.30 (3.4)	0.31 (3.5)	0.34 (3.6)	0.36 (3.8)	0.40
Leucine	1.03 (11.3)	0.90 (10.3)	1.05 (11.9)	1.11 (11.7)	1.31 (13.7)	0.81
Tyrosine	0.20 (2.2)	0.20 (2.3)	0.22 (2.5)	0.23 (2.4)	0.26 (2.7)	0.15
Phenylalanine	0.43 (4.7)	0.39 (4.5)	0.43 (4.9)	0.46 (4.9)	0.50 (5.2)	0.59
Hydroxylysine	0.02 (0.2)	0.02 (0.2)	0.02 (0.2)	0.02 (0.2)	0.02 (0.2)	0.17
Ornithine	0.01 (0.0)	0.01(0.1)	0.01 (0.1)	0.01 (0.1)	0.01 (0.1)	0.00
Lysine	0.33 (3.6)	0.35 (4.0)	0.29 (3.5)	0.33 (3.5)	0.28 (2.9)	0.29
Histidine	0.33 (3.6)	0.34 (3.9)	0.29 (3.3)	0.33 (3.5)	0.27 (2.8)	0.35
Arginine	0.46 (5.1)	0.49 (5.6)	0.42 (4.7)	0.46 (4.9)	0.39 (4.1)	0.60
Tryptophan	0.07 (0.8)	0.08 (0.9)	0.07 (0.8)	0.06 (0.6)	0.05 (0.5)	0.05
Total	9.10	8.77	8.86	9.46	9.59	
Crude protein	9.83	9.45	9.55	10.25	9.89	

**The values are expressed in w/w = grams per 100 grams of sample. The number is parentheses represent the percent (%) of individual amino acid in the crude protein. AA = Amino Acid. Longe 5 QPM and DMR-ESR-W-QPM are parental lines for MUDISHI 1 and MUDISHI 3, respectivel*

Overall, the total basic acids, which include lysine, arginine, and histidine constituent 12.3% and 11.2 % of the total amino acids for MUDISHI 1 and MUDISHI 3, respectively. These values were 11.9% and 13.5% for DMR-ESR-W QPM and Longe 5-QPM varieties, respectively. They were lower (9.8%) in normal maize Salongo 2. In general, the total basic acids are considerably lower than the acidic amino acids (aspartic acid and glutamic acid), which represent around 25% of the total amino acid residue for both MUDISHI 1 and MUDISHI 3. The acidic amino acid levels were 25.2%, 24.4%, and 26%, for DMR-ESR-W QPM, Longe 5 QPM, and Salongo 2, respectively.

3.3 Molecular Analysis

3.3.1 ISSR and RAPD primer analysis

The ISSR and RAPD primers used are described in Tables 3 and 4. Out of the 70 primers screened, 24 ISSR and 46 RAPD primers generated amplified products. One of the 17 ISSR primers HB 15 generated a diagnostic marker at 480 bp for MUDISHI 3 (Fig. 1) that was selected for further analysis. The other primers either generated poor amplification or did not produce unique band for QPM variety identification.

3.3.2 Identification of variety-diagnostic markers

The variety-diagnostic marker of 480 bp size that was diagnostic for MUDISHI 3 DNA sample was cloned and sequenced. The consensus sequence described in Fig. 2 has been registered in the National Center for Biotechnology Information (NCBI) Genbank in Bethesda (Maryland, USA) under the accession number KM360096. BLAST search results reveal 88% matching with a deoxyribonuclease from *Pantoea vagans* C9-1. The sequence showed also 73% and 78% similarity with deoxyribonuclease from *Yersinia enterocolica* LC20 and *Serratioa marcescens*, respectively.

A primer pair targeting the insert was designed to produce a sequence characterized amplified region (SCAR) marker (Table 5). The primer pair amplified the targeted band in all the DNA samples from the QPM and normal maize varieties screeed. The identified marker is therefore diagnostic for MUDISHI 3 but not variety-specific when converted to a SCAR marker (Fig. 3).

4. DISCUSSION

4.1 General Characteristics

The two newly developed QPM varieties (MUDISHI 1 and MUDISHI 3) are the results of open pollinations of their parental lines with several QPM and normal maize varieties that were grown in the same location for few seasons. Based on amino acid profile, they have all the characteristics of quality protein maize varieties. The agronomic evaluation of these two lines revealed that they are adapted to several agro-ecological conditions in the DR-Congo and they are resistant to local maize diseases. In facts, hundreds of different open-pollinated varieties were developed by farmers in the United States during the 19th and early 20th centuries using the same approach described for MUDISHI 1 and MUDISHI 3 development. The method consisted in selecting for different plant characteristics in different environments. Some of the more famous of these American corn varieties were Krug, Lancaster Sure Crop, Leaming, Midland, and Reid [20].

4.2 Amino Acid Analysis

In several reports, lysine levels have been associated with tryptophan levels. The data reported in the present study are in accord with the lysine values reported by Mbuya et al. [2] and Kniep and Mason [21]. The significant increase of basic totally charged and hydrophilic amino acids in MUDISHI 1 and MUDISHI 3 compared to normal maize is consistent with other QPM varieties analyzed and suggest an increase in nonzein protein and hydrophobicity in QPM [2,22]. The concentration of lysine in the maize endosperm has been shown to be highly correlated with the content of a single nonzein protein called the protein synthesis factor EF-1α [23,24]. All this data indicate that MUDISHI 1 and MUDISHI 3 are QPM maize varieties.

Even though lysine content in MUDISHI 1 and MUDISHI 3 proteins were significantly higher than normal maize, it is still below the recommended FAO/WHO reference lysine standard value of 58 mg/g of dietary protein for a 2 – 5 year child [5]. Specifically, MUDISHI 1 and MUDISHI 3 supply 62% and 60% of human lysine requirements, respectively compared to 48% for Salongo 2.

Although normal maize is not deficient in isoleucine or threonine, the presence of large

amount of leucine in human diet can cause both amino acid imbalances and interference of isoleucine absorption. The ratio of leucine / isoleucine found in MUDISHI 1 and MUDISHI 3 were 3.2 and 3.3, respectively. This indicates that these two new varieties provide proteins with a better EAA balance compared to normal maize. This is consistent with Huang et al. [25] stating that a pleiotropic increase in non-zein proteins is contributing to an improved amino acid balance.

4.3 Molecular Analysis

To date, most studies on QPM in relation to molecular markers have been restricted to identifying genetic distance and diversity among QPM and normal maize [26,27]. Nkongolo et al. [26], studied genetic diversity among QPM and normal maize accessions from Africa, and found low genetic distance and diversity between accessions which have also been reported in other studies [28].

In the present study, only 50% of the RAPD primers generated PCR amplification products compared to 74% of ISSR primers that amplified DNA samples. This could be due to more sensitivity of RAPD primers to PCR contaminants compared to ISSR primers [29].

We have identified an ISSR marker that is diagnostic for MUDISHI 3 in a breeding program. This ISSR marker was not specific once converted to a SCAR marker. This suggests that it is present in other varieties but in a low copy number. The marker will be useful as a diagnostic tool to track MUDISHI 3 genome in progenies derived from crosses involving this variety.

There exists couple of reasons that could explain why diagnostic marker such as the 480 bp sequence in MUDISHI 3 once converted to a SCAR marker is not specific. The appearance of a band in one species or a variety and its absence in another could be the result of competition among DNA fragments during amplification [30]. Amplified products which are complementary to each other are stabilized by internal base pairing that could prevent amplification by out-competing the binding of random primers [30,31]; this is the most serious problem that leads to the incorrect interpretation of results. Formation of secondary structures including hair pin, by DNA fragments. Since the SCAR system is a more sensitive technique, the above problems will not interfere with the amplification of sequences even if they are present in a low copy number.

Table 3. The 3' anchored nucleotide sequences of ISSR primers used to screen DNA samples from all the maize varieties

ISSR Primer	Nucleotide sequence (5'-3')	Fragment size range (bp)
809	(AG)8G	250-850
818	(CA)8G	250-100
823	(TC)8C	-
827	(AC)8G	100-650
829	(TG)8C	300-850
834	(AG)8YT	300-1650
835	(AG)8YC	200-1000
841	GAA GGA GAG AGA GAG AYC	300-1000
844	(CT)8RC	300-850
849	(GT)8YA	200-1000
873	(GACA)4	-
879	(CTTCA)3	100-2000
17898B	(CA)6GT	200-1650
ECHT 3	(AAC)3GC	-
ECHT 4	(AAG)3GC	-
HB 15	(GTG)3GC	200-1000
SC ISSR 3	(GAC)4G	400-5000
SC ISSR 4	(CGT)4C	-
SC ISSR 5	(ACG)4AC	300-5000
SC ISSR 6	(TTG)5CB	-
SC ISSR 7	(AGG)5GY	-
SC ISSR 8	(AGAT)5GY	-
SC ISSR 9	(GATC)3GC	300-2000
SC ISSR 10	(CTT)5(CCT)6CT	-

Table 4. Nucleotide sequences of RAPD primers used to screen DNA samples from all the maize varieties

RAPD primers	Nucleotide sequence	Fragment size (bp)
C10	TGTCTGGGTG	400-2000
D8	GTGTGCCCCA	-
E12	TTATCGCCCC	500-5000
F10	GGAAGCTTGG	500-2000
Grasse 1	CCGCCCAAAC	-
Grasse 2	GTGGTCCGCA	200-1650
Grasse 3	GTGGCCGCGC	-
Grasse 4	GAGGCGCTGC	400-1650
Grasse 5	CGCCCCCAGT	-
Grasse 7	CACGGCGAGT	-
Grasse 9	GTGATCGCAG	1000-2000
OPA 1	CAGGCCCTTC	400-1000
OPA 3	AGTCAGCCAC	400-1000
OPA 8	GTGACGTAGG	-
OPA 11	CAATCGCCGT	400-5000
OPA 12	TCG GCGATAG	-
OPA 14	TCTGTGCTGG	-
OPA 15	TTCCGAACCC	-
OPA 16	AGCCAGCGAA	-
OPA 17	GACCGCTTGT	-
OPA 18	AGGTGACCGT	400-2000
OPA 19	CAAACGTCGG	-
OPA 20	GTTGCGATCC	300-2000
OPB 1	GTTTCGCTCC	500-1650
OPB 2	TGATCCCTGG	100-1650
OPB 3	CATCCCCCT G	-
OPB 4	GGACTGGAGT	200-1000
OPB 6	TGCTCTGCCC	400-1650
OPB 7	GGTGACGCAG	300-1000
OPC 10	TGTCTGGGTG	-
OPE 9	CTTCACCCGA	-
OPT 17	CCAACGTCGT	300-5000
OPX 4	CCGCTACCGA	400-5000
OPY 9	AGCAGCGCAC	300-2000
PINUS 23	CCCGCCTTCC	-
PINUS 146	ATGTGTTGCG	-
UBC 48	TTAACGGGGA	-
UBC 78	GAGCACTAGC	-
UBC 186	GTGCGTCGCT	300-1650
UBC 214	CATGTGCTTG	-
UBC 270	TGCGCGCGGG	300-850
UBC 402	CCCGCCGTTG	-
UBC 486	CCA GCATCAG	-
UBC 494	TGATGCTGTC	-
UBC 551	GGAAGTCCAC	400-1000
UBC 561	CATAACGACC	-

Table 5. Nucleotide sequences of the designed primers targeting variety-diagnostic ISSR marker

Primer name	Bases	Sequence (5'-3')
Maize 480 6F	20	TCA TTG TTC ACA CCC GTG AT
Maize 480 6R	20	GCC AGC GTT TCT AAA TCC AC

GTGGTGGTGG CTCTGGGGGA AACCGgGCTG GATTATCACT ATCAGCCAGA AACAAAAGAT CAGCAGCAGC GCTCGTTCCT GGAACATATC CGTACCGGTA TTGCGCTGAA CAAACCGA**TC ATTGTTCACA CCCGTGATGC CGCGAAGAT ACCCTGACGA TTCTGCGTGA AGAGCAGGTT GAACGTTGCG GCGGCGTGCT GCACTGCTTC ACTGAGGATC AGCCCACCGC AGCAAAACTG CTGGATATGG GCTTTTACAT CTCTTTTTCC GGCATCGTCA CATTCCGCAA TGCCGAGCAG TTACGTGAAG CCGCACGCTA TGTGCCGCTG GATCGGATGC TGGTGGAAAC GGATTCGCCT TATCTGGCAC CGGTGCCTTT CCGTGGTAAA GAGAATCAGC CCGCTTATAC GCGCGATGTT GCCGAATATC TGGCTATCCT GAAAGGGGTG GATTTAGAAA CGCTGGC**AGCCACCACCAC

Fig. 2. Consensus sequence of variety-diagnostic ISSR marker of 480 bp from MUDISHI 3 generated by the ISSR primer HB 15; Underlined region indicates the ISSR HB 15 primer consensus sequence. Bolded region indicates the SCAR marker region 349 bp

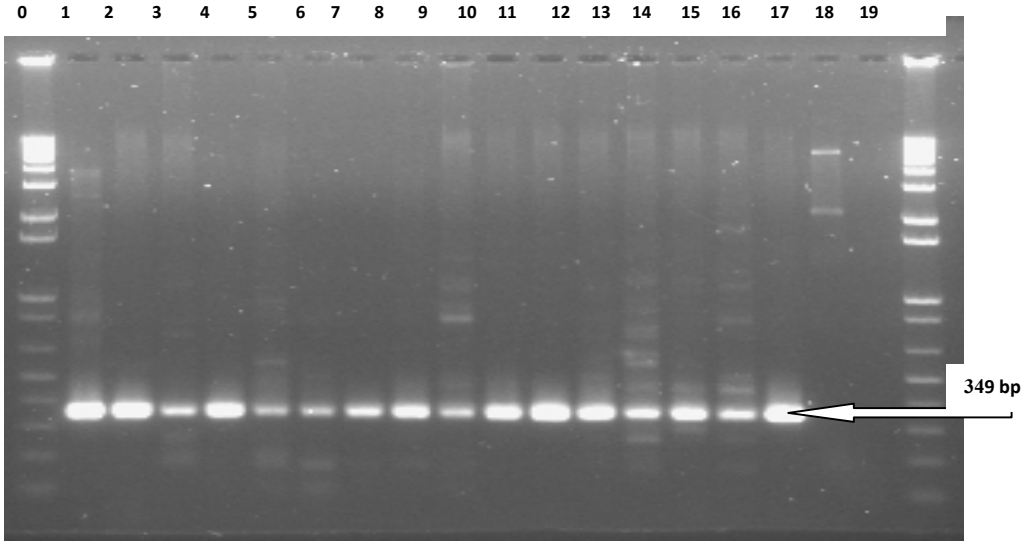

Fig. 3. SCAR bands generated using a designed pair of primers targeting the MUDISHI 3 diagnostic marker. Lane 0 and 19 contain 1-Kb plus ladder; lane 1, Recombinant plasmid with 480 bp marker; lane 2, MUDISHI 3; lane 3-16, maize varieties 15 contain GPS-5, SALONGO-2, ECAQVE-6, AK9331-DMR-ESR-Y, MUS-1, LOCALE-1, QPM-LONGE-5, ECAQVE-3, ECAQVE-4, DMR-ESR-W**, LOCALE-2, QPM-SRSYNTH***, SUSUMA, MUDISHI 1 and MUDISHI 3 1-15 (Table 1); lane 17, background plasmid (no insert); lane 18, blank. The arrows indicate the SCAR marker that is present in all the DNA samples**

The need for molecular identification of maize variety by using variety-specific markers such as SCAR has increased recently in maize breeding programs. This is partly due to the development of QPM hybrid adapted to local regions that require the application of molecular tools that are more reliable than other methods. Another reason would be due to the open pollinated nature of maize plant that leads to contamination of elite QPM maize lines if not properly handled [1]. Protein analysis which indicates correctly the presence of improved protein is very expensive and not recommended for most breeding programs [32]. The application of diagnostic markers could be quite expensive and time consuming. It can be only used if a SCAR marker cannot be developed. Hence, for molecular breeding, SCAR marker remains the most effective method to track QPM genome in maize hybrid background [19].

The low level of genetic variation among accessions that made it difficult to develop more diagnostic markers could be explained by the effect of bottle neck among African maize accessions [26]. More studies would need to be carried out to develop SCAR markers for QPM by possibly using more unanchored ISSR primers.

5. CONCLUSION

The premise of this study was to determine the amino acid profile of a new QPM and to develop variety diagnostic/ specific molecular markers for quality protein and normal maize using ISSR and

RAPD primers. Protein analysis data revealed that MUDISHI 1 and MUDISHI are QPM varieties that are distinct from their original populations (Longe 5 QPM and DMR-ESR-W-QPM). One primer revealed a diagnostic marker for QPM MUDISHI 3. The remaining primers showed relatively good amplification and a high level of polymorphism. Primers flanking the diagnostic marker sequence were developed. However the SCAR marker amplified was present in all the maize accessions analyzed. Although the diagnostic marker that we have developed is useful in tracking MUDISHI 3 genome in progenies, further analysis of several other ISSR and RAPD primers is required to achieve the main goal of developing variety-specific markers in the targeted breeding program.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Prasanna BM, Vasal SK, Kassahun B, Singh NN. Quality protein maize. Curr Sci. 2001;81(10):1308-1318.
2. Mbuya K, Nkongolo KK, Kalonji-Mbuyi A. Nutritional analysis of quality protein maize varieties selected for Agronomic characteristics in a breeding program. Int J Plant Breed Genet 2011;5(4):317-327.
3. FAO Agrostat, Food Balance Sheets, FAO, Rome, Italy; 1992.
4. Gaziola SA, Alessi ES, Guimaraes PEO, Damerval C, Azevedo RA. Quality protein maize (QPM): A biochemical study of enzymes involved in lysine metabolism. J Agric Food Chem. 1999;47:1268-1275.
5. FAO/WHO. Protein Quality Evaluation. Expert Consultation, Report Series 51, Food and Agriculture Organization/ World Health Organization Nutrition Meetings, Rome, Italy; 1991.
6. Akalu G, Taffesse S, Gunaratna NS, De Groote H. The effectiveness of quality protein maize in improving nutritional status of young children in Ethiopian Highlands. Food Nutr Bull. 2010;31(3):418-430.
7. Nuss ET, Tanumihardjo ST. Closing the protein inadequacy gap in vulnerable populations. Adv Nutr. 2011;2:217-224.
8. Bjarnason M, Vasal SK. Breeding for quality protein maize. Plant Breed Rev. 1992;9:181-216.
9. Greetha KB, Lending CR, Lopes MA, Wallace JC, Larkins BA. Opaque-2 modifiers increase y-zein synthesis and alter its special distribution in maize endosperm. Plant Cell. 1991;3:1207-1219
10. Bostwick E, Larkins BA. Opaque-15, a maize mutation with properties of a defective opaque-2 modifier (y zein/Quality Protein Maize/storage proteins). Proc Natl Acad Sci USA. 1995;92:1931-1935.
11. Dannenhoffer JM, Bostwick DE, Or E, Larkins BA. Opaque-15, a maize mutation with properties of a defective opaque-2 modifier (y-zein/Quality Protein Maize/ storage proteins). Proc Natl Acad Sci. USA. 1993;92:1931-1935.
12. Vasal SK, Villegas E, Bjarnason M, Gelaw B, Goertz P. Genetic Modifiers and Breeding Strategies in Developing Hard Endosperm opaque-2 Materials, pp. in Improvement of Quality Traits of Maize for Grain and Silage Use, edited by Pollmer W. G., Phipps R. H., MartinusNijhoff, London. 1980;37-73.
13. Paes MC, Bicudo MH. Proceedings of the international symposium on quality protein maize (eds Larkins, B. A and Mertz, E. T.), EMBARAPA/CNPMS, Sete Lagaos, Brazil. 1995;65-78.
14. Mbuya K, Nkongolo KK, Kalonji-Mbuyi A, Kizungu R. Participatory, selection and characterization of quality protein maize (QPM) varieties in Savanna agro ecological region of DR-Congo. J. Plant Breed Crop Sci. 2010;2(11):325-332.

15. AOAC. Official Methods of Analysis. 18th Edn., Association of Official Analytical Chemists, Gaithersburgs, MD; 2006.
16. Nkongolo KK. RAPD and cytological analysis of *Picea* spp. from different provenances: Genomic relationships among taxa. Hereditas. 1999;130:137-144.
17. Nkongolo KK, Gervais S, Zhou Y. Comparative analysis of inter simple sequence repeats and simple sequence repeats markers: genetic analysis of *Deschampsia cespitosa* populations growing in metal contaminated regions in Canada. Amer J Biochem Biotechnol. 2014;10(1):69-80.
18. Mehes M, Nkongolo KK, Michael P. Genetic variation in Pinusstrobus and P. Monticola populations from Canada: development of genome- specific markers. Plant Syt Evol. 2007;267:47-63.
19. Vaillancourt A, Nkongolo KK, Michael M. Identification, characterization, and chromosome locations of rye and wheat specific ISSR and SCAR markers useful for breeding purposes. Euphytica. 2008;159:297-306.
20. Russell WK, Sandall L. Corn breeding: types of cultivars. J Nat Resour Life Sci Educ. 2006;35(1):242.
21. Kniep KR, Mason SC. Lysine and protein content of normal and opaque-2 maize grain as influenced by irrigation and nitrogen. Crop Sci. 1991;31:177-181.
22. Zarkadas CG, Hamilton RI, Yu ZR, Choi VK, Khanizadeh S, Rose NGW, Pattison PL. Assessment of the protein quality of 15 new Northern adapted cultivars of quality protein maize using amino acid analysis. J Agric Food Chem. 2000;48:5351-5361.
23. Habben JE, Moro GL, Hunter BG, Hamaker BR, Larkins BA. Elongation factor 1 α concentrations is highly correlated with the lysine content of maize endosperm. Proc Natl. Acad Sci. USA. 1995;92:8640-8644.
24. Moro GL, Habben JE, Hamaker BR, Larkins BA. Characterization of the variability in lysine content for normal and opaque-2 maize endosperm. Crop Sci. 1996;36:1651-1659.
25. Huang S, Frizzi, A, Florida CA, Kruger DE, Luethy MH. High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD alpha-zeins. Plant Mol Biol. 2006;61:525-535.
26. Nkongolo KK, Mbuya K, Mehes-Smith M, Kalonji-Mbuya A. Molecular characterization of quality protein maize (QPM) and normal maize varieties from the DR-Congo breeding program. Afr J Biotechnol. 2011;10(65):14293-14301.
27. Mbuya K, Nkongolo KK, Narendrula R, Kalonji-Mbuyi A, Kizungu RV. Development of quality protein maize (QPM) inbred lines and genetic diversity assessed with ISSR markers in maize breeding program. Am J Exp Agric. 2012;2(4):626-640.
28. Semagn K, Bjornstad A, Ndiondjop MN. An overview of molecular markers methods for plants. Afr J Biotechnol. 2006;5(25):2540-2568.
29. Liu Y, Li S, Zhu T-h, Baolin S. Specific markers for detection of bacterial canker of kiwifruit in Sichuan, China. Afr J Microbiol Res. 2012;6(49):7512-7519.
30. Nkongolo KK, Deverno L, Michael P. Genetic validation and characterization of RAPD markers differentiating black and red spruces: molecular certification of spruce trees and hybrids. Plant Syst Evol. 2003;236:151-163.
31. McGrath A, Higgins DG, McCarthy TV. Sequence analysis of DNA randomly amplified from the Saccharomyces cerevisiae genome. Mol Cell Probes. 1998;12:397-405.
32. Krivanek AF, DeGroote H, Gunaratna NS, Diallo AO, Friesen D. Breeding and disseminating quality protein maize (QPM) for Africa. Afr J Biotechnol. 2007;6(4):312-324.

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