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Prolamins and Glutelins as protein markers to distinguish normal lines from QPM germplasm

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Abstract
Maize protein quality is poor due to high content of zein protein lacking essential amino acids: lysine and tryptophan. *Opaque2* mutants were discovered having high lysine, tryptophan due to reduction zeins with pleotropic effects such as soft and chalky kernel. Quality protein maize (QPM) were developed with high protein quality and vitreous kernel texture. Present study was planned to establish protein markers to distinguish normal maize from QPM. Kernels were harvested from normal and QPM lines for estimation of protein fractions from extracted endosperm. Results revealed that prolamin and glutelin are the two major fractions affected by *opaque-2* mutation. Prolamin is highest in normal (44.9%) and least in QPM (8.94%) whereas, glutelin is more in QPM (32.9%) than normal lines (17.6%). Hence out of all protein fractions, prolamin and glutelin show maximum variation, so can be used as precise and cost effective protein markers to differentiate normal lines from QPM.

Keywords: Normal, QPM, protein, prolamin, glutelin

1. Introduction
Maize (*Zea mays*) is the world's third leading cereal crop, after wheat and rice and is a staple food for a large segment of population [1]. A mature maize kernel constitutes 80-85% of endosperm, followed by germ (9-10%) and pericarp (5-6%). Endosperm the major edible part of maize kernel is composed of 70% starch and 8-10% protein and relatively low fat content [2]. Maize endosperm consists of two types of protein i.e., zein and non-zein protein. Zein, the alcohol soluble protein (prolamin, prolamin-like protein), is the major seed storage protein of maize kernel and constitutes approximately 50-70% of maize endosperm [3]. The non-zein protein consists of globulins (3%), glutelins (34%) and albumins (3%). Protein quality of maize endosperm is poor because it is devoid of essential amino acids, particularly lysine and tryptophan due to higher proportion of nutritionally poor zein protein, whereas non-zein fraction has balanced proportion of essential amino acid but there content in normal maize is comparatively low which decreases the overall nutritional quality of maize protein [4]. Discovery of *opaque-2* mutant led to, the development of nutritionally improved maize [5]. *Opaque-2* gene codes for a transcriptional factors that belongs to basic region-leucine zipper family [6] and controls the expression of other genes. The nutritional quality of *opaque-2* mutants was increased due to overall reduction of (50-70%) zein protein, specifically the 22-kDa alpha zeins and subsequently the increase in non-zein fractions including globulin, albumin and glutelins [7]. However, the pleotropic effects such as kernel opaqueness, yield loss and disease susceptibility halted the success of *opaque-2* mutant varieties [8]. Later, combination of *opaque-2* mutants with endosperm modifiers led to the development of quality protein maize (QPM) which has similar kernel texture as that of normal maize but possess higher protein quality as that of *opaque-2* mutants. Breeding programs have led to development of high quality maize varieties and there screening for protein quality is necessary, traditional screening method involve lysine and tryptophan estimation by papain hydrolysis method which is costly so there is demand to develop substitutes to the available method. Present study is targeted to investigate the protein fractions which are majorly effected by *opaque-2* mutation and to develop cost effective method of QPM screening.

2 Materials and Methods

2.1 Materials

Experimental material was procured from Indian Agricultural Research Institute, New Delhi, Indian Institute of Maize Research, Ludhiana and Punjab Agricultural University, Ludhiana. 16 normal and 7 QPM lines with different genetic background were grown in the experimental fields of Indian Institute of Maize Research, Ludhiana during kharif 2017. The complete detail including modification score, based on the opaqueness level assigned to the experimental material is presented in Table 1. Modification score refers to degree of opaqueness estimated through light box screening of matured kernel. Normal maize has a rigid matrix and therefore have 0% opaqueness (modification score 1), whereas *opaque-2* mutants showed 100% opaqueness (modification score 5) and QPM have varying degree of opaqueness (modification score from 2 to 4) due to differential expression of endosperm modifiers as in Figure1. Kernels were extracted from the center of each cob; the endosperm was dissected from the embryo. To minimize the effect of biological variation between ears, equal numbers of dissected endosperms from 4 ears of each line were pooled and treated as one sample; and a minimum of three replicated samples were used for each experiment. Extracted endosperms were finely ground and de-fatted using petroleum ether (40-60^{O}C).

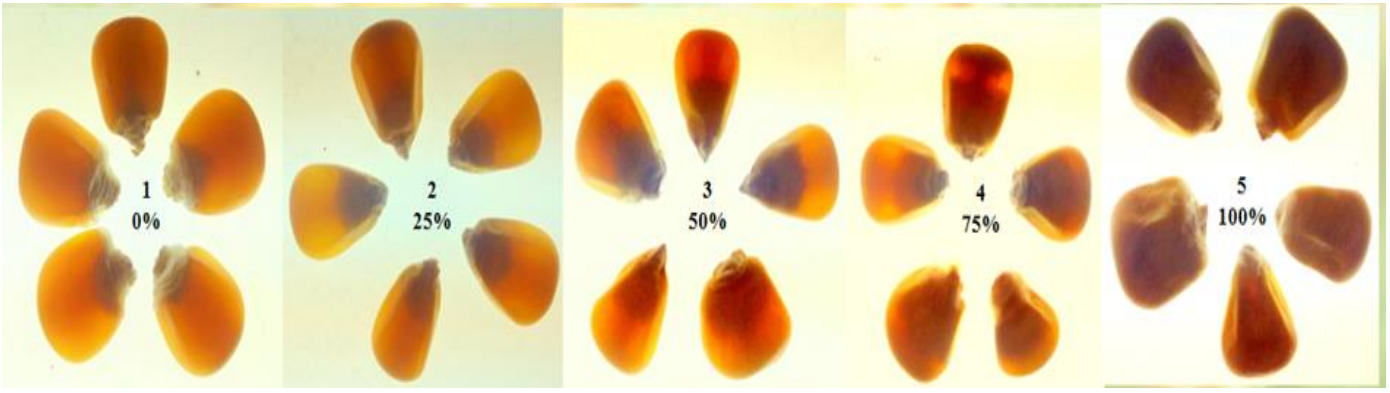

Fig 1: Light box screening of maize kernel having variable degree of opaqueness from 0 to 100% with subsequent modification score 1 to 5.

2.2 Total protein estimation

Total protein content (dry weight basis) was estimated by analyzing the nitrogen content using an automated nitrogen analyzer (Gerhardt) by micro-kjeldahl method [9]. Protein percent was obtained by multiplying the nitrogen content by conversion factor 6.25.

2.3 Protein fractionation and estimation

Protein fractionations were estimated as per the method of Landry and Mourex (1970) with certain modifications [10]. Based on the solubility characteristics maize endosperm proteins are broadly classified into 5 fractions including water soluble albumins, salt soluble globulins, alcohol soluble (prolamin, prolamin-like), and alkali soluble (glutelins, glutelin-like) and residue (insoluble) proteins. Nitrogen is estimated in all the fractions after following micro-kjeldahl method. The protein was calculated by multiplying with factor 6.25 as specified earlier.

2.4 Statistical analysis

Repeated measure analysis of descriptive statistics, and Analysis of variance (ANOVA) among different experimental genotypes was done using SPSS software. Dendrogram analysis to describe genetic distance between thirty experimental lines for each parameter was performed using group average squared eucledian method by using statgraphia 18 software (Statistical Graphics Corp. Manugistics Inc., Cambridge, MA).

3 Results and Discussion

Total protein content was found to be nearly same in normal (12.41%) and QPM (11.91%) as in Figure 2. Considering total protein content as 100%, protein concentration of different fractions was calculated. Protein fractions can be classified into nutritionally poor zein and nutritionally rich non-zeins. The major endosperm storage protein, the prolamin and prolamin-like fractions, are collectively referred to as the zeins. These proteins contain large amounts of the amino acids such as glutamine, proline, leucine, and alanine, and are of relatively poor nutritional quality [11]. The comparison in normal and QPM lines on the basis of prolaimn and glutelin content is described in Table.1. Prolamin fraction was observed to be retained maximally in normal as compared to QPM (3.71-fold decrease) genotypes (Figure 2). Direct correlation between zein content and kernel vitreousness has been reported depicting that normal maize retains more zein content as compared to *opaque-2* mutants [12].

Glutelin content is observed to be retained maximally in QPM (32.9%) whereas normal lines were found to retain the least concentration of glutelin (17.63%) (Figure 2). Similar findings have been reported earlier showing that glutelin content was increased from 17% to 44% in *opaque-2* mutants as compared to its normal counterpart [13]. Genetic variation again found to influence glutelin conent as significant variation has been observed normal 11.12% (CML 266) to 19.65% (HKI 323N), as well as QPM lines 31.13% (VQL-2) to 34.43% (LM11-288).

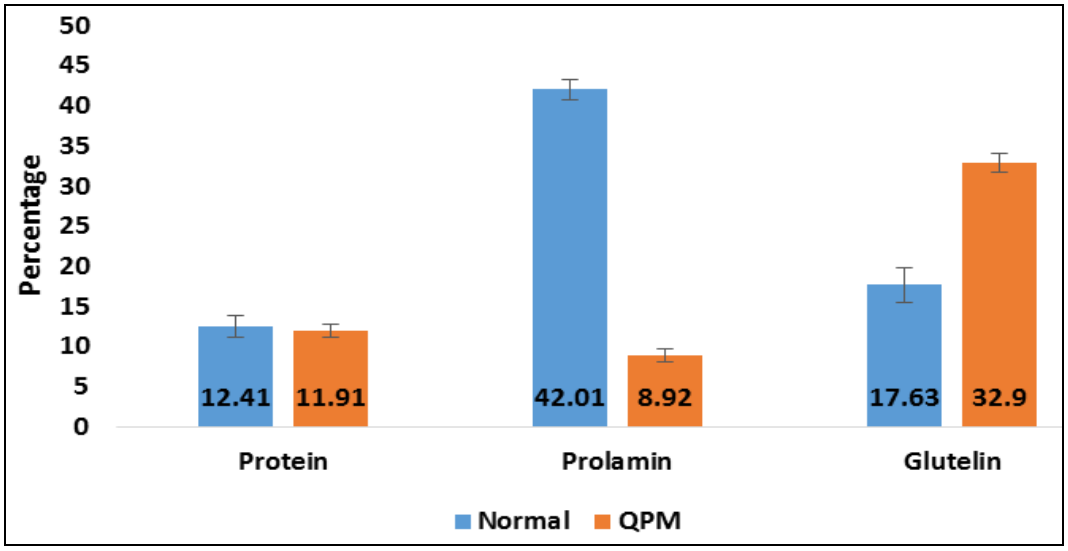

Fig 2: Comparison of total protein, prolamin and glutelin content between normal and QPM lines.

From the above it is observed that *opaque-2* mutation affect prolamin and glutelin fraction mainly as a sufficient changes in QPM genotype has been observed from the normal lines in (Fig.2). It has been reported that *opaque-2* mutation leads to overall reduction of zein protein, specifically the prolamins (22-kDa α-zein) and compensatory mechanism subsequently leads to non-zein synthesis majorly glutelin which reveals that *opaque-2* and normal lines are majorly differentiated from each other in terms of prolamin and glutelin content [13, 14].

Table 1: Protein, prolamin and glutelin content in different normal and QPM lines along with modification score.

Genotype	Varieties	Modification score	Protein (%)	Prolamin (%)	Glutelin (%)
Normal	CML479	1	11.43 ± 0.02Cop	41.22± 0.03Ak	18.65 ± 0.02Ar
	CML334	1	11.26 ± 0.02Cs	41.15 ± 0.03Ag	18.41 ± 0.02Au
	CML266	1	12.04 ± 0.02Cm	42.22 ± 0.03Aa	11.12 ± 0.02Aaa
	CML172	1	11.47 ± 0.015Cr	40.26 ± 0.03Ae	14.42 ± 0.02Az
	CML169	1	13.65 ± 0.015Cd	43.15 ± 0.03Ad	17.99 ± 0.02Aq
	CML163	1	10.37 ± 0.010Ct	44.07 ± 0.01Ab	17.01 ± 0.02Aw
	CML117	1	10.24 ± 0.041Cv	45.22 ± 0.01Ac	16.69 ± 0.03Ay
	CML114	1	11.35 ± 0.025Cm	41.75 ± 0.02Am	18.42 ± 0.02As
	CML44	1	12.26 ± 0.015Cno	42.27 ± 0.01Ai	17.98 ± 0.02At
	LM11-1275	1	15.03 ± 0.026Ca	42.26 ± 0.02Ah	17.42 ± 0.02Av
	LM-12	1	13.62 ± 0.015Ch	41.24 ± 0.02An	17.49 ± 0.03Ax
	CM-145	1	13.05 ± 0.015Cf	42.24 ± 0.03Af	17.78 ± 0.03Aw
	CM-212	1	13.63 ± 0.025Ce	41.96 ± 0.03Al	18.86 ± 0.04Av
	HKI-323(N)	1	12.73 ± 0.035Cd	41.05 ± 0.03Ao	19.65 ± 0.01Ap
	HKI-1105(N)	1	12.43 ± 0.025Cq	40.17 ± 0.01Ap	20.24 ± 0.02An
	HKI-1128(N)	1	14.05 ± 0.037Cb	41.94 ± 0.03Aj	19.92 ± 0.02Ao
Total			12.41 ± 1.35	42.01±1.3	17.63 ± 2.2
QPM	DQL 1019	2	11.94 ± 0.03Cjk	9.24 ± 0.02Ay	33.13± 0.02Aj
	LM11-236B	2	10.34 ± 0.02Co	8.64 ± 0.02Ax	34.42± 0.01Ad
	LM11-288	2	12.03 ± 0.02Cjk	8.15 ± 0.02Aaa	34.43± 0.03Ac
	LM12-205	1	11.36 ± 0.01Cu	8.23 ± 0.02Az	33.05± 0.03Ab
	LM12-177	2	12.95 ± 0.04Cg	8.19 ± 0.005Ay	32.18 ±0.01Ag
	VQL-2	2	12.43 ± 0.01Ck	9.95 ± 0.02At	31.13± 0.04Af
	HKI-1105	2	12.42 ± 0.01Cq	10.23 ± 0.01Aq	32.25± 0.04Ae
Total			11.91 ± 0.84	8.92±0.8	32.9±1.2

*(Modification score 1-5 is given for varied degree of opaqueness from 0-100% i.e. 1-0%, 2-25%, 3-50%, 4-75%, 5-100%). Values are mean ± SD of three replicates, values for total protein fractions (prolamin and glutelin) are expressed as protein 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).

The Dendrogram analysis of experimental genotypes (Fig.3) conducted on the basis of group average revealed that prolamins and glutelins can be used as markers to distinguish normal from QPM lines. Dendrogram analysis also shows that genetic background plays an important role in modifying the nutritional quality of maize. The converted QPM lines express versatile pattern in accumulating protein fractions, which proves *opaque-2* mutation acts on broadly and its outcome is dependent on the genetic background along with it endosperm modifiers have more complex mode of inheritance and these modifiers are variably expressed under different genetic background. Endosperm modifier genes are quantitative trait loci (QTL) which show additive effect and its influence varies with respect to biochemical composition of maize and genetic backgrounds [15].

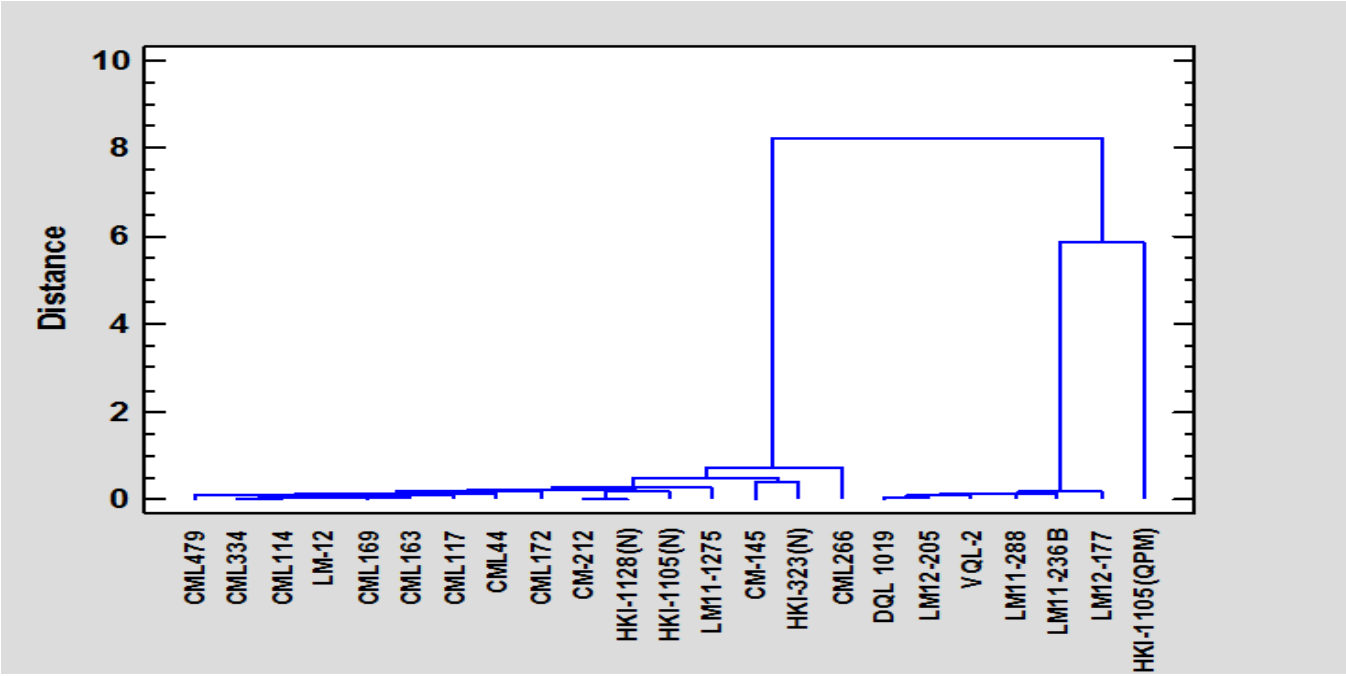

Fig 3: Dendrogram of normal and QPM lines on the basis of prolamin and glutelin content.

The broad comparison of zein and non-zein fractions in normal and QPM lines at kernel maturity revealed that normal lines retain higher concentration of zein and low concentration of non-zein proteins, whereas QPM show high concentration of non-zein and least concentration of zein proteins. As in Figure 4 a significant positive correlation is observed between glutelin and lysine, tryptophan content, whereas a significantly negative correlation is observed between prolamin and lysine, tryptophan content. It was reported that *opaque-2* mutation reduces zein synthesis, which leads to accumulation of non zeins and as zeins do not contain lysine, all the protein-bound lysine comes from the non zein fraction [16]. Hence, the reduction in zein and the increased synthesis of non zein proteins both contribute to the enhanced percentage of lysine in the grain protein.

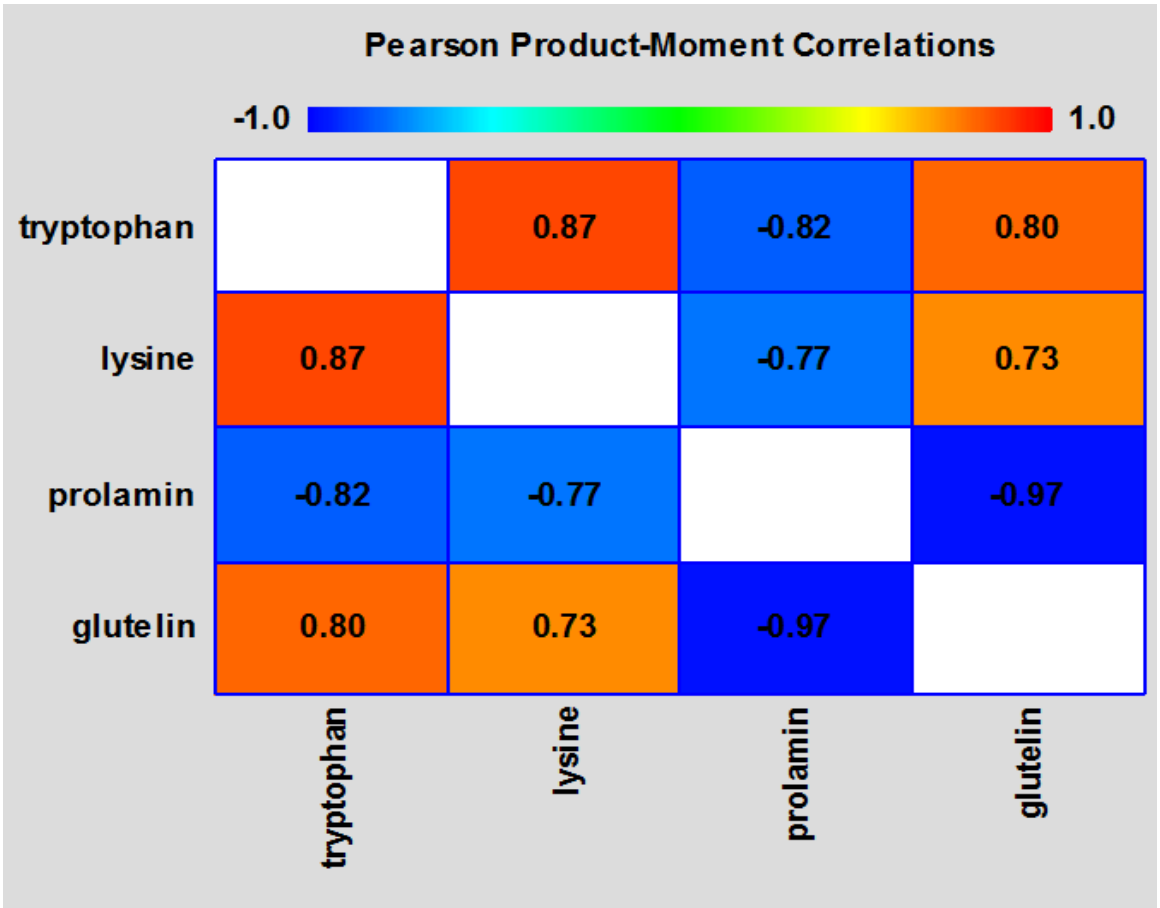

Fig 4: correlation between lysine, tryptophan, prolamin and glutelin content of normal and QPM lines.

4. Conclusions

From the above results it is concluded that nutritionally poor zein fraction is a major storage protein and is found to be in highest concentration in normal maize whereas QPM retain nutritionally rich non-zein fraction. Dendrogram analysis shows that genetic background significantly effects the nutritional status of all maize types. Dendrogram analysis also revealed that prolamin and glutelin can be used as precise markers to distinguish normal from QPM lines. Overall it is concluded that genetic background immensely affects the protein fraction accumulation and continuous monitoring of protein quality is important while converting a normal to its QPM counterparts as introgression of *opaque-2* mutation and endosperm modifiers have diverse effects on maize protein quality and kernel appearance and for this continuous monitoring prolamin and glutelin estimation can replace the traditional cost effective method involving lysine and tryptophan estimation as a strong correlation exist between these two methods.

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