Indian J. Genet., 77(1): 59-64 (2017)
DOI: 10.5958/0975-6906.2017.00008.6

Exploration of novel *opaque16* mutation as a source for high lysine and tryptophan in maize endosperm

Konsam Sarika, Firoz Hossain*, Vignesh Muthusamy, Aanchal Baveja, Rajkumar Zunjare, Rajat Goswami, Nepolean Thirunavukkarasu, Supradip Saha and Hari Shanker Gupta

Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi 110 012

(Received: May 2016; Revised: December 2016; Accepted: December 2016)

Abstract

In the present study, two F_2 populations derived by crossing wild type- (CML533 and CML537) and *o16*-donor line (QCL3024) were raised and genotyped. The recessive *o16o16* possessed nearly two-fold more lysine (0.247%) and tryptophan (0.072%) in mutants, than wild type (0.125% lysine and 0.035% tryptophan) across populations. However, *o16o16* showed wide variation for both lysine (0.111-0.376%) and tryptophan (0.027-0.117%) across populations, suggesting the role of modifier loci. The study did not show any significant correlation (r=0.14) between lysine and tryptophan, however some segregants with high-lysine (>0.300%) and -tryptophan (>0.090%) were identified. Some of the segregants with *o16o16* possessed comparable lysine and tryptophan with *o2o2* genotypes. These *o16o16* segregants may serve as novel genetic resource in the QPM breeding programme, and some of them can be used for enhancement of both lysine and tryptophan in place of *o2* mutant. This is the first ever report of influence of *o16* on accumulation of tryptophan in maize.

Key words: lysine, *opaque16*, *opaque2*, QPM, tryptophan

Introduction

Prolamin also known as zein, constitutes 70% of storage proteins in maize, but is characterized by low concentration of essential amino acids especially lysine and tryptophan (Nelson, 1969). As opposed to the 0.5% of lysine requirement (in flour) recommended for optimal human consumption by the Food and Agriculture Organization (FAO), United Nations, maize contains only 0.15-0.20% lysine in the endosperm flour. Human, pigs and monogastric animals like poultry birds cannot synthesize lysine and tryptophan, and are required to be supplemented through diet (Prasanna et al. 2001). Maize is an important staple crop in sub-Saharan African, Latin America and Asia too, where it serves as a vital source of food and energy (Gupta et al. 2015). Besides, more than half of the production in Asia is also used as poultry and livestock feed (Yadav et al. 2015). Maize being poor in nutritional quality warrants genetic amelioration to enhance the content of these essential amino acids in the endosperm.

Attempts to improve grain quality through focused efforts and painstaking research began at the later part of nineteenth century. In the 1960s, researchers found that the mutation, *opaque2* (*o2*) located on chromosome 7L, makes grain protein nearly twice as nutritious as those found in normal maize endosperm (Mertz 1964; Nelson 1965). However, in addition to two-fold increase in lysine and tryptophan, *opaque2* mutation also had pleiotropic effects which make the endosperm soft and opaque that in turn causes enhanced susceptibility to insect-pest infestation, inferior food processing and low yield, thus making the maize grains undesirable to the stakeholders (Bjarnason et al. 1992). Several other mutations such as *o1*, *o5*, *o9-11*, *o13*, *o17*, *floury1*, *fl2*, *fl3*, *Mucronate* and *Defective endosperm* B30 were discovered (McWirter 1971; Salamini et al. 1983). Several of these mutants have been experimentally tried singly or in combinations but resulted in severe yield losses due to negative effects of the individual mutation (Huangs et al. 2004; Gibbon and Larkin 2005). The soft, chalky and opaque phenotype of *o2* kernel was modified by the accumulation of 'endosperm modifiers' leading to the birth of 'Quality Protein Maize' (QPM) (Vasal et al. 1980; Pandey et al. 2015). The deployment of *o2* along

*Corresponding author's e-mail: fh_gpb@yahoo.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; http://epubs.icar.org.in/journal/index.php/IJGPB

with the endosperm modifiers have led to the successful commercialization of diverse QPM hybrids worldwide with enhancement of both lysine (from 0.15 to 0.37% in flour) and tryptophan (from 0.04 to 0.08% in flour) (Gupta et al. 2013; Tufchi et al. 2015).

The search for a novel mutation that can be successfully utilized to develop high lysine maize continued in the new millennium, till Yang et al. (2005) reported another recessive mutant from Robertson's Mutator stocks and named it as *opaque16* (*o16*). This mutant is located on chromosome 8L, and *o16* along with *o2* is reported to increase lysine by 30% over *o2o2* or *o16o16* alone (Zhang et al. 2010; Zhang et al. 2013). So far only research group led by Dr. Wenpeng Yang at Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China has reported the utilization of *o16* in their breeding programme. However, the efforts are confined to the effects of *o16* only on lysine accumulation in Chinese genetic background, and no information on its effect on tryptophan accumulation is available. For the first time the *o16* mutation has been used in the maize breeding programme of India, and in the present investigation, attempts have been made to (i) validate the nutritional benefit of *o16* in a new genetic background, (ii) understand the effects of *o16* on tryptophan accumulation, (iii) study the influence of modifier loci on accumulation of lysine and tryptophan, and (iv) estimate the correlation of lysine and tryptophan in *o16* genetic background.

Materials and methods

Plant materials

The experimental materials consisted of two CIMMYT-based normal inbred lines, CML533 (a yellow line) and CML537 (a white line), and *o16o16*-donor line (QCL3024, a yellow line) of Chinese origin. F_1 seeds derived from CML533 × QCL3024 and CML537 × QCL3024 were obtained from Guizhou Institute of Upland Food Crops, China. F_1s were raised at Indian Agricultural Research Institute-Experimental Field during *kharif* 2014, and selfed to generate F_2 seeds. 150- and 146- F2 plants in CML533 × QCL3024 and CML537 × QCL3024, respectively were raised at Indian Institute of Maize Research-Winter Nursery Centre, Hyderabad during *rabi* 2014-15. Selfed seeds harvested from *o16o16*- F_2 segregants were subjected to biochemical analysis. The parental inbreds could not be obtained from China; therefore, they could not be raised along with the F_2 populations. Since, Ultra Performance Liquid Chromatography (UPLC) analyses involves high resource, five randomly selected individuals with wild type allele from each of the crosses were taken as control. Heterozygous individuals (*O16o16*) were not considered for the present analyses as the gene exhibits complete dominance, and likely to possess low lysine and tryptophan. Further, genotypes with *o2o2*-genetic constitution *viz*., MGUQ-102 and HKI163 (inbred) along with wild type genotype (HM4) were included as checks in the biochemical analysis.

Identification of o16o16 and O16 segregants

True hybridity test of F_1s and genotyping of individual plants in F_2 generations were carried out by targeting *o16* locus specific SSR marker, *umc1149*. Genomic DNA was extracted from three weeks old young tender leaves using CTAB method (Murray and Thompson 1980). The PCR amplicons were resolved in 4% agarose gel and the profiles were visualised in a gel documentation system (AlphaInnotech, California, USA).

Estimation of lysine and tryptophan

Amino acids were estimated by using UPLC (Dionex Ultimate 3000, Thermo Scientific). 20mg of degermed endosperm flour per sample with three replications was used. For lysine, acid hydrolysis was done overnight for 16 hours at 110^{o}C with 800µl of 6N HCl, 100µl 0.1N HCl, 100µl of Nor-leucine and 10µl of phenol. 500µl of 12.5M NaOH solution was added to the hydrolysed sample and the volume was made up to 10ml with 0.1N HCl. For derivatization process, to the 100µl of syringe filtered sample, 900µl of boric acid and 1ml fluorenyl methoxy carbonyl chloride (FMOC) were added to it. Further 4ml of n-pentane was added and vortexed continuously for 45 seconds and the lower layer was taken for quantification of lysine. The mobile phase comprised of a buffer phase and an organic phase. The buffer phase consisted of tetra-methyl ammonium chloride and sodium acetate trihydrate with a pH of 3.5 and organic phase of acetonitrile and methanol in the ratio 49:1. The samples were run in anisocratic mode with 90:10 (Solvent A: Solvent B). Solvent A and B had the buffer and organic phase in the ratio of 9:1 and 1:9, respectively. For tryptophan, endosperm flour was base hydrolysed with 2ml of 4M NaOH. 200µl of 0.1% ascorbic acid was added and kept overnight for 16 hours at 120^{o}C. After cooling, the hydrolysed sample was neutralized with 6N HCl to maintain a pH of 7-8, and the volume was made up to 10ml with water. After filtering with Whatman^{TM} filter

papers 42, volume was further made up to 25ml with water. Water and acetonitrile were used as buffer. For both lysine and tryptophan, the samples were eluted through Acclaim^{TM} 120 C_{18} column (5µm, 120 Å, 4.6×150mm, Thermo Scientific) and detected with a RS photodiode array detector (PDA) with absorbance at 265 and 280nm wavelength, respectively. The injected volume was fixed at 40µl with a flow rate of 1ml min^{-1}. Eight dilutions of amino acids standard (HiMedia for lysine and SIGMA ALDRICH for tryptophan) were used for constructing the standard curve. The concentration of the amino acids in the each sample was estimated by standard regression using external amino acid standards.

Results and discussion

Effect of o16 on lysine and tryptophan

F_1s of the two cross combinations showed heterozygosity with *umc1149*, thereby depicting true nature of hybrids. F_2 populations exhibited both types of homozygotes and heterozygotes. Recessive *o16* allele is rare in the germplasm, and is currently available in Chinese genetic background. Since, recessive *o16* allele has not been reported in CIMMYT inbreds, we used CML161 and CML537 (obtained from CIMMYT) as the reference for wild type (dominant *O16*) allele, and amplicon of the favourable allele (recessive *o16*) could be easily identified. The respective amplicon size of the marker in wild allele and *o16* mutant were approximately 130 and 140 bp, respectively (Fig. 1). The recessive F_2 *o16o16* possessed 0.270% and 0.224% lysine as compared to 0.134% and 0.117% in corresponding wild *O16O16* of crosses CML533 × QCL3024 and CML537 × QCL3024, respectively (Table 1). The concentration of tryptophan also exhibited the similar pattern with *o16o16* having 0.075% and 0.070%, and dominant homozygotes possessing 0.044% and 0.026% tryptophan in CML533 × QCL3024 and CML537 × QCL3024, respectively. The recessive *opaque16* allele thus caused almost two-fold increase in both lysine and tryptophan. Yang et al. (2005) reported similar degree of increase only for lysine with 0.206% in *O16O16* and 0.447% in *o16o16* genotypes. Further, Yang et al. (2013) introgressed the *o16* allele into two waxy inbreds and reported a lower degree of increase (16-28%) in lysine. Thus, the earlier reports of enhancement of lysine by *o16* in Chinese genetic background are also validated in these two new populations. In addition, it is also established here that in some segregants, *o16* also plays important role in higher accumulation of tryptophan as well in maize endosperm.

Fig. 1. Marker segregation of *o16*-linked SSR, *umc1149* among F_2 individuals of CML533 × QCL3024. Star marked individuals possess favourable *o16* allele in homozygous state

Variation for lysine and tryptophan in o16

The *o16o16* families identified in the present study showed wide variation with lysine ranging from 0.111-0.376% and 0.128-0.317%, and tryptophan varying from 0.027-0.117% and 0.032-0.108%, respectively in CML533 × QCL-3024 and CML537 × QCL-3024 (Table 1; Fig. 2). Among *O16O16* segregants, lysine varied from 0.097-0.197% in CML533 × QCL-3024, and 0.107-0.124% in CML537 × QCL-3024. Tryptophan in *O16O16* segregants of CML533 × QCL-3024 varied from 0.034-0.055%, while the same in CML537× QCL-3024 was 0.010-0.045%. It was thus amply clear that though recessive *o16* plays major role in enhancement of lysine and tryptophan, the modifier genes in the background could be an important factor in deciding

Table 1. Variation for lysine and tryptophan content in *o16o16* and *O16O16* genotypes of crosses CML533 × QCL3024 and CML537 × QCL3024, and the checks

S. No.	Pedigree	Genotype	No. of segregants	Per cent lysine in sample		Per cent tryptophan in sample	
				Range	Mean	Range	Mean
1.	CML533 × QCL3024	*O2O2/o16o16*	29	0.111-0.376	0.269±0.014	0.027-0.117	0.074±0.005
2.	CML533 × QCL3024	*O2O2/O16O16*	5	0.097-0.197	0.134±0.013	0.034-0.055	0.044±0.005
3.	CML537 × QCL3024	*O2O2/o16o16*	21	0.128-0.317	0.223±0.011	0.032-0.108	0.069±0.004
4.	CML537 × QCL3024	*O2O2/O16O16*	5	0.107-0.124	0.117±0.003	0.010-0.045	0.026±0.005
5.	HM4	*O2O2/O16O16*	-	-	0.181±0.011	-	0.023±0.003
7.	HKI163	*o2o2/O16O16*	-	-	0.342±0.010		0.084±0.007
8.	MGUQ-102	*o2o2/O16O16*	-	-	0.378±0.009	-	0.927±0.017

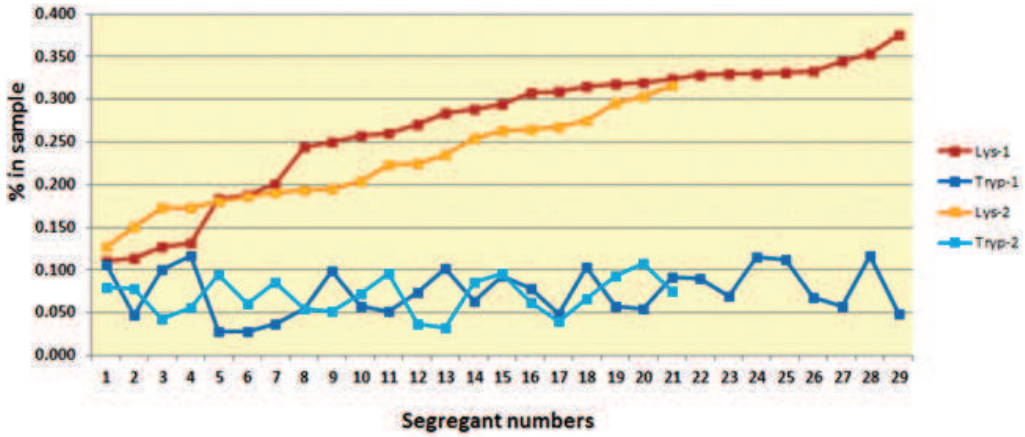

Fig. 2. Variation in lysine and tryptophan among *o16o16* segregants of two F_2 populations (1: CML533 × QCL3024; 2: CML537 × QCL3024)

the final nutritive value. Yang et al. (2005) reported transgressive segregation of lysine in F_2 and suggested that accumulation of lysine is also governed by modifier loci. It is worth mentioning here, that though *o2* causes significant increase of lysine and tryptophan, it showed wide variation for lysine (0.27-0.45%) and tryptophan (0.05-0.11%) in different genetic background (Vivek et al. 2008), and suggesting the importance of favourable amino acid modifier in *o2o2* genetic background for realizing maximum gain (Pandey et al. 2015). Among the two populations, *o16o16* segregants of CML533 × QCL3024 in general possessed higher lysine and tryptophan than CML537 × QCL3024. This is possibly because CML533 contributed more favourable modifier loci (than CML537) that alone or in interaction with modifiers from QCL3024 caused higher accumulation of lysine and tryptophan.

Comparison of o16 with o2 for nutritive quality

The increase in the lysine accumulation in some of the families was comparable with the *o2o2* genotypes (Table 1). For example, selection-29 of CML533 × QCL3024 possessed 0.376% lysine as compared to MGUQ-102 (0.378%) and HKI163 (0.342%). Similarly, selection-1, -3, -4, -9, -13, -18, -24, -25, -28 of CML533 × QCL3024 possessed comparable tryptophan than the *o2o2* genotypes. Though none of the segregants in CML537 × QCL3024 could possess similar lysine as that of the *o2o2*, selection-5, -11, -15, -19 and -20 had the comparable tryptophan. It is worth mentioning here, that the average levels of lysine and tryptophan in *o16o16* was less than the *o2o2*, but were much higher than the wild type (*O16O16*). The recessive *o16* is a major locus that can enhance lysine and tryptophan in normal maize by almost two fold over the corresponding wild type. The mutant *o16* allele can therefore be utilized in the breeding programme for improving the protein quality of normal maize, and some of the *o16o16* genotypes can be effectively utilized in the QPM breeding programme in place of *o2o2* genotypes.

Relationship of lysine and tryptophan in o16o16

The findings thus suggested that *o16* possesses a significant effect in accumulation of both lysine and tryptophan in maize endosperm. However, no correlation (r = 0.14, across populations) between the lysine and tryptophan could be observed among *o16o16* genotypes. This was apparent as selection-23, -27 and -29 of CML533 × QCL3024 possessed higher lysine (0.318-0.376%) and lower tryptophan (0.049-0.058%). On the other hand, selection-1, -3 and -4 had higher tryptophan (0.101-0.171%) but low lysine (0.111-0.132%). In contrast, tryptophan and lysine possess strong correlation (r = 0.99) in *o2* genetic background, with concentration of lysine being four times that of tryptophan (Vivek et al. 2008). Though *o16o16* possesses higher average lysine and tryptophan than wild type, the simultaneous enhancement may not be always achieved due to their poor correlation. This could be due to the fact that mechanism of *o2* and *o16* in enhancing these essential amino acids could be different. Selection of QPM genotypes (possessing *o2o2*) in early segregating generations is undertaken based on tryptophan only since lysine estimation is cumbersome, while tryptophan can be easily estimated through

colorimetric method. However for *o16o16*, it warrants estimation of both lysine and tryptophan in order to select individuals high in both the amino acids. In the present study, despite having no relationships, selection-28 (0.354% lysine, 0.117% tryptophan), selection-24 (0.332% lysine, 0.113% tryptophan), selection-25 (0.330% lysine, 0.116% tryptophan), selection-21 (0.328% lysine, 0.090% tryptophan), selection-22 (0.323% lysine, 0.092% tryptophan) and selection-18 (0.315% lysine, 0.103% tryptophan) of CML533 × QCL3024 possessed both the amino acids in higher concentration (Fig. 2). For CML537 × QCL3024, though the frequency was low, selection-20 (0.323% lysine, 0.092% tryptophan) and selection-19 (0.296% lysine, 0.094% tryptophan) were superior in both the essential amino acids. Promising segregants having *o16o16* were selected, and found to have wide variation in cob- and grain- characteristics (Fig. 3). These segregants with high lysine and tryptophan could serve as novel genetic resource in the breeding programme, as these newly-derived lines would further diversify QPM genetic base which has traditionally used *o2* for the nutritional enhancement. Further, *o16* has been pyramided with *o2*, and effects have been synergistic with accumulation of more lysine in *o2o2/o16o16* than *o2o2* alone (Yang et al. 2005, Zhang et al. 2013). Thus, *o16* mutant alone or in combination with *o2* would provide ample opportunity to enhance the nutritional quality of maize grains.

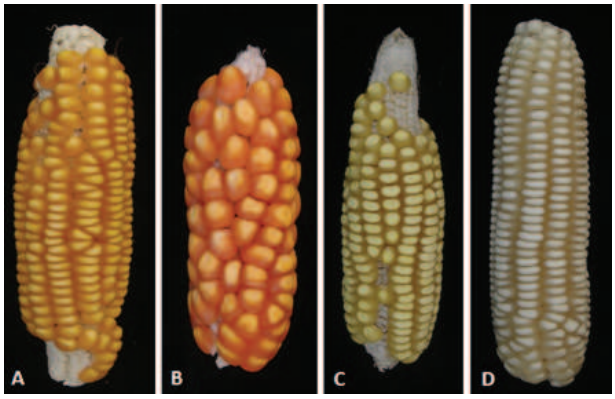

Fig. 3. Cob- and grain-characteristics of *o16o16* segregants selected from F_2 populations of CML533 × QCL3024 (A & B) and CML537 × QCL3024 (C & D)

Recessive *opaque2* has been traditionally used for the enrichment of lysine and tryptophan in QPM genotypes. Though several other mutations have been discovered, none could be successfully utilized due to more of the negative pleiotropic effects. Here, we analyzed the effects of a novel mutation, *opaque16* on accumulation of lysine and tryptophan in maize endosperm using two F_2 populations. Enhancement of both lysine and tryptophan was recorded among *o16o16* segregants. The newly developed *o16o16*-based progenies developed here would serve as a valuable genetic resource in the QPM breeding programme. This is the first report on effect of *o16* on accumulation of tryptophan in maize endosperm. In India, the mutant has been analyzed for the first time, and information generated here would be immensely useful in the nutritional enhancement of maize.

Authors' contribution

Conceptualization of research (FH, HSG, KS); Designing of the experiments (FH, HSG); Contribution of experimental materials (RUZ, RG); Execution of field/lab experiments and data collection (KS, AB, VM, RG); Analysis of data and interpretation (KS, NT, AB, VM, SS, FH); Preparation of manuscript (KS, FH, VM).

Declaration

The authors declare no conflict of interest.

Acknowledgments

We acknowledge DBT-network projects (BT/PR11708/AGR/02/649/2008 & BT/PR10922/AGII/106/944/2014) for the financial support. The authors sincerely thank Dr. Wenpeng Yang, Guizhou Institute of Upland Food Crops, Guizhou Academy of Agricultural Sciences, China for making initial crosses and providing the F_1s. First author is thankful to the Council of Scientific and Industrial Research-University Grant Commission for Junior Research Fellowship during the doctoral programme.

References

Bjarnason M. and Vasal S. K. 1992. Breeding of quality protein maize. Plant Breed. Rev., **9**: 181-216.

Gibbon B. C. and Larkins B. A. 2005. Molecular genetic approaches to developing quality protein maize. Trends Genet., **21**: 227-233.

Gupta H. S., Raman B., Agrawal P. K., Mahajan V., Hossain F. and Nepolean T. 2013. Accelerated development of quality protein maize hybrid through marker-assisted introgression of *opaque2* allele. Plant Breed., **132:** 77-82.

Gupta H. S., Hossain F. and Muthusamy V. 2015. Biofortification of maize: An Indian perspective. Indian J. Genet., **75**: 1-22.

Huang S., Adams W. R., Zhou Q., Malloy K. P., Voyles D. A. and Anthony J. 2004. Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. J. Agric. Food Chem., **52**: 1958-1964.

McWhirter K. S. 1971. A floury endosperm, high lysine locus on chromosome 10. Maize Genet. Coop. Newsletter, **45**: 184.

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.

Murray M. G. and Thompson W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res., **8**: 4321-4326.

Nelson O. E., Mertz E. T. and Bates L. S. 1965. Second Mutant Gene Affecting the Amino Acid Pattern of Maize Endosperm Proteins. Science, **150**: 1469-1470.

Nelson O. E. 1969. Genetic modification of protein quality in maize. Adv. Agron., **21**: 171-194.

Pandey N., Hossain F., Kumar K., Vishwakarma A. K, Muthusamy V., Saha S., Agrawal P. K., Guleria S. K., Reddy S. S., Thirunavukkarasu N. and Gupta H. S. 2015. Molecular characterization of endosperm- and amino acids-modifications among quality protein maize inbreds. Plant Breed. doi:10.1111/pbr.12328

Prasanna B. M., Vasal S. K., Kassahun B. and Singh N. N. 2001. Quality protein maize.Curr. Sci., **81**: 1308-1319.

Salamini F., di Fonzo N., Fornasari E., Gentinetta E., Reggland R. and Soave C. 1983. *Mucronate*, Mc a dominant gene of maize which interacts with *opaque2* to suppress zein synthesis. Theor. Appl. Genet., **65**: 123-128.

Tufchi M., Rashmi, Jha S. K. and Singh N. K. 2015. Effect of *opaque-2* allele on accumulation of tryptophan among backcross-derived introgressed progenies of maize. Indian J. Genet., **75**(4): 453-458. DOI: 10.5958/0975-6906.2015.00073.5.

Vasal, S. K., Villegas E., Bajarnason M., Gelaw B. and Geirtz P. 1980. Genetic modifiers and breeding strategies in developing hard endosperm opaque-2 materials. In: W. G. Pollmer, and R. H. Philips (eds), Improvement of Quality Traits for Silage Use, 37-71. Martinus Nijhof publishers, The Hague, The Netherlands.

Vivek B. S., Krivanek A. F., Palacios-Rojas N., Twumasi-Afiriye S. and Diallo A. O. 2008. Breeding quality protein maize (QPM) cultivars: protocols for developing QPM cultivars. CIMMYT, Mexico.

Yadav O. P., Hossain F., Karjagi C. G., Kumar B., Zaidi P. H., Jat S. L., Chawla J. S., Kaul J., Hooda K. S., Kumar O., Yadava P. and Dhillon B. S. 2015. Genetic improvement of maize in India: Retrospect and prospects. Agric. Res., **4**: 325-338.

Yang L., Wang W., Yang W. and Wang M. 2013. Marker-assisted selection for pyramiding the waxy and *opaque16* genes in maize using cross and backcross schemes. Mol. Breed., **31**: 767-775.

Yang W., Zheng Y., Zheng W. and Feng R. 2005. Molecular genetic mapping of a high-lysine mutant gene (*opaque16*) and the double recessive effect with *opaque2* in maize. Mol. Breed., **15**: 257-269.

Zhang W. L., Yang W. P., Chen Z. W., Wang M. C., Yang L. Q. and Cai Y. L. 2010. Molecular marker-assisted selection for o2 introgression lines with *o16* gene in corn. Acta Agron Sin., **36**: 1302-1309.

Zhang W., Yang W., Wang M., Wang W., Zeng G., Chen Z. and Cai Y. 2013. Increasing lysine content of waxy maize through introgression of *opaque2* and *opaque16* genes using molecular assisted and biochemical development. PLoS One, **8**: 1-10.