Indian J. Genet., 75(4): 453-458 (2015)
DOI: 10.5958/0975-6906.2015.00073.5

Effect of *opaque-2* allele on accumulation of tryptophan among backcross-derived introgressed progenies of maize

Mahak Tufchi, Rashmi[1], S. K. Jha[2] and N. K. Singh[1]*

Department of Molecular Biology and Genetic Engineering; [1]Department of Genetics and Plant Breeding, G. B. Pant University of Agriculture and Technology, Pantnagar 263 145, Uttarakhand; [2]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012

(Received: June 2015; Revised: October 2015; Accepted: October 2015)

Abstract

The tryptophan concentration of the 85 $BC_2F_{2:3}$ progenies derived from a cross between normal maize inbred line, Pant 10k1375 and o2 donor line, CML 161 were determined. The genic marker phi057 positive progenies showed significant variation for tryptophan concentration in $BC_2F_{2:3}$ progenies with tryptophan as low as 0.046 per cent in $BC_2F_{2:3}$-80, whereas $BC_2F_{2:3}$-78 possessed maximum tryptophan concentration of 0.082 per cent. Normal maize line Pant10k1375 possessed tryptophan concentration of 0.040 per cent whereas o2 donor line CML161 had tryptophan concentration of 0.089 per cent. All the $BC_2F_{2:3}$ progenies had tryptophan concentration higher than the normal maize inbred, whereas none of the progenies had tryptophan concentration higher than the donor line CML161. Twelve lines of $BC_2F_{2:3}$ populations had tryptophan concentration 0.075 per cent or more, the level used to determine quality protein maize. Variability in amino acid modifier genes in the background and/or the insertion of transposable element at the regulatory sites indicated by unusual segregation pattern of *phi057* and *umc1066* in backcross progenies could be the possible reasons for such high level of variation in tryptophan concentration. The progenies with tryptophan concentration $\geq$ 0.075 per cent may serve as potential germplasm in development of quality protein maize hybrids.

Key words: *Zea mays*, *opaque-2*, tryptophan, SSR markers, backcross

Introduction

Novel gene and its alternate form have contributed significantly in development of speciality corn. Allele frequencies in a population may change due to gene flow, genetic drift, natural selection and mutation. But it is only through mutation that a new genetic variation can be created. It was in 1964, that an important event of mutation was discovered. Mertz et al. (1964) reported that the *opaque-2 (o2)* mutant gene can alter the amino acid composition in maize endosperm and alleviate the deficiency of essential amino acids tryptophan and lysine. In 1990s, the mechanism through which the *o2* gene regulates protein expression was intensively studied and it got revealed that *o2* gene encodes a protein that has structural homologies to transcriptional activators (Schmidt et al. 1990; Lohmer et al. 1991; Bass et al. 1992). It has a 'leucine-zipper' motif that can bind to zein DNA and actually recognizing a specific target site on the 22-kD alpha zein gene. The mutant gene greatly reduces storage zein protein, thus changing the endosperm texture and has pleiotropic effect with complex function. The *o2* mutant maize lines acted as donor stocks to develop populations, inbred lines and hybrids with high tryptophan and lysine concentration. The *o2* allele introgressed populations had variation in concentration of tryptophan and lysine probably because of many genetic factors with small effect and popularly called as modifier genes (Mertz et al. 1964; Krivanek et al. 2007). Latter, it revealed that high tryptophan and lysine lines were as a result of the cumulative effect of many genes/quantitative trait loci (QTLs) along with the *o2* gene and together it was a polygenic trait (Babu et al. 2005). With the rapid advances in genome research and molecular technology, it became possible to use genomic markers mainly SSR (simple sequence repeats) in marker-assisted selection (MAS) for the development of high tryptophan and lysine lines. It

*Corresponding author's e-mail: narendraksingh2@gmail.com
Published by the Indian Society of Genetics & Plant Breeding, F2, First Floor, NASC Complex, PB#11312, IARI, New Delhi 110 012
Online management by indianjournals.com

has recently become possible to use MAS to accelerate selection for the *o2* allele in Quality Protein Maize (QPM) breeding programs. In 2001, three important genic and functional SSR markers namely, *phi057, umc1066* and *phi112* were documented (www.agron.missouri.edu) which facilitated the speedy transfer of recessive *o2* allele and development of *o2* based population with high lysine and tryptophan. Structurally, the *phi112* SSR is located between the G box and 3 upstream open reading frames (uORFs) (Schmidt et al. 1990) in the leader sequence of the *o2* gene and its mutation can affect transcription of the *o2* gene. The *umc1066* and *phi057* SSRs are located in exon 1 and exon 6, respectively. These two are the largest of the 6 exons known within the *o2* gene. Therefore, using *phi112*, *umc1066* and *phi057* markers, the allelic SSR variations in the leader region, the 5' region and 3' region of the *o2* gene can represent the variation within the *o2* gene. QPM inbreds are being created at a much faster pace and therefore providing diverse germplasm for development of QPM hybrids (Babu et al. 2005; Manna et al. 2005; Danson et al. 2006; Magulama et al. 2009; Jompuk et al. 2011). Thus the present investigation was planned to develop marker aided $BC_2F_{2:3}$ progenies by crossing a normal maize line, Pant 10k1375 with *o2* donor line, CML161 to study the effect of *o2* introgression on kernel tryptophan concentration and also to develop new inbreds for further use in breeding programme for development of QPM hybrid.

Materials and methods

Normal maize inbred line Pant10k 1375 deficient in tryptophan and lysine was used as recipient parent (RP) while CML161, a QPM line developed by CIMMYT was used as donor parent (DP) for *o2* allele. These two lines were sown during winter 2010-2011 at Norman E Borloug Crop Research Centre of G. B. Pant University of Agriculture and Technology, Pantnagar (Uttarakhand), India for making cross and determine polymorphism at *o2* locus using gene specific SSR markers *phi057, phi112* and *umc1066.*

DNA isolation, PCR amplification and gel electrophoresis

Leaf samples at seedling stage (15 days old) were collected from field grown maize lines and were stored at –20^{o}C. The DNA isolation was carried out using the CTAB method (Doyle and Doyle 1990) with some modifications and quantified using dual beam spectrophotometer. The PCR reaction mixture of 25 µl was consisted of distilled water (16.7 µl), Taq buffer (2.5 µl), dNTP (0.5 µl), Taq polymerase (0.34 µl), forward and reverse primer (1.5 µl each) and genomic DNA (2 µl). Polymerization reaction was performed in Thermal Cycler (Applied Bio Systems, U.S.A) with amplification cycling of initial denaturation at 94^{o}C for 2 min, followed by 35 cycles of amplification at 94^{o}C for 40 s, 55-60^{o}C for 40 s, and 72^{o}C for 1 m. A final extension step was kept at 72^{o}C for 7 m. The amplified products were resolved on a 3.5% Metaphor gel at 110 V for 3-4 h (Sambrook et al. 1989; Senior et al. 1998).

Development of backcross (BC) populations

The inbred line Pant 10k 1375 used as seed parent and crossed with CML161 used as pollen parent during winter 2010-11. The F_1s were backcrossed with Pant 10k1375 twice to obtain BC_1F_1 and BC_2F_1 populations during rainy season 2011 and winter season 2011-12, respectively. The BC_2F_1 population was selfed during rainy season of 2012 and promising plants were selected from BC_2F_2 population grown during winter 2012-13. Of the 183 BC_2F_1 plants, 85 were selected, selfed and raised in ear to row fashion in BC_2F_3 generation during rainy season of 2013. The 85 $BC_2F_{2:3}$ progenies were harvested separately and seeds were used for determination of tryptophan concentration.

Determination of tryptophan concentration in BC_2F_3 families

The BC_2F_3 seeds were dried and after removal of embryo the endosperm was ground to very fine powder using grinding mill. The sieved (0.5 mm) fine flour of each genotype was packed in water proof seed packets and stored at 4^{o}C. The milled flour was then defatted with hexane in a Soxhlet-type continuous extractor for 6 h. After hexane evaporation, 80 mg of powder was digested using 3 mL of 4 mg/mL papain. Further processing of the hydrolysate was done using protocol of Nurit et al. (2009). The optical density (OD) of samples at 560 nm was recorded in a UV-Visual Double Beam Spectrophotometer. Each sample was analyzed in triplicate to ensure accuracy. A stock solution of 100 µg/mL of DL-tryptophan was prepared weekly in 0.165M sodium acetate at pH 7 and stored at 4^{o}C. The solution was diluted in the same sodium acetate to 0, 10, 15, 20, 25, and 30 µg of tryptophan per mL to develop a standard curve. The data on tryptophan was recorded thrice on each sample and statistical significance was determined (Snedecor and Cochran 1989).

Results and discussion

Of the three *o2* gene specific SSR markers, *phi112* exhibited dominant polymorphism between the normal and *o2* donor line. Approximately 180bp PCR fragment was observed with normal maize line while in *o2* donor line, the allele was absent. Thus, the marker *phi112* clearly exhibited dominant inheritance and cannot be used in tracking *o2* allele in QPM breeding programme since the marker is unable to distinguish plants heterozygote for *o2* locus from other plants homozygous for *o2* locus in backcross population. The markers namely *phi057* and *umc1066* exhibited co-dominant polymorphism between normal maize line Pant 10K 1375 and donor inbred line CML 161. Allelic size observed with *phi057* was ~170 bp in the normal inbred line Pant 10K 1375 whereas the same marker amplified an allele of around 180 bp in CML 161. Marker *umc1066* amplified alleles of 150 bp and 160 bp in the *o2* donor inbred line CML 161 and normal inbred line Pant10K 1375, respectively (Fig. 1). Of the two genes

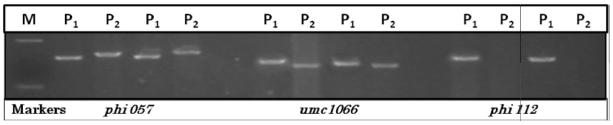

Fig. 1. Polymorphism at *o2* locus between parents using SSR markers. M-Ladder (100bp), P_1-Pant 10K 1375; P_2-CML 161

targeted co-dominant SSR markers, only *phi057* was used to determine the polymorphism at *o2* locus since the marker *phi057* exhibited stable and discrete phenotype in the present investigation and therefore considered to be more reliable whereas, variable and ambiguous phenotype with *umc1066* indicated that this marker cannot be used reliably in selection of plants in QPM breeding programme. Babu et al. (2005) also noted similar observations and used only *phi057* in tracking of *o2* allele in backcross population. Magulama et al. (2009) noted polymorphism at *o2* locus with *phi057* and *umc1066* however they applied only *phi057* in marker-assisted selection for the development of BC populations. Because of the reliability and discrete polymorphism, *phi057* SSR marker has also been used earlier in marker-assisted backcrossing programme (Manna et al. 2005; Danson et al. 2006). Jompuk et al. (2011) observed allele size of 160bp and 170bp in *o2* and normal maize lines, respectively when *o2* locus was genotyped using marker *phi057*. Recently, Gupta et al. (2013) used successfully *umc1066* marker in foreground selection to identify plants heterozygote at o2 locus in backcross generations and also to select the homozygotes in selfed generation for QPM hybrid development. The allele size of this marker in recurrent parents (RPs) was 460 bp, and the same marker produced the allele size of 480 bp in *o2* donors.

Breeding programme involved development of F_1 seeds from the cross between (Pant 10k 1375 x CML 161) during *rabi* 2010-11. At the time of maturity, ears on normal maize line consisted of crossed seed (F_1 of Pant 10k 1375 x CML 161 line) were harvested. The BC_1F_1 seeds were developed by crossing F_1s with normal maize line Pant 10k 1375. Population of 158 BC_1F_1 plants out of the 200 seeds sown, after 30 days of sowing was noted. Individual plants were screened using SSR marker *phi057* before flowering and identified 45 plants with *o2* locus in heterozygous condition (Fig. 2). The Chi square value of 29.26

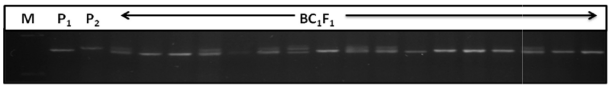

Fig. 2. Screening of BC_1F_1 population using SSR marker *phi057*. M-Ladder (100bp), P1-Pant 10K 1375; P2-CML 161; BC_1F_1: Backcrossed Progenies of F_1

indicates rare probability (0.001) of co-linearity of observed and expected segregation (1:1) of dominant homozygotes and heterozygous *o2* allele. The segregation distortion from 1:1 is probably because of the rejection of approximately 20 per cent of the plants, sampling error and relatively low plant population. The plants identified to be dominant homozygous at *o2* locus based on genotyping using SSR marker *phi057* were rouged out before flowering. Pollen from recurrent parent Pant 10k 1375 were collected and transferred to stigma of the BC_1F_1 plants identified to be in heterozygous condition for *o2* allele. The forty five BC_1F_1 ears were further screened using kernels and cob phenotype as selection criteria and finally 24 BC_1F_1 ears consisted of BC_2F_1 seeds were selected. Seeds from 24 selected ears were bulked and 200 BC_2F_1 seeds were sown. The plant counts after 30 days of sowing were 183 plants. Before flowering, all these plants were genotyped at *o2* locus using marker *phi057*. Of the 183 plants, 85 plants possessed *o2* allele in heterozygous condition. The chi square test revealed chi square value of 0.92 which is statistically non-significant however probability of co-linearity of observed and expected segregation is in between 0.5-0.3. These 85 plants were maintained by placing pollen

of the plant on the protected stigma of the same plant to effect self pollination. All plants which were dominant homozygous for *o2* allele were rejected. Kernel modification due to expression of recessive *o2* allele in BC_2F_2 seed of 85 BC_2F_1 plants was quantified using light box and kernels with modified endosperm of about 25 per cent opaqueness were selected. The light box based selected kernels of each of the 85 lines were planted separately, self pollinated at the time of flowering and BC_2F_2 derived BC_2F_3 seeds were obtained for determining tryptophan concentration.

Effect of gene is studied through its expression by means of protein product that the gene encodes. In the present investigation, the effect of expression of *o2* gene on tryptophan concentration in maize kernel was quantified. Analysis of variance revealed significant variance for tryptophan concentration in $BC_2F_{2:3}$ progenies. The minimum tryptophan concentration of 0.046 per cent was determined in $BC_2F_{2:3}$-80 line while the maximum tryptophan concentration of 0.082 per cent was observed in $BC_2F_{2:3}$-78 line. The two parents namely Pant10k1375 and CML161 used to generate population had tryptophan concentration of 0.040 and 0.089 per cent, respectively (Fig 3). None the $BC_2F_{2:3}$ progenies had

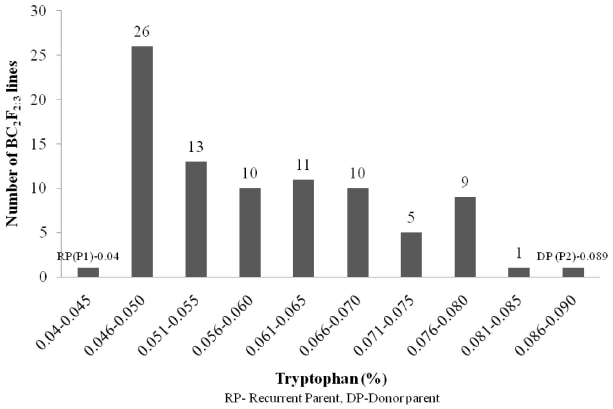

Fig. 3. Tryptophan concentration in parents (P1, P2) and its derived $BC_2F_{2:3}$ population of maize

tryptophan concentration numerically or statistically *at par* or higher than the concentration of *o2* donor line CML161. All the $BC_2F_{2:3}$ progenies exhibited tryptophan concentration significantly higher than 0.040 per cent, the level of tryptophan observed in normal maize line, Pant10k1375. The $BC_2F_{2:3}$ progenies exhibited tryptophan concentration 0.075 per cent or more were $BC_2F_{2:3}$-9, $BC_2F_{2:3}$-10, $BC_2F_{2:3}$-11, $BC_2F_{2:3}$-24, $BC_2F_{2:3}$-25, $BC_2F_{2:3}$-39, $BC_2F_{2:3}$-40, $BC_2F_{2:3}$-53, $BC_2F_{2:3}$-55, $BC_2F_{2:3}$-71, $BC_2F_{2:3}$-78 and $BC_2F_{2:3}$-79 (Fig. 3). These *o2* introgressed $BC_2F_{2:3}$ lines have potential as parents in breeding maize cultivars for high tryptophan concentration in maze kernel.

The wide variation for tryptophan concentration was observed in recombinant $BC_2F_{2:3}$ lines derived from the cross between Pant10k1375 and CML161. The *O2* regulates the expression of the 22-kDa α-zeins and several other genes (Kodrzycki et al. 1989; Schmidt et al. 1990) including lysine ketoglutarate reductase (LKR) (Brochetto-Braga et al. 1992). An *o2* mutant typically shows a marked increase in non-zein proteins, which in association with the decrease in lysine-poor α-zein proteins, leads to a greater percentage of lysine and tryptophan. Furthermore, the loss of LKR activity results in increased levels of free lysine (Arruda et al. 2000). Lower α-zein concentration in *o2* endosperm results in protein bodies that are about one-fifth to one tenth the normal size; this, in turn, is presumed to alter packing of starch grains during seed desiccation, thereby conferring a characteristic soft texture to the kernel (Babu and Prasanna 2014). Depending upon the gradation in kernel opaqueness, the tryptophan concentration varies. With the reduction of α-zeins in the endosperm due to *o2* mutation, there is a concomitant increase in the level of γ-zeins. Transcription factors of bZIP type frequently function as heterodimers. Heterodimerizaton between *O2* and another bZIP protein, OHP1, has been demonstrated, suggesting the involvement of multiple bZIP proteins in transcriptional control of zein genes (Habben et al. 1993) which results in variable expression. Another factor influencing the expression of *o2* gene is the role of endosperm modifiers or quantitative trait loci (QTLs), which alleviate the negative effects of *o2* gene without losing the high-lysine trait. One of the QTLs is linked to the 27-kDa γ-zein locus on chromosome 7S. Consistent association between endosperm modification and enhanced accumulation of the gamma-zein storage protein suggested that they are essential for endosperm modification. Elimination of γ-zeins disrupts endosperm modification by endosperm modifiers, indicating their hypostatic action to γ-zeins (Wu et al. 2010). Activities of endosperm modifier genes are also influenced by the genetic backgrounds (Lopes and Larkin 1995). In addition to these two genetic systems that is, *o2* and endosperm modifiers, amino acid modifier genes a third genetic system that affects relative levels of lysine and tryptophan (Krivanek et al. 2007). Thus a number of factors control the expression of *o2* gene as discussed above.

During the marker assisted transfer of recessive *o2* allele, we noticed some interesting events. The markers *phi057* and *umc1066* are genic in nature and located on the same locus in exon 6 and exon 1, respectively. Because of their location, ideally these two markers must exhibit similar pattern in terms of alleles among different genotypes of BC_1F_1 and BC_2F_1 populations. However, we noted variation in the results observed with *phi057* and *umc1066* SSR genic markers in the present investigation. In some of the cases we observed presence of *phi057* marker whereas we did not observe any amplification with another marker *umc1066*. Similar situation was also observed where we noticed a desirable allelic form with *umc1066* but no amplification with *phi057*. The third situation consisted of genotypes positive for both *phi057* and *umc1066* markers. One possibility we assume about the present observation is the involvement of intragenic recombination. However, this assumption requires further experimentation for validation. The other possibility includes the inactivation of marker sites by insertion of transposons. Such events at gene level might be responsible for differential level of tryptophan in *phi057* based *o2* gene introgressed $BC_2F_{2:3}$ families. More recently, Chen et al. (2014) demonstrated that the presence of transposable element within the *o2* gene has been the cause of variable *o2* expression (Fig. 4). Transposable element *rbg* induces the differential expression of *o2* mutant gene in two maize o2 NILs derived from the same inbred line. However,

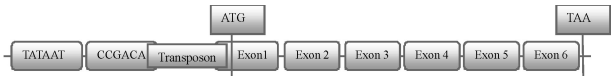

Fig. 4. Insertion of transposon within the *o2* locus (Adapted from Chen et al. 2014)

the kernel phenotypes of the two *o2* NILs were different; one had the wild-type vitreous endosperm and normal lysine concentration, while the endosperm of other was opaque with doubled lysine content. Sequence and transcript abundance analyses indicated that the coding DNA sequence (CDS) of two *o2* NILs had different promoters and the *O2* transcript of opaque line is largely inhibited because of an *rbg* transposable element inserted between the TATA box and initiator codon. They concluded that different crossing-over patterns during the process of *o2* NIL construction resulted in the different kernel phenotypes of the two *o2* NILs during introgression and backcrossing (Chen et al. 2014).

In the present investigation, we noticed variable expression of *o2* gene as evident from the variability in tryptophan concentration of the 85 BC_2F_3 progenies derived from the cross between Pant10k1375 and CML161. Though, we did not identified any lines out of the 85 BC_2F_3 progenies having tryptophan content higher than the donor line CML 161, yet great possibilities exist to use marker effectively along with limited back crossing in diversification and development of QPM lines. The BC_2F_3 progenies namely $BC_2F_{2:3}$-9, $BC_2F_{2:3}$-10, $BC_2F_{2:3}$-11, $BC_2F_{2:3}$-24, $BC_2F_{2:3}$-25, $BC_2F_{2:3}$-39, $BC_2F_{2:3}$-40, $BC_2F_{2:3}$-53, $BC_2F_{2:3}$-55, $BC_2F_{2:3}$-71, $BC_2F_{2:3}$-78 and $BC_2F_{2:3}$-79 identified in the investigation may prove to be potential germplasm in QPM hybrid development programme.

Acknowledgement

Director Experiment Station, G. B. Pant University of Agriculture & Technology, Pantnagar (India) and AICRP Maize are duly acknowledged for support during the course of investigation. Indian Council of Agricultural Research (ICAR) New Delhi is duly acknowledged for awarding Ph. D. Fellowship to senior author.

References

Arruda P., Kemper E. L., Papes F. and Leite A. 2000. Regulation of lysine catabolism in higher plants. Trends Plant Sci., **5**: 324-330.

Babu R., Nair S. K., Kumar A., Venkatesh S., Sekhar J. C., Singh N. N., Srinivasan G. and Gupta H. S. 2005. Two-generation marker aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor. Appl. Genet., **111**: 888-897.

Babu R. and Prasanna B. M. 2014. Molecular Breeding for Quality Protein Maize (QPM). *In*: R. Tuberosa edited book Genomics of Plant Genetic Resources, Springer Science, Dordrecht, pp. 489-505.

Bass H. W., Webster C., O'Brien G. R., Roberts J. K. M. and Boston R. S. 1992. A maize ribosome inactivating protein is controlled by the transcriptional activator *opaque-2.* Plant Cell, **4**: 225-234.

Brochetto-Braga M. R., Leite A. and Arruda P. 1992. Partial purification and characterization of lysine-ketoglutarate reductase in normal and *opaque-2* maize endosperms. Plant Physiol., **98**: 1139-1147.

Chen Y., Zhou Z., Zhao G., Li X., Song L. et al. 2014. Transposable element rbg induces the differential expression of *opaque-2* mutant gene in two maize o2 NILs derived from the same inbred line. PLoS ONE 9: e85159.

Danson J. W., Mbogori M., Kimani M., Lagat M., Kuria A. and Diallo A. 2006. Marker assisted introgression of *opaque2* gene into herbicide resistant elite maize

inbred lines. African J. Biotech., **5**: 2417-2422.

Doyle J. J. and Doyle J. L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. Focus, **12**: 13-15.

Gupta H. S., Babu R., Agrwal P. K., Mahajan V., Hossain F. and Thirunavukkarasu N. 2013. Accelerated development of quality protein maize hybrid through marker-assisted introgression of *opaque-2* allele. Plant Breed., **132**: 77-82.

Habben J. E., Kirleis A. W. and Larkins B. A. 1993. The origin of lysine-containing proteins in *opaque-2* maize endosperm. Plant Mol. Biol., **23**: 825-838.

Jompuk C., Cheuchart P., Jompuk P. and Apisitwanich S. 2011. Improved Tryptophan Content in Maize with *Opaque-2* gene Using Marker Assisted Selection (MAS) in Backcross and Selfing Generations. Kasetsart J., **45**: 666-674.

Kodrzycki R., Boston R. and Larkins B. A. 1989. The *opaque-2* mutation of maize differentially reduces zein gene transcription. Plant Cell, **1**: 105-114.

Krivanek A., Groote H., Gunaratna N., Diallo A. and Freisen D. 2007. Breeding and disseminating quality protein maize for Africa. Afr. J. Biotech., **6**: 312-324.

Lohmer S., Maddaloni M., Motto M., Difonzo N., Hartings H., Salamini F. and Thomson R. D. 1991. The maize regulatory locus *opaque-2* encodes a DNA binding protein which activates the transcription of the B-32 gene. EMBO J., **10**: 617-624.

Lopes M. and Larkins B. 1995. Genetic-analysis of *opaque 2* modifier gene activity in maize endosperm. Theor. Appl. Genet., **91**: 274-281.

Magulama E. E. and Sales E. K. 2009. Marker-assisted introgression of *opaque* 2 gene into elite maize inbred lines. USM R & D., **17**: 131-135.

Manna R., Okello D. K., Imanyhowa J., Pixley K. and Edema R. 2005. Enhancing introgression of *opaque-2* trait into elite maize lines using simple sequence repeats. Afr. Crop Sci. J., **13**: 215-226.

Mertz E. T., Bates L. S. and Nelson O. E. 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science, **145**: 279-280.

Nurit E., Tiessen A., Pixley K. and Palacios-Rojas N. 2009. Reliable and inexpensive Colorimetric Method for Determining Protein-Bound Tryptophan in Maize Kernels. J. Agric. Food Chem., **57**: 7233-7238.

Sambrook J., Fritsch E. F. and Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, vol. I. 2nd edition. Cold Spring Harbor Laboratory Press, ISBN 0-87969-309-6.

Schmidt R. J., Burr F. A., Aukerman M. J. and Burr B. 1990. Maize regulatory gene *opaque-2* encodes protein with a ‘‘leucine zipper’’ motif that binds to zein DNA. Proc. Natl. Acad. Sci., **87**: 46-50.

Senior M. L., Murphy J. P., Goodman M. M. and Stuber C. W. 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. Crop Sci., **38**: 1088-1098.