RESEARCH ARTICLE

Temporal profiling of essential amino acids in developing maize kernel of normal, *opaque-2* and QPM germplasm

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Abstract Maize, an important cereal crop, has a poor quality of endosperm protein due to the deficiency of essential amino acids, especially lysine and tryptophan. Discovery of mutants such as *opaque-2* led to the development of nutritionally improved maize with a higher concentration of lysine and tryptophan. However, the pleiotropic effects associated with *opaque-2* mutants necessitated the development of nutritionally improved hard kernel genotype, the present-day quality protein maize (QPM). The aim of present study was to analyze and compare the temporal profile of lysine and tryptophan in the developing maize kernel of normal, *opaque-2* and QPM lines. A declining trend in protein along with tryptophan and lysine content was observed with increasing kernel maturity in the experimental genotypes. However, *opaque-2* retained the maximum concentration of lysine (3.43) and tryptophan (1.09) at maturity as compared to QPM (lysine-3.05, tryptophan-0.99) and normal (lysine-1.99, tryptophan-0.45) lines. *Opaque-2* mutation affects protein quality but has no effect on protein quantity. All maize types are nutritionally rich at early stages of kernel development indicating that early harvest for cattle feed would ensure a higher intake of lysine and tryptophan. Two promising lines (CML44 and HKI 1105) can be used for breeding high value corn for cattle feed or human food in order to fill the protein inadequacy gap. Variation in lysine and

tryptophan content within QPM lines revealed that differential expression of endosperm modifiers with varying genetic background significantly affects nutritional quality, indicating that identification of alleles affecting amino acid composition can further facilitate QPM breeding program.

Keywords Maize · *Opaque-2* · QPM · Lysine · Tryptophan

Introduction

Maize (*Zea mays* L.) is the third leading cereal crop, after wheat and rice (Sleper and Poehlman 2006). A typical kernel contains 68–73% starch, 8–13% protein, 2–4% oil and 12–15% other constituents (mainly fibers) (Ranum et al. 2014). The germ contains high levels of oil (30%) and protein (18%), whereas the economically and nutritionally valuable endosperm is a starch-rich tissue (71%) but have low protein content (8–9%) (Prasanna et al. 2001). Endosperm protein is nutritionally poor as zein, the major storage protein that lacks two essential amino acids viz., lysine and tryptophan (Vasal 2000). During the early seventies, it was found that the composition of maize endosperm protein was affected by several spontaneous and induced mutations and *opaque-2* (*o2*) is one of the most effective mutation which increased the nutritional quality of endosperm protein (Lazzari et al. 2002; Henry et al. 2005). *Opaque-2* gene encodes a transcription factor, which binds to target DNA sequence with leucine zipper motifs (Arruda et al. 2000; Schmidt et al. 1990). The increased level of lysine and tryptophan in *opaque-2* mutants is due to several factors which include the reduction of nutritionally poor alpha-zeins in the maturing endosperm (Jia et al. 2013), increase in accumulation of nutritionally rich non-zeins (Wang and Larkins 2001), and

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an increase in level of free lysine due to reduced action of lysine-ketoglutarate reductase, an enzyme for lysine catabolism (Azevedo et al. 2003; Fornazier et al. 2003). However, breeding programs using *opaque-2* mutants were hampered due to pleiotropic effects associated with these mutant lines, which include soft and chalky kernel prone to mechanical damage, yield loss and susceptibility towards fungal and insects attack (Gupta et al. 2013). Discovery of endosperm modifiers made it possible to alter the kernel texture of *opaque-2* mutants from soft and chalky to hard and vitreous, which resemble the normal maize kernel (Paez et al. 1969). These hard endosperm *opaque-2* mutants were known as quality protein maize (QPM) (Vasal 2000). The QPM, thus refers to the maize homozygous for the *o2* allele with increased lysine and tryptophan content but without the negative secondary effects of the soft and chalky endosperm. It has been noted that genetic background affects the composition of kernel in terms of both quality and quantity of protein throughout the kernel development (unpublished data). Endosperm development is a complex process and goes through four main stages of development: syncytial, cellularization, cell fate specification, and differentiation (Becraft and Asuncion-Crabb 2000). Immature kernels contain relatively higher levels of active metabolites such as sugars and free amino acids whereas it has lesser amounts of inert storage molecules, which include starch, protein and oil, which accumulate during development (Sabelli and Larkins 2009). The storage products of the maize seed, mainly starch and proteins are synthesized, beginning around 15th DAP, in the sub aleurone and starchy cell layers of the endosperm. Starch accumulation starts at amyloplast whereas zein storage protein accumulated as protein bodies in the rough endoplasmic reticulum (Becraft et al. 2002; Oleson 2004; Sabelli and Larkins 2009) and these storage products are accumulated until the metabolic activity is prevented by desiccation at seed maturity (after 40th DAP) (Kladnik et al. 2004). Analysis of mutants related to development and appearance of kernel has been extensively done in endosperm of maize (Consonni et al. 2005). Although a lot of information is available regarding development and storage product (Becraft et al. 2002; Oleson 2004; Sabelli and Larkins 2009; Kladnik et al. 2004) but the pattern of the accumulation of essential amino acid in normal and *opaque-2* counterparts in developing kernel is not well understood in maize endosperm. The objective of the present study was to analyze normal, *opaque-2* and QPM lines for protein quality at different stages of kernel development under different genetic backgrounds in maize. The outcome will give important insights to deploy maize for offering potential solutions in human and animal nutrition.

Materials and methods

Experimental material

The experimental material consists of genetically pure maize inbred lines including 16 of normal maize, 7 *opaque-2* and 7 QPM. The material was produced from Indian Agricultural Research Institute, New Delhi, Indian Institute of Maize Research, Ludhiana and Punjab Agricultural University, Ludhiana. The experimental lines were screened on the basis of kernel opaqueness (modification score 1–5) and were grown at the experimental field of Indian Institute of Maize Research, Ludhiana during *Kharif* 2017 in a plot size of 21 m², with a row length of 3 m. A minimum of 8 well-filled selfed ears from each genotype were collected at 15th, 30th and 45th DAP (days after pollination) and stored in liquid nitrogen. The endosperms were extracted immediately and dried using lyophilizer (Freezone 2.5) and stored at – 20 °C. Samples were ground to fine powder and defatted using petroleum ether (40–60 °C) for further analysis. Hundred kernel weight (HKW) and specific gravity of harvested kernel were estimated. The yield potential (t/ha) of each genotype was estimated separately in order to identify the agronomically superior genotypes.

Protein estimation

Total nitrogen concentration of the defatted endosperm samples was analyzed using the micro-kjeldahl method (AOAC 1975) in automated nitrogen analyzer (Gerhardt). Protein content (%) was calculated by multiplying the estimated nitrogen content by a conversion factor of 6.25.

Tryptophan and lysine estimation

Tryptophan and lysine concentration (% endosperm protein) was estimated by the method of Hernandez and Bates (1969) and Tsai et al. (1972), respectively, whereby enzymatic (papain) hydrolysis of endosperm releases free amino acids.

Statistical analysis

All experiments include three technical replicates and data represents the average value of repeated measure analysis of descriptive statistics. Analysis of variance (ANOVA) among different experimental genotypes at different stages of kernel development was done using SPSS software. Statgraphics 18 (Statistical Graphics Corp. Manugistics Inc., Cambridge, MA) was used to analyze frequency histograms and descriptive statistics. Dendrogram analysis

was performed to describe the genetic distance between 30 genotypes under consideration for each variable. Pearson product movement correlation between variables under consideration at each stage of kernel development was analyzed using statgraphics 18 software.

Results and discussion

Accumulation pattern of protein, tryptophan and lysine in developing kernel

The data pertaining to protein, lysine and tryptophan concentrations at different stages of endosperm development, along with modification score is presented in Table 1. It is observed that protein content showed a declining trend with kernel maturity in all the maize types. Maximum protein content (13.83%), is observed at 15th DAP, followed by (12.89%) at 30th DAP and minimum protein content (12.20%) is observed at 45th DAP (Fig. 1). Trend of protein accumulation with maturity can be attributed to the fact that high protein is needed for the actively dividing tissue, i.e. at early stages of kernel development, whereas mature seed is metabolically dormant with reduced protein content as towards maturity, tissues are stabilized with consistent storage products, especially starch which decreases the demand of protein synthesis in comparison to immature stages (Sabelli and Larkins 2009). Similar results were reported by Ortega et al. (1991), that protein content decrease with kernel maturity due to the increase in starch synthesis and enzymatic nitrogen breakdown. Jia et al. (2013) reported that the numbers of protein isoforms decreases from 439 to 383 from 17th to 30th DAP. Protein is an important nutritional component and high protein content increases the nutritional value of maize and is a cost-effective way of protein intake. The higher concentration of protein observed in the early stages of kernel development proves the nutritional superiority of baby corn, sweet corn and green maize used for silage making. Tryptophan and lysine content also shows a decreasing trend with endosperm maturity as maximum concentration is observed at 15th DAP (tryptophan 2.18%, lysine 5.28%), intermediate concentration at 30th DAP (tryptophan 1.27%, lysine 3.09%) and least concentration at 45th DAP (tryptophan 0.71%, lysine 2.61%) irrespective of the maize type. Since amino acids are major building blocks of protein, so a decrease in protein content with kernel maturity is correlated with lysine and tryptophan reduction in the maturing kernel. Another major reason for decrease in lysine and tryptophan is an increase in zeins with kernel maturity as zeins are deficient in both lysine and tryptophan and their reduction is negatively correlated with increased zein accumulation in maturing kernel irrespective of maize

type. Although, there is significant variation in lysine and tryptophan accumulation between normal, *opaque-2* and QPM lines, as discussed in “[Comparison of normal, opaque-2 and QPM lines for protein quality assessment in developing kernel](#)” section, the overall trend of lysine and tryptophan accumulation remains same irrespective of the maize type. It has been reported that both free as well as protein bound lysine decreases at kernel maturity due to increased activity of lysine degrading enzyme and lysine deficient zein accumulation, respectively (Woo et al. 2001; Li et al. 2014; Fornazier et al. 2003). Ren et al. (2018) also reported that zein synthesis is directly correlated with the activation of lysine degrading enzyme (LKR-SDH) indicating that lysine degradation is mandatory for zein synthesis. Thus, towards kernel maturity zein increases which subsequently decreases the lysine content.

Comparison of normal, *opaque-2* and QPM lines for protein quality assessment in developing kernel

The protein concentration in normal, *opaque-2* and QPM lines estimated at different stages of endosperm development (Fig. 2a) indicates that no significant differences are observed among normal, *opaque-2* and QPM lines for total protein content. However, our findings are in contrast to the previously published results, which reported that normal maize exhibited more protein content as compared to *opaque-2* and QPM lines (Hunter et al. 2002; Ortega et al. 1991). Andrés-Meza et al. (2017) reported that normal maize have higher total protein content towards maturity as compared to its quality improved counterparts involving *opaque-2* and QPM lines. Zarkadas et al. (2000) explained that high protein in normal lines is associated with the accumulation of higher amount of zein, especially alpha zeins in comparison to *opaque-2* and QPM. However present study deciphers that protein content is not influenced by *opaque-2* mutation as well as endosperm modifiers. The two genetic systems majorly affect protein quality whereas protein quantity changes in accordance with the developmental stage and genetic background. In support of the present study, it is reported that *opaque-2* mutation negatively affects the zein accumulation, which is counter balanced, with non-zein accumulation, which maintains the similar protein content in normal, and quality improved counterparts (Frizzi et al. 2010; Jia et al. 2013; Azevedo et al. 2003). Ren et al. (2018) also reported similar trend for protein accumulation supporting the present study whereby they described that while developing quality protein popcorn (QPP) protein quantity remain similar as that of parental genotypes.

Tryptophan and lysine concentration show significant variation at each developmental stage as in Fig. 2b, c, respectively. Comparison of normal, *opaque-2* and QPM

Table 1 Total protein, tryptophan and lysine content (% endosperm protein) along with modification score of Normal, *Opaque-2* and QPM genotype at different stages of kernel development

Modification score (opaqueness %)		Protein	Tryptophan			Lysine				
		15th DAP	30th DAP	45th DAP	15th DAP	30th DAP	45th DAP			
Normal										
1	CML479	14.05 ± 0.02 ^{Aop}	11.73 ± 0.02 ^{Bop}	11.43 ± 0.02 ^{Cop}	1.31 ± 0.01 ^{At}	0.75 ± 0.02 ^{Bt}	0.42 ± 0.01 ^{Ct}	4.95 ± 0.03 ^{Akl}	2.14 ± 0.02 ^{Bkl}	2.08 ± 0.01 ^{Ckl}
1	CML334	12.36 ± 0.03 ^{As}	11.41 ± 0.01 ^{Bs}	11.26 ± 0.02 ^{Cs}	1.56 ± 0.02 ^{Aq}	1.04 ± 0.01 ^{Bq}	0.45 ± 0.03 ^{Cq}	4.13 ± 0.03 ^{Ann}	2.52 ± 0.01 ^{Bnn}	2.26 ± 0.03 ^{Cnn}
1	CML266	13.44 ± 0.01 ^{Am}	12.75 ± 0.02 ^{Bm}	12.04 ± 0.02 ^{Cm}	1.73 ± 0.02 ^{Aq}	1.04 ± 0.02 ^{Bq}	0.37 ± 0.01 ^{Cq}	5.17 ± 0.01 ^{Ak}	2.25 ± 0.02 ^{Bk}	1.84 ± 0.03 ^{Ck}
1	CML172	12.65 ± 0.03 ^{Ar}	11.67 ± 0.01 ^{Br}	11.47 ± 0.01 ^{Cr}	1.43 ± 0.01 ^{Ast}	0.72 ± 0.005 ^{Bst}	0.44 ± 0.01 ^{Cst}	4.92 ± 0.01 ^{Aj}	2.46 ± 0.02 ^{Bj}	2.23 ± 0.02 ^{Cj}
1	CML169	14.49 ± 0.02 ^{Ad}	13.85 ± 0.01 ^{Bd}	13.65 ± 0.01 ^{Cd}	1.85 ± 0.02 ^{An}	1.32 ± 0.005 ^{Bn}	0.53 ± 0.03 ^{Cn}	4.64 ± 0.03 ^{Aj}	2.50 ± 0.01 ^{Bj}	2.46 ± 0.02 ^{Cj}
1	CML163	12.85 ± 0.01 ^{At}	11.04 ± 0.02 ^{Bt}	10.37 ± 0.01 ^{Ct}	1.95 ± 0.02 ^{An}	1.34 ± 0.03 ^{Bn}	0.45 ± 0.02 ^{Cn}	3.96 ± 0.03 ^{Ann}	2.66 ± 0.01 ^{Bnn}	2.35 ± 0.03 ^{Cnn}
1	CML117	11.97 ± 0.02 ^{Av}	10.64 ± 0.02 ^{Bv}	10.24 ± 0.04 ^{Cv}	1.56 ± 0.02 ^{Aq}	1.05 ± 0.02 ^{Bq}	0.53 ± 0.01 ^{Cq}	3.95 ± 0.02 ^{Am}	2.51 ± 0.01 ^{Bm}	2.35 ± 0.03 ^{Cm}
1	CML114	13.96 ± 0.02 ^{Am}	12.87 ± 0.03 ^{Bm}	11.35 ± 0.02 ^{Cm}	1.63 ± 0.04 ^{Ar}	0.74 ± 0.02 ^{Br}	0.46 ± 0.02 ^{Cr}	5.03 ± 0.01 ^{Ahi}	2.64 ± 0.02 ^{Bhi}	2.23 ± 0.03 ^{Chi}
1	CML44	12.97 ± 0.01 ^{5Auo}	12.64 ± 0.03 ^{1Buo}	12.26 ± 0.01 ^{5Cuo}	2.36 ± 0.02 ^{Al}	1.16 ± 0.02 ^{Bl}	0.64 ± 0.02 ^{Cl}	4.44 ± 0.04 ^{Ahi}	2.95 ± 0.04 ^{Bhi}	2.51 ± 0.01 ^{Chi}
1	LM11-1275	16.15 ± 0.03 ^{0Aa}	15.51 ± 0.03 ^{7Ba}	15.03 ± 0.02 ^{6Ca}	3.25 ± 0.02 ^{Al}	0.55 ± 0.02 ^{Bl}	0.35 ± 0.02 ^{Cl}	5.74 ± 0.03 ^{Al}	2.02 ± 0.03 ^{Bk}	1.60 ± 0.01 ^{Ck}
1	LM-12	12.27 ± 0.01 ^{5Ah}	14.26 ± 0.03 ^{7Bh}	13.62 ± 0.01 ^{5Ch}	1.85 ± 0.01 ^{Ap}	1.03 ± 0.01 ^{Bp}	0.45 ± 0.02 ^{Cp}	4.69 ± 0.01 ^{Ahi}	2.95 ± 0.01 ^{Bhi}	2.32 ± 0.01 ^{Chi}
1	CM-145	14.60 ± 0.13 ^{8Af}	13.67 ± 0.01 ^{5Bf}	13.05 ± 0.01 ^{5Cf}	1.54 ± 0.03 ^{As}	0.76 ± 0.02 ^{Bs}	0.35 ± 0.02 ^{Cs}	5.96 ± 0.02 ^{Am}	1.34 ± 0.01 ^{Bo}	1.04 ± 0.02 ^{Co}
1	CM-212	14.14 ± 0.04 ^{5Ae}	13.95 ± 0.03 ^{0Be}	13.63 ± 0.02 ^{5Ce}	2.46 ± 0.02 ^{Am}	1.05 ± 0.03 ^{Bm}	0.44 ± 0.03 ^{Cm}	4.72 ± 0.22 ^{Alm}	2.63 ± 0.01 ^{Bim}	1.66 ± 0.02 ^{Cim}
1	HKI-323(N)	15.06 ± 0.02 ^{5Ad}	14.14 ± 0.01 ^{5Bd}	12.73 ± 0.03 ^{5Cd}	2.46 ± 0.03 ^{Ap}	0.66 ± 0.02 ^{Bp}	0.22 ± 0.01 ^{Cp}	6.04 ± 0.03 ^{Al}	2.51 ± 0.02 ^{Bi}	1.26 ± 0.03 ^{Ci}
1	HKI-1105(N)	12.33 ± 0.02 ^{6Aq}	12.07 ± 0.01 ^{5Bq}	12.43 ± 0.02 ^{5Cq}	2.6 ± 0.01 ^{Al}	1.03 ± 0.02 ^{Bl}	0.53 ± 0.02 ^{Cl}	4.27 ± 0.02 ^{Ann}	2.62 ± 0.01 ^{Bnn}	1.94 ± 0.03 ^{Cnn}
1	HKI-1128(N)	16.46 ± 0.03 ^{Ab}	14.85 ± 0.01 ^{5Bb}	14.05 ± 0.03 ^{7Cb}	2.32 ± 0.01 ^{An}	0.85 ± 0.02 ^{Bn}	0.46 ± 0.03 ^{Cn}	5.92 ± 0.01 ^{Ah}	2.51 ± 0.02 ^{Bh}	1.65 ± 0.03 ^{Ch}
		13.74 ± 1.3	12.92 ± 1.44	12.41 ± 1.35	1.98 ± 0.53	0.94 ± 0.23	0.45 ± 0.09	4.92 ± 0.70	2.45 ± 0.38	1.99 ± 0.43
Opaque-2										
5	CML269	13.73 ± 0.04 ^{Al}	12.71 ± 0.02 ^{Bi}	12.66 ± 0.02 ^{Ci}	1.97 ± 0.01 ^{Aj}	1.52 ± 0.005 ^{Bj}	1.34 ± 0.02 ^{Cj}	5.06 ± 0.03 ^{Ae}	3.93 ± 0.03 ^{Be}	3.52 ± 0.03 ^{Ce}
5	DQL1001	15.04 ± 0.03 ^{Ac}	13.85 ± 0.02 ^{Bc}	13.43 ± 0.02 ^{Cc}	2.32 ± 0.01 ^{Al}	1.74 ± 0.01 ^{Bl}	0.91 ± 0.01 ^{Cl}	6.43 ± 0.03 ^{Ab}	3.54 ± 0.08 ^{Bb}	3.96 ± 0.04 ^{Cb}
4	DQL 1005	14.05 ± 0.04 ^{Af}	13.61 ± 0.01 ^{Bf}	13.66 ± 0.03 ^{Cf}	1.55 ± 0.03 ^{Ao}	0.94 ± 0.03 ^{Bo}	0.97 ± 0.01 ^{Co}	5.52 ± 0.03 ^{Ae}	3.83 ± 0.03 ^{Be}	3.12 ± 0.01 ^{Ce}
4	DQL 1020	13.49 ± 0.01 ^{An}	12.07 ± 0.02 ^{Bn}	12.35 ± 0.02 ^{Cn}	3.73 ± 0.02 ^{Aa}	2.03 ± 0.005 ^{Ba}	0.95 ± 0.03 ^{Ca}	5.03 ± 0.02 ^{Ag}	2.86 ± 0.02 ^{Bg}	3.44 ± 0.01 ^{Cg}
3	VQL-1	14.65 ± 0.03 ^{Aij}	13.15 ± 0.04 ^{Bij}	12.05 ± 0.03 ^{Cij}	2.63 ± 0.02 ^{Aef}	1.86 ± 0.02 ^{Bef}	0.93 ± 0.01 ^{Cef}	5.93 ± 0.03 ^{Ad}	3.93 ± 0.02 ^{Bd}	3.04 ± 0.02 ^{Cd}
5	HKI-323	14.36 ± 0.01 ^{Ap}	11.59 ± 0.03 ^{Bp}	11.15 ± 0.02 ^{Cp}	2.91 ± 0.01 ^{Ac}	1.96 ± 0.02 ^{Bc}	1.21 ± 0.01 ^{Cc}	6.04 ± 0.02 ^{Ac}	3.31 ± 0.02 ^{Bc}	3.86 ± 0.01 ^{Cc}
5	HKI-1128	14.85 ± 0.03 ^{Ai}	13.34 ± 0.04 ^{Bi}	11.73 ± 0.005 ^{Cl}	2.22 ± 0.01 ^{Ad}	2.02 ± 0.01 ^{Bd}	1.28 ± 0.01 ^{Cd}	6.15 ± 0.03 ^{Ac}	3.96 ± 0.02 ^{Bc}	3.05 ± 0.02 ^{Cc}
		14.3 ± 0.58	12.8 ± 0.84	12.42 ± 0.90	2.48 ± 0.69	1.73 ± 0.38	1.09 ± 0.16	5.73 ± 0.56	3.61 ± 0.41	3.43 ± 0.45
QPM										
2	DQL 1019	14.14 ± 0.04 ^{Ajk}	13.63 ± 0.01 ^{Bjk}	11.94 ± 0.03 ^{Cjk}	2.21 ± 0.01 ^{Ak}	1.31 ± 0.02 ^{Bk}	1.02 ± 0.01 ^{Ck}	5.82 ± 0.03 ^{Ad}	3.93 ± 0.02 ^{Bd}	2.99 ± 0.01 ^{Cd}
2	LM11-236B	14.28 ± 0.01 ^{Ao}	12.74 ± 0.04 ^{Bo}	10.34 ± 0.02 ^{Co}	2.18 ± 0.02 ^{Ah}	1.92 ± 0.01 ^{Bh}	0.86 ± 0.03 ^{Ch}	5.81 ± 0.03 ^{Ad}	4.08 ± 0.01 ^{Bd}	2.68 ± 0.03 ^{Cd}
2	LM11-288	14.06 ± 0.03 ^{Ajk}	13.65 ± 0.04 ^{Bjk}	12.03 ± 0.02 ^{Cjk}	2.57 ± 0.02 ^{Af}	1.96 ± 0.02 ^{Bf}	0.8 ± 0.01 ^{Cf}	5.91 ± 0.01 ^{Ad}	4.02 ± 0.02 ^{Bd}	2.74 ± 0.03 ^{Cd}
1	LM12-205	11.74 ± 0.04 ^{Au}	10.93 ± 0.03 ^{Bu}	11.36 ± 0.01 ^{Cu}	2.75 ± 0.01 ^{Ag}	1.55 ± 0.05 ^{Bg}	0.76 ± 0.01 ^{Cg}	4.49 ± 0.01 ^{Ag}	4.06 ± 0.02 ^{Bg}	2.9 ± 0.02 ^{Cg}
2	LM12-177	13.94 ± 0.02 ^{Ag}	13.63 ± 0.03 ^{Bg}	12.95 ± 0.04 ^{Cg}	1.81 ± 0.02 ^{Am}	1.19 ± 0.05 ^{Bm}	0.83 ± 0.03 ^{Cm}	5.12 ± 0.03 ^{Af}	3.94 ± 0.01 ^{Bf}	2.78 ± 0.02 ^{Cf}
2	VQL-2	13.95 ± 0.03 ^{Ak}	13.25 ± 0.03 ^{Bk}	12.43 ± 0.01 ^{Ck}	2.25 ± 0.02 ^{Ak}	1.35 ± 0.03 ^{Bk}	0.97 ± 0.02 ^{Ck}	5.18 ± 0.03 ^{Af}	4.18 ± 0.01 ^{Bf}	3.24 ± 0.03 ^{Cf}

Table 1 continued

Modification score (opaqueness %)	Protein	Tryptophan			Lysine		
		15th DAP	30th DAP	45th DAP	15th DAP	30th DAP	45th DAP

2 HKI-1105

12.35 ± 0.03 ^{Aq}	12.08 ± 0.01 ^{Bq}	12.42 ± 0.01 ^{Cq}	2.98 ± 0.02 ^{Ab}	1.94 ± 0.02 ^{Bb}	1.63 ± 0.01 ^{Cb}	6.74 ± 0.02 ^{Aa}	4.09 ± 0.01 ^{Ba}	3.64 ± 0.03 ^{Ca}
13.48 ± 1.03	12.8 ± 1.03	11.91 ± 0.84	2.39 ± 0.39	1.6 ± 0.33	0.99 ± 0.27	5.58 ± 0.72	4.04 ± 0.08	3.05 ± 0.38
13.83 ± 2.28	12.89 ± 1.73	12.20 ± 3.07	2.18 ± 0.58	1.27 ± 0.47	0.71 ± 0.33	5.28 ± 0.75	3.09 ± 0.79	2.61 ± 0.91

Values are mean ± SD of three replicates, values for tryptophan and lysine are expressed as percentage with respect to total protein content whereas total protein content is expressed as dry weight protein percentage. Values with same letter(s) in a column are not significantly different at $P \leq 0.05$ (Tuckey's post-hoc test). (Score 1–5 is given for varied degree of opaqueness from 0 to 100% i.e. 1–0%, 2–25%, 3–50%, 4–75%, 5–100%)

lines for tryptophan content in developing kernel depicts that *opaque-2* accumulate high concentration of tryptophan in comparison to normal lines at 15th (1.25 fold change), 30th (1.83 fold change) and 45th DAP (2.2 fold change). Similarly, while comparing QPM and normal lines, QPM retained higher tryptophan content at all stages of kernel development with 1.21 fold increase at 15th DAP, 1.70-fold increase at 30th DAP and 2.2 fold increase at 45th DAP. It is observed that in both *opaque-2* and QPM lines, the fold change for tryptophan accumulation in comparison to normal lines increase, which can be correlated to increased zein accumulation with kernel development in normal lines (De Groote et al. 2013). Therefore, the effect of the *opaque-2* mutation in terms of tryptophan accumulation can be well observed at initial stages but this enhances with kernel development indicating that although tryptophan content decreases with maturity (“[Accumulation pattern of protein, tryptophan and lysine in developing kernel](#)” section) but reduction is more in normal lines in comparison to *opaque-2* and QPM counterparts. Among *opaque-2* and QPM, it is observed that *opaque-2* accumulate slightly higher tryptophan in comparison to QPM lines with 1.04 fold change at 15th DAP, 1.08 fold change at 30th DAP and 1.10 fold change at 45th DAP. Lysine follows similar accumulation pattern in the developing kernel as that of tryptophan, with *opaque-2* retaining higher lysine in comparison to normal at 15th (1.16 fold increase), 30th (1.46 fold increase) and 45th DAP (1.72 fold increase). The comparison of QPM and normal lines shows that QPM outreaches in terms of lysine accumulation at each developmental stage with a fold difference of 1.12 at 15th DAP, 1.64 at 30th DAP and 1.52 at 45th DAP. From the above, it is observed that in normal and *opaque-2* lines, fold changes increases towards maturity whereas in normal and QPM lines, fold change is maximum at 30th DAP and then decreases at 45th DAP explaining that endosperm modifiers expressed in QPM lines mediate their effect towards maturity. This information is further established by comparing *opaque-2* and QPM lines, as *opaque-2* retained more lysine than QPM lines at 15th DAP (fold increase 1.03), 30th DAP (1.19 fold change), whereas at 45th DAP, fold difference was slightly reduced (1.11 fold change), proving our observation that endosperm modifiers expressed in QPM, majorly come into play towards kernel maturity as their major role is to retain the vitreous kernel texture in the maturing kernel. In subsequent process of establishing vitreous kernel texture, QPM slightly compromises with protein quality in comparison to *opaque-2* counterparts. It has been reported that in *opaque-2* mutant, a specific class of heat shock protein is increased which was reduced in QPM due to expression of endosperm modifiers while stabilizing vitreous kernel texture, which may be correlated to variability in terms of lysine content

Fig. 1 Protein, tryptophan and lysine concentration at different stages of kernel development. *Protein concentration is represented as percent endosperm protein whereas tryptophan and lysine concentration is represented with respect to percent endosperm protein

among *opaque-2* and QPM lines towards maturity (Morton et al. 2016).

In support to our findings, several studies have reported that tryptophan and lysine content increased in *opaque-2* and QPM in comparison to normal lines, majorly due to non-zein fractions, which are rich in lysine and tryptophan (Andrés-Meza et al. 2017). It has also been reported that lysine content is increased in *opaque-2* mutants due to increase in non-zein protein, specifically elongation factor 1 α (eEF1A), whose concentration is positively correlated with lysine content in maize endosperm (Huang et al. 2006; Jia et al. 2013). *Opaque-2* mutation is also responsible for the increase in free amino acid in maize kernel (Azevedo et al. 2003), thus leading to high lysine and tryptophan content in *opaque-2* in comparison to normal lines. Free amino acids increased as the *opaque-2* gene codes for

transcription factors with multiple targets including lysine degrading enzyme, lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR-SDH) which further enhances lysine accumulation in quality improved varieties (Ren et al. 2018). Morton et al (2016) also reported that several factors add to the increased concentration of lysine in *opaque-2* mutant including increased non-zein fraction and qualitative changes in the protein composition of non-zeins. Overall, the maximum concentration of lysine and tryptophan is observed in *opaque-2* lines, followed by QPM and least concentration was observed in normal lines irrespective of the developmental stage. The increased accumulation of non-zeins and reduction in zein protein, along with an increased amount of free amino acids also enhances the nutritional quality of *opaque-2* and QPM lines as reported previously (Ren et al. 2018; Galili and Amir 2013).

Effect of genetic background on protein quantity and quality

Genetic background also adds to variability in protein, tryptophan and lysine accumulation. As discussed earlier, no significant difference exists between normal, *opaque-2* and QPM lines in terms of protein content but significant differences exist within normal lines, as the protein content ranges from 10.4% (CML117) to 15.03% (LM11-1275) with 1.46 fold change. Similarly, in *opaque-2* lines, protein content ranges from 11.15% (HKI-323) to 13.66% (DQL-1005) with 1.22 fold difference, whereas in QPM lines, protein content ranges from 10.34% (LM11-236B) to 12.95% (LM12-177) with 1.25 fold change. Tryptophan content ranges from 0.22% (HKI-323N) to 0.64% (CML-

Fig. 2 Comparison of protein (a), tryptophan (b), lysine (c) content among normal, *opaque-2* and QPM lines at different stages of kernel development

44) with 2.90-fold change and lysine content varies from 1.26% (HKI-323N) to 2.51% (CML-44) with 1.99-fold change within normal lines. Among *opaque-2*, tryptophan content ranges from 0.91% (DQL1001) to 1.34% (CML 269) with 1.46-fold change, whereas lysine content ranges from 3.04% (VQL-1) to 3.96% (DQL-1001) with a 1.30-fold change. Among QPM, tryptophan content varies from 0.76% (LM12-205) to 1.63% (HKI-1105) with the 2.13-fold change; lysine content varies from 2.68% (LM11-236B) to 3.64% (HKI-1105) with 1.35-fold change. Normal lines shows maximum variability, followed by QPM and *opaque-2* lines in terms of protein, tryptophan and lysine accumulation which revealed that in addition to *opaque-2* mutant and endosperm modifiers, some other factors might be affecting both protein quality and quantity and such factors can further be targeted for future studies in order to develop improved maize genotypes.

The dendrogram analysis of experimental genotypes conducted on the basis of group average, squared Euclidean method for the above parameter is depicted in Fig. 3, labels 1–16 represents normal, 17–23 *opaque-2* and 24–30 represents QPM lines. The dendrogram for protein analyzed at different stages of kernel development revealed that protein content is not affected by *opaque-2* mutation at any stage of development, as no considerable differences were observed between normal, *opaque-2* and QPM lines in the dendrogram (Fig. 3a–c). However, the dendrogram for tryptophan (Fig. 3d–f) and lysine content (Fig. 3g–i), displays that normal, *opaque-2* and QPM lines are indistinguishable at initial stages of kernel development, but as the kernel matures, normal lines are clearly separated from *opaque-2* and QPM lines because the *opaque-2* mutation negatively influences the accumulation of nutritionally poor zein proteins at later stages of kernel development. The dendrogram analysis revealed that *opaque-2* mutation affects lysine and tryptophan accumulation only at kernel maturity, whereas at early stages, even certain normal lines outnumber the *opaque-2* and QPM in terms of lysine and tryptophan accumulation, which presumes that genetic background immensely affects the nutritional quality of maize along with the *opaque-2* mutation. The Pearson's product-moment correlation matrix between protein, lysine and tryptophan at 15th, 30th and 45th DAP (Fig. 4), showed that at the early stage, a significant positive correlation exists between lysine and protein content ($P \leq 0.05$) whereas no significant relationship exists between tryptophan-protein content and tryptophan-lysine concentration ($P \geq 0.05$). However, at advanced stages of kernel maturity, lysine and tryptophan content are found to be positively correlated to each other ($P \leq 0.05$), whereas no significant correlation was observed between protein-tryptophan and protein-lysine content ($P \geq 0.05$). It has been reported that a decrease in lysine content is coordinated

with the rate of zein accumulation during endosperm development (Li et al. 2014), which revealed that normal lines having high zein content retain low lysine as compared to *opaque-2* counterparts.

Yield potential and physical parameters of normal, *opaque-2* and QPM lines

Yield potential, hundred kernel weight and specific gravity are estimated (Table 2) in the experimental genotypes in order to evaluate the agronomic performance and kernel characteristics. Yield potential (t/ha) is found to be maximum in normal (2.05 t/ha), intermediate in QPM (1.72 t/ha) and least in *opaque-2* lines (1.57 t/ha). Reduction of grain yield in *opaque-2* is one of the major pleiotropic effects, which generated the need for developing quality protein maize (Gupta et al. 2013). In QPM, grain yield is higher as compared to *opaque-2* because of endosperm modifiers in QPM which help to combat the pleiotropic effects associated with *opaque-2* lines including yield performance. While comparing normal and QPM lines, it is observed that certain QPM lines are at par to normal lines in terms of yield potential. Genetic background is responsible for adding variability among varieties for grain yield trait (Andrés-Meza et al. 2017). Yield potential varies from 1.59 (LM11-1275) to 2.58 t/ha (CML44) with a fold change of 1.61 in normal lines. Similarly, in *opaque-2* lines, yield potential varies from 1.34 (DQL-1001) to 1.98 t/ha (DQL-1020) with a fold change of 1.47, whereas in QPM lines, it varied 1.24 (LM-12-205) to 2.25 t/ha (HKI 1105) with fold change 1.81. Maximum variation for yield potential is reported in QPM lines, depicting that endosperm modifier may have different responses under different genetic backgrounds. Hundred kernel weight (HKW) and specific gravity are important traits that contribute in breeding high yielding quality enriched maize varieties. Hundred kernel weight is found to be less in *opaque-2* as compared to normal and QPM counterparts with a fold change of 1.30 and 1.29, respectively. HKW expression is associated with a QTL region qGW4.05, which defines its variability among different genetic backgrounds (Chen et al. 2016a, b). HKW is linked to kernel number, a trait which is further associated with ear row number, ear length and kernel number per row (Chen et al. 2016a, b). Specific gravity is another important trait that is related to kernel hardness, the tendency of seed breakage and the probability of disease development (Chang 1987). Specific gravity is maximum in normal (1.24%), intermediate in QPM (1.18%) and least in *opaque-2* lines (1.14%). The breeding programs need to consider quality along with the performance of physical traits in order to develop nutritionally improved high yielding maize genotypes. The present study immensely helps towards the identification of

Fig. 3 Dendrogram of protein (a–c), tryptophan (d–f) and lysine (g–i) content estimated at different stages of kernel development in normal, opaque-2 and QPM lines

promising QPM lines. The study revealed that CML44 is a high yielding (2.58 t/ha) line, possessing the highest tryptophan (0.64%) and lysine (2.51%) among normal lines

under study. This line can be further used in the breeding program for the development of high yielding QPM lines for the Indian subcontinent. In addition, HKI 1105 was

Fig. 4 Correlation matrix between protein, tryptophan and lysine content at different stages of kernel development. *Correlation analysis with * represents ($P \leq 0.05$) statistically significant, whereas ** represents ($P \geq 0.05$) not statistically significant at 95% confidence interval

found to be the most effective QPM line in terms of essential amino acid abundance viz; tryptophan (1.63%) and lysine (3.64%) along with vitreous kernel texture and high yield potential (2.33 t/ha). Further, new areas where high lysine- and high tryptophan-maize can be deployed have been implicated. One such area is cattle feed, where high-moisture grain, also referred to as rocket fuel because of its importance in cattle nutrition, is an upcoming opportunity, as the dairy industry requires the availability of better nutrition choices (Naik et al. 2012). Normal maize harvested before maturity has an advantage as both lysine and tryptophan were found to be higher at the initial stages of kernel development. To further enhance the level of nutrients, QPM, with an extra 20% tryptophan and 13% lysine at the early stages of kernel development, may be utilized as a better source of cattle feed and animal nutrition. In order to overcome the protein malnutrition, which has found renewed interest in recent years; it is necessary to include QPM in food diets of affected populations (Nyakurwa et al. 2017).

Conclusions

From the above, it is concluded that maize endosperm contains the highest content of protein, lysine and tryptophan at 15th DAP and least at 45th DAP, concluding that best protein quality was observed at 15 DAP as the nutritionally deficient zein are synthesized during later stages of kernel development which subsequently decreases the protein quality of the mature kernel. Protein quantity is also maximum during the early stages of kernel development. Lysine and tryptophan were retained maximally in *opaque-*

2, followed by QPM and the least concentration was observed in normal lines irrespective of kernel developmental stage. No considerable differences were observed for protein content among normal, *opaque-2* and QPM lines. Dendrogram analysis revealed that tryptophan and lysine content can separate normal lines from *opaque-2* and QPM but it can't distinguish *opaque-2* from QPM lines at kernel maturity. To distinguish QPM from *opaque-2* maize, screening of maize kernel under lightbox along with biochemical analysis for lysine and tryptophan could be best employed. At initial stages of kernel development, different maize types are indistinguishable and even certain normal lines retain higher lysine and tryptophan content than *opaque-2* and QPM, which shows that along with *opaque-2* mutation, developmental stage and genetic background strongly affect lysine and tryptophan accumulation in different maize types. It has also been concluded that although *opaque-2* mutations affect protein quality, it has no appreciable effect on protein concentration. Lysine and tryptophan content are important benchmarks for protein quality parameters and are highly correlated at later stages of kernel development, but no significant correlation was observed between lysine and tryptophan at the early stages of kernel development. The genetic background adds to variability in lysine and tryptophan content and maximum variability is for the accumulation of tryptophan content as appreciable differences were observed in the protein quality among 14 normal lines. Grain yield and physical parameters are important and valuable traits in developing quality protein maize with consistent agronomic performance. Overall, it is revealed that for the development of high protein quality genotypes, genetic background assessment is necessary, as certain

Table 2 Physical parameters (yield potential, hundred kernel weight and specific gravity) of the experimental genotypes

	Yield potential (t/ha)	Hundred kernel weight (HKW)	Specific gravity
Normal			
CML479	2.05 ± 0.02	23.48 ± 0.02	1.23 ± 0.02
CML334	2.36 ± 0.03	17.66 ± 0.01	1.19 ± 0.02
CML266	2.11 ± 0.01	26.36 ± 0.02	1.19 ± 0.02
CML172	2.38 ± 0.036	27.36 ± 0.011	1.24 ± 0.015
CML169	1.94 ± 0.021	19.96 ± 0.015	1.14 ± 0.015
CML163	2.21 ± 0.012	21.63 ± 0.020	1.27 ± 0.010
CML117	1.99 ± 0.026	18.12 ± 0.025	1.29 ± 0.041
CML114	1.78 ± 0.021	19.70 ± 0.032	1.23 ± 0.025
CML44	2.58 ± 0.015	26.67 ± 0.031	1.21 ± 0.015
LM11-1275	1.59 ± 0.030	19.12 ± 0.037	1.15 ± 0.026
LM-12	1.62 ± 0.015	21.78 ± 0.037	1.21 ± 0.015
CM-145	1.60 ± 0.138	23.67 ± 0.015	1.29 ± 0.015
CM-212	1.74 ± 0.045	21.95 ± .030	1.32 ± 0.025
HKI-323(N)	2.06 ± 0.025	20.14 ± 0.015	1.24 ± 0.035
HKI-1105(N)	2.33 ± 0.026	22.07 ± 0.015	1.28 ± 0.025
HKI-1128(N)	2.46 ± 0.03	20.85 ± 0.015	1.31 ± 0.037
Average (normal)	2.05 ± 1.3	21.90 ± 1.64	1.24 ± 1.07
Opaque-2			
CML269	1.43 ± 0.04	20.81 ± 0.02	0.99 ± 0.02
DQL1001	1.34 ± 0.03	19.22 ± 0.02	1.20 ± 0.02
DQL 1005	1.51 ± 0.04	18.77 ± 0.01	1.08 ± 0.03
DQL 1020	1.98 ± 0.01	16.81 ± 0.02	0.98 ± 0.02
VQL-1	1.61 ± 0.03	13.15 ± 0.04	1.18 ± 0.03
HKI-323	1.36 ± 0.01	14.59 ± 0.03	1.21 ± 0.02
HKI-1128	1.85 ± 0.03	13.34 ± 0.04	1.16 ± 0.005
Average (opaque-2)	1.57 ± 0.58	16.67 ± 0.84	1.14 ± 0.90
QPM			
DQL 1019	2.14 ± 0.04	22.30 ± 0.01	1.17 ± 0.03
LM11-236B	1.28 ± 0.01	24.01 ± 0.04	1.20 ± 0.02
LM11-288	1.64 ± 0.03	21.11 ± 0.04	1.20 ± 0.02
LM12-205	1.24 ± 0.04	20.93 ± 0.03	1.14 ± 0.01
LM12-177	1.54 ± 0.02	21.63 ± 0.03	1.16 ± 0.04
VQL-2	1.95 ± 0.03	19.25 ± 0.03	1.24 ± 0.01
HKI-1105	2.25 ± 0.03	22.08 ± 0.01	1.21 ± 0.01
Average (QPM)	1.72 ± 1.61	21.61 ± 1.03	1.18 ± 0.84
Overall average	1.78 ± 3.28	20.06 ± 2.73	1.19 ± 1.07

Values are mean ± SD of three replicates, values for yield potential are expressed in t/ha

normal lines are more responsive towards *opaque-2* introgression. Yield assessment and biochemical parameters must be monitored continuously while converting a low-quality normal line to high yielding quality protein maize.

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