Opaque16, a high lysine and tryptophan mutant, does not influence the key physico-biochemical characteristics in maize kernel

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Abstract

The enhancement of lysine and tryptophan in maize is so far based on opaque2(o2) mutant, that along with the endosperm-modifiers led to development of Quality Protein Maize [QPM]. Though many mutants improving the endospermic protein quality were discovered, they could not be successfully deployed. Recently discovered opaque16 (o16) mutant enhances the lysine and tryptophan content in maize endosperm. In the present study, the influence of o16 on the endosperm modification was analyzed in four F2 populations, two each segregating for o16 allele alone and in combination with o2. The recessive o16o16 seed endosperm was found to be vitreous phenotypically similar to wild-O16O16. The mutant did not influence the degree of kernel opaqueness in o2o2 genetic background as opaqueness in o2o2/O16O16 and o2o2/o16o16 was similar. Grain hardness of o16o16 was comparable with the normal and QPM maize. The pattern of microscopic organization of proteinaceous matrix and starch granules, and zein profiling of the storage protein in o16o16 were found to be similar with normal maize endosperm, but distinct from the o2o2-soft genotype. The pattern in o2o2/o16o16 was unique and different from o2o2 and o16o16 as well. Here we demonstrated the effects of o16 on physico-biochemical characteristics of endosperm and report of o16 possessing negligible influence on kernel modification and hardness, which holds a great significance in maize quality breeding programme.

Introduction

Maize is one of the most important food crops in sub-Saharan African, Latin American and many of the Asian countries[1]. It is also an important source of poultry and livestock feed worldwide[2]. Storage protein of maize, prolamin also known as zein, constitutes about 70% of the total protein. Prolamin is characterized by limiting level of two essential amino acids, lysine and tryptophan[3,4]. Maize, therefore, being poor in nutritional quality does not provide balanced nutrition to human and mono-gastric animals such as poultry and pig. A mutation, opaque2 (o2) discovered in 1920s was found to be nutritionally superior in lysine and
tryptophan compared to normal maize [5]. However, the improvement in the quality was deterred by the pleiotropic effects of the mutant that causes soft endosperm making the kernel more prone to insect infestation and pathogen susceptibility with poor processing quality and reduced yield [6]. Several other genetic mutations viz., floury1 (fl1), floury2 (fl2), floury3 (fl3), opaque5 (o5), opaque6 (o6), opaque7 (o7), opaque15 (o15), Defective endosperm (Def-B30), Mucronate (Mc) that affect the lysine content in maize endosperm, have been discovered [7]. Different combinations of these mutants to further increase the lysine and tryptophan were also tried, but could not succeed due to adverse pleiotropic effect that imposed severe constraints in implementing them [8, 9].

Researchers found that the opaqueness caused due to o2 can be overcome with the accumulation of o2-modifiers and led to the development of Quality Protein Maize (QPM) with improved lysine content from 0.15 to 0.37% and tryptophan from 0.04 to 0.08% on average [10, 11]. The exact mechanism of the o2 endosperm modification in QPM is not known but a possible role of 27-kDa γ-zein in recovering the vitreous phenotype has been put forward [12]. Genetic mapping of o2 modifiers in QPM was found to be the locus encoding linked with 27-kDa γ-zein storage proteinson chromosome 7. Wu and Messing [13] later demonstrated that silencing of 27- and 16-kDa γ-zein genes resultin clumping of protein bodies and thus opacity of QPM seeds. Yang et al. [14] discovered a recessive mutant from Robertson’s Mutator stocks and named it temporarily as opaque16 (o16). The o16 located on chromosome 8 induces higher lysine content compared to normal maize. The locus o16 in o2o2 genetic background increases lysine by ~30% over o2o2 or o16o16 alone. In our earlier studies, genotype with o16o16 possessed nearly on average two-fold more lysine (0.247%) and tryptophan (0.072%) compared to normal maize (0.125% lysine and 0.035% tryptophan) [15]. The effect of o16 on higher accumulation of lysine was also reported by Zhang et al. [16, 17]. Yang et al. [14] reported the presence of opaque phenotype in two o16-based inbreds. However, the effects of o16 on degree of influence on endosperm opaqueness, hardness, zein profile and organization of starch granules with proteinaceous matrix in kernel in segregating populations have not been yet investigated. It is therefore, pertinent here to evaluate the performance of o16 mutant on general endosperm attributes, as o2 despite its nutritional superiority could not be initially accepted due to induction of soft endosperm. In the present study, we attempted to study the influence of o16 on grain hardness and different physico-biochemical characteristics.

Materials and methods

Plant materials

The experimental materials consisted of four populations derived from two CIMMYT-based o2o2 inbreds (CML161, CML193) and two CIMMYT-based normal (CML533 and CML537) inbreds crossed with an o16o16-donor line (QCL3024, a yellow line of Chinese origin). Derived F1s from the crosses were obtained from Guizhou Institute of Upland Food Crops, China. F1s of the four crosses were grown at the Indian Agricultural Research Institute, New Delhi, India during rainy season-2014. The F2 populations were raised at Winter Nursery Centre, Hyderabad of Indian Institute of Maize Research, New Delhi- during winter season 2014–15. Each of the F2 plants was selfed to generate F3 seeds. The derived F3 seeds along with three other inbreds: a CIMMYT-based normal inbred-CML543, a soft and opaque endosperm inbred-MGUQ-102 (o2o2 based without endosperm modifiers), and a QPM inbred-HKI193-1 (o2o2 based with endosperm modifiers), were subjected for the studies.
DNA isolation, PCR amplification and gel electrophoresis

Genomic DNA was extracted from young tender leaves by using CTAB method [18]. The PCR (Bio-Rad, California, USA) reaction was carried out applying 'touch down' procedure for 15 μl reaction mixture using REDtaq ReadyMix™ PCR Reaction Mix (SIGMA-ALDRICH). 15 μl reaction mixture consists of 7.5 μl of REDtaq reaction mix, 3.5 μl water, 2 μl of DNA and 1 μl each of forward and reverse primers. The 'touch down' procedure consisted of three steps. The first step was set for 12 cycles: denaturation at 94˚C for 30s, annealing at 62˚C for 30s (reducing the annealing temperature subsequently by 0.5˚C per cycle), and extension at 72˚C for 45s. The second step was set for 45 cycles: denaturation at 94˚C for 30s, annealing at 58˚C for 45s, and extension at 72˚C for 45s. The third step final extension was carried out at 72˚C for 7 min. The PCR amplicons of CML533-, CML537- and CML161-based populations were resolved in 4% agarose gel, while CML193-based population was resolved in 8% native PAGE acrylamide gel. The amplicon profiles were visualized in a gel documentation system (AlphaInnotech, California, USA).

Genotyping

The genotyping of individual plant in each generation of all populations for o2 was carried out using gene-based SSR markers, phi112, phi057 and umc1066 [19] and for o16, linked markers, umc1141 and umc1149 were used [14]. The test for hybridity of F1(s) and genotyping of individual plants in F2 generations were carried out by targeting these SSRs. Chi-square test was performed using MS-Excel 2010 for testing the goodness of fit between the segregation pattern at 5% level of significance.

Endosperm modification

One hundred randomly selected seeds in each population were used for analyses of endosperm modification. The degree of opaqueness of seeds was analysed by using standard 'light box' with the formula: Degree of opaqueness = [(N100×100) + (N75×75) + (N50×50) + (N25×25) + (N0×0)]/100, where N100, N75, N50, N25 and N0 are the numbers of seeds with 100%, 75%, 50%, 25% and 0% opacity, respectively (Hossain et al. 2008). For observing the ratio of inner soft and outer hard endosperm, seed kernels were transversely cut through the centre by a sharp cutter exposing both the embryo and the surrounding tissue of endosperm.

Grain hardness

Nine genotypic classes could be obtained in F2 derived F3 seeds of both crosses, CML161 × QCL3024 and CML193 × QCL3024 since the progenies are segregating for o2 and o16. For the crosses, CML533 × QCL3024 and CML537 × QCL3024, where only o16 was segregating, three classes could be obtained in F2 populations. Derived F3 families from F2 double homozygotes viz. o2o2/o16o16, o2o2/O16o16, O2O2/o16o16, and O2O2/O16O16 were performed for grain hardness studies along with normal inbred CML543 (O2O2/O16O16), soft endosperm MGUQ-102 (o2o2/O16O16) and QPM line HKI193-1 (o2o2/O16O16) as checks. Five randomly selected kernels per line were used for measuring grain hardness (GH) using Texture Analyzer (Scientific Microsystem, UK). The hardness was measured at grain moisture content of ~14%. A cylindrical probe of 75 mm diameter (P75 mm compression platen) was used. Individual seeds were placed centrally beneath the probe with the embryo facing down. The test speed of the probe was fixed at 2 mm/s and the compression distance at 70% with a trigger load cell of 500 kg. The first peak force (N, newton) in the force deformation curve was noted as GH of the seeds [20]. t-test was performed if the difference in hardness between the
different classes and with the corresponding \(O2O2/O16O16\) in each population is significant by using Microsoft Excel.

**Scanning electron microscopy of maize endosperm**

Maize kernels were decapped and degermed with a razor blade and cut through the centre of the kernel giving a fracture with rough surface rather than a clean cut. A small piece from the central region of endosperm was used for study and was coated with an alloy of gold and palladium and documented in Zeiss EVO MA 10 Scanning electron microscope at 20kV/EHT and 80 Pa with a magnification of 1.50 KX.

**Protein profiling**

The total protein and the zein fractions \(\alpha\), \(\beta\), \(\gamma\) and \(\delta\) zein fractions of different samples maize endosperm protein were extracted from 50 milligram of maize flour in accordance with Yue et al.[21]. The 10 \(\mu\)l of extracted alcohol soluble zein protein fractions were profiled in 15% SDS-PAGE.

**Results**

**Segregation of \(o2\) and \(o16\) through SSR markers analyses**

The three reported \(o2\) gene-based SSR markers viz., \(phi112\), \(phi057\) and \(umc1066\) were used for testing the polymorphism between the female parents (CML161, CML193, CML533 and CML537) and the respective \(F1\) (s). Of the three, \(umc1066\) showed distinct polymorphism in 4% agarose gel, thus used for genotyping the \(F2\) individual plants (Fig 1A). In the case of \(o16\), Yang et al.[14] reported three linked SSRs viz. \(umc1121\), \(umc1141\) and \(umc1149\).

In CML193 × QCL3024, \(umc1141\) showed a distinct polymorphism in 8% native PAGE and in the remaining three populations viz. CML161 × QCL3024, CML533 × QCL3024 and CML537 × QCL3024, \(umc1149\) was polymorphic in 4% agarose (Fig 1B). The \(F2\) populations of all the crosses exhibited a co-dominant segregation of both \(o2\) and \(o16\) as per Mendelian ratio of 1:2:1 (\(p < 0.05\)) (Table 1).

**Effect of \(o16\) on maize endosperm opaqueness**

One hundred randomly selected \(F2\) seeds per cross were grouped into five classes with the scores in degree of opaqueness as 100%, 75%, 50%, 25% and 0%[22]. In CML161 × QCL3024 and CML193 × QCL3024 (segregating for both \(o2\) and \(o16\)), the opaqueness in \(F2\) generation was found to be 26.09% and 28.98%, respectively (Fig 2, Table 2). However, CML533 × QCL3024 and CML537 × QCL3024 segregating only for \(o16\) displayed a mere 2.25% and 0% opaqueness, respectively (Table 2). The extent of opaqueness in CML161 × QCL3024 and CML193 × QCL3024 \(F2\)-derived \(F3\) seeds of genotype \(o2o2/o16o16\) (98.24% and 96.34%, respectively) was comparable to \(o2o2/O16O16\) (97.65% and 95.81%, respectively); genotype \(O2O2/o16o16\) (2.15% and 3.55%, respectively) and \(O2O2/O16O16\) (1.23% and 1.72%, respectively) displayed negligible opaqueness (Fig 3). In the case of CML533 × QCL3024 and CML537 × QCL3024, the opaqueness observed in \(o16o16\) (4.30% and 0.35%, respectively) and \(O16O16\) (2.03% and 1.49%, respectively) was of similar degree (Table 3). The ratio of inner soft and outer hard endosperm of \(o16o16\) line was also found to be similar with the one observed in wild line CML543 and HKI193-1 QPM inbred (Fig 4).
Effect of o16 on maize endosperm

The endosperm of genotypes O2O2/o16o16 and O2O2/O16O16 were hard, as reasonably force of higher degree was required to break the F3-grains of CML161 × QCL3024 (399.73N and 414.97N, respectively) and CML193 × QCL3024 (332.89N and 337.18N, respectively) compared to o2o2/o16o16 and o2o2/O16O16 (CML161 × QCL3024: 213.65N and 267.85N; CML193 × QCL3024: 205.52N and 246.96N), respectively (Table 4). Further,

![Marker segregation of o16-linked SSR umc1149.](https://doi.org/10.1371/journal.pone.0190945.g001)

**Effect of o16 on grain hardness**

The endosperm of genotypes O2O2/o16o16 and O2O2/O16O16 were hard, as reasonably force of higher degree was required to break the F3-grains of CML161 × QCL3024 (399.73N and 414.97N, respectively) and CML193 × QCL3024 (332.89N and 337.18N, respectively) compared to o2o2/o16o16 and o2o2/O16O16 (CML161 × QCL3024: 213.65N and 267.85N; CML193 × QCL3024: 205.52N and 246.96N), respectively (Table 4). Further,

**Table 1. Segregation pattern of SSRs associated with opaque16 and opaque2.**

|                  | CML161 × QCL3024 | CML193 × QCL3024 | CML533 × QCL3024 | CML537 × QCL3024 |
|------------------|------------------|------------------|------------------|------------------|
| **opaque16**     |                  |                  |                  |                  |
| Population size  | 119              | 150              | 159              | 143              |
| o16o16           | 30               | 39               | 41               | 40               |
| O16o16           | 56               | 76               | 81               | 69               |
| O16O16           | 33               | 35               | 37               | 34               |
| $\chi^2$        | 0.563            | 0.3061           | 0.2579           | 0.6783           |
| p value          | 0.7546 ns        | 0.8581 ns        | 0.879 ns         | 0.7124 ns        |
| **opaque2**      |                  |                  |                  |                  |
| o2o2             | 28               | 32               | Na               | na               |
| O2o2             | 58               | 81               | Na               | na               |
| O2O2             | 33               | 37               | Na               | na               |
| $\chi^2$        | 0.4958           | 1.2933           | Na               | na               |
| p value          | 0.7804 ns        | 0.5238 ns        | Na               | na               |

ns: non-significant

Top row indicates the F2 populations derived from the respective crosses as mentioned; Genotyping was carried out by using o2-based marker umc1066 and o16-linked marker umc1149 in CML161 × QCL3024, CML533 × QCL3024, and CML537 × QCL3024 and umc1141 in CML193 × QCL3024. ns- non significant; na- not applicable

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CML533 × QCL3024 and CML537 × QCL3024, segregating only for o16, showed a similar degree of hardness among families O2O2/O16O16 and O2O2/o16o16 and also with the normal line CML543 (O2O2) requiring 426.45N to break its grain. The same for HKI193-1 (QPM-o2o2) and MGUQ-102 (full opaque-o2o2) was 301.46 and 188.19N, respectively (Table 4).

**Effect of o16 on organization of starch granules and proteinaceous matrix**

The morphological arrangement of the starch granules and proteinaceous matrix were compared among O2O2 (CML543), o2o2(MGUQ-102), o2o2-modified (HKI193-1), and o16o16 and o2o2/o16o16 F3 seeds. It revealed that the starch granules of normal line had an angular polygonal shape with proteinaceous matrix surrounding them, and characterized by a tightly packed structure with no air space (Fig 5A). But a significant reduction in the proteinaceous matrix adhering to the starch granules was observed in the soft endosperm line, MGUQ-102 (Fig 5B); the starch granules were loosely packed with relatively large intergranular space between starch granules. In HKI193-1, though the starch granules were spherical and smooth, a relatively more proteinaceous matrix adhered to the starch granules with lesser air space revealing a tighter interaction among the starch granules of seed endosperm (Fig 5C). The o16o16 line had more or less similar microscopic arrangement with that of a normal line with angular polygonal shape starch granules and air tight packed structure with proteinaceous matrix (Fig 5D). The structure of starch granules of the genotype o2o2/o16o16 (Fig 5E) was

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**Table 2. Average degree of opaqueness (%) in F2 seeds.**

| F2 populations | Parental genotypes | Opaqueness | 0% | 25% | 50% | 75% | 100% | Average (%) |
|----------------|--------------------|------------|----|-----|-----|-----|------|-------------|
| CML161 × QCL3024 | o2o2/O16O16 × O2O2/o16o16 | 67 | 4 | 7 | 0 | 22 | 26.09 |
| CML193 × QCL3024 | 66 | 0 | 5 | 11 | 18 | 28.98 |
| CML533 × QCL3024 | O2O2/O16O16 × O2O2/o16o16 | 91 | 5 | 2 | 0 | 0 | 2.25 |
| CML537 × QCL3024 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |

Hundred F2 seeds derived from selfed F1s of crosses mentioned in the left column were subjected to light box testing and scoring was done based on the degree of opacity.

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intermediate between o2o2 (Fig 5B) and o16o16 (Fig 5D), having semi-polygonal shape with spare proteinaceous matrix and less packed compared to o16o16.

**Effect of o16 on zein protein fractions**

The variation in zein protein profile among o2, o16 and wild type genotypes could be observed in Fig 6. The fully opaque-o2o2 (MGUQ-102) showed a considerable reduction in both 19- and 22-kDa α-zein. We could also observe a nearly two-fold increase in the expression of 16-, 27- and 50-kDa γ-zein in modified-o2o2 (QPM: HK193-1) compared to fully opaque o2o2-soft line, MGUQ-102. The o16o16 genotypes showed a very similar profile with that of the normal line, CML543 but with a slight reduction of 50-kDa γ-zein and 15-kDa β-zein. However, it showed a completely different pattern from MGUQ-102 with a higher level of expression in 19- and 22-kDa α-zein, but a similar expression of 27-kDa γ-zein. The zein profile of o2o2/o16o16 was unique with intermediate levels of 19- and 22-kDa α-zein as compared to o2o2-soft and o16o16. However, it possessed less 50-kDa γ-zein compared to o2o2-soft, and more levels of 15-kDa β-zein as found in o16o16. The 16- and 27-kDa γ-zein were similar to both o2o2-soft and o16o16 type.

**Discussion**

Recessive o2 gene-based SSR umc1066 confirmed the true hybridity of F1s with a perfect Mendelian segregation of 1:2:1 in F2 populations (p<0.05). It has been relied upon for genotyping individual plant positive for o2 allele in earlier studies of several breeding programme [11, 14]. o16 linked-SSR, umc1149 showed perfect segregation in CML161 × QCL3024, CML533 × QCL3024 and CML537 × QCL3024 but failed to do so in CML193 × QCL3024.

**Table 3. Average degree of opaqueness (%) of F3 seeds.**

| Population          | o2o2/o16o16 (%) | o2o2/O16O16 (%) | O2O2/o16o16 (%) | O2O2/O16O16 (%) |
|---------------------|----------------|----------------|----------------|----------------|
| CML161 × QCL3024   | 98.24          | 97.65          | 2.15           | 1.23           |
| CML193 × QCL3024   | 96.34          | 95.81          | 3.55           | 1.72           |
| CML533 × QCL3024   | NA             | NA             | 4.30           | 2.03           |
| CML537 × QCL3024   | NA             | NA             | 0.35           | 1.49           |

The F3 seeds derived from the F2 populations of crosses mentioned in the first column and their respective genotypes as mentioned in the top row were subjected for the light box testing and scoring was done based on the degree of opacity.

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However, *umc1141* showed a distinct polymorphism in CML193 × QCL3024 in 8% native PAGE and were therefore used for further genotyping. Yang et al. [23] and Zhang et al. [17] used *umc1141* for selecting the individuals possessing *o16* allele. The *o2* based *umc1066* and *o16*...

### Table 4. Force (N) required in breaking F₃ seeds.

| Populations           | Genotypes                   | Newton (N)   | p-value wrt to corresponding O2O2/O16O16 |
|-----------------------|------------------------------|--------------|-----------------------------------------|
| CML161 × QCL3024      | o2o2/o16o16                  | 213.65± 6.15 | 0.015<sup>s</sup>                       |
|                       | o2o2/O16O16                  | 267.85 ± 5.18| 0.002<sup>s</sup>                       |
|                       | O2O2/o16o16                  | 399.73± 20.45| 0.852<sup>ns</sup>                      |
|                       | O2O2/O16O16                  | 414.97± 20.11| na                                      |
| CML193 × QCL3024      | o2o2/o16o16                  | 205.52± 3.16 | 0.002<sup>s</sup>                       |
|                       | o2o2/O16O16                  | 246.96± 12.45| 0.005<sup>s</sup>                       |
|                       | O2O2/o16o16                  | 332.89± 11.45| 0.789<sup>ns</sup>                      |
|                       | O2O2/O16O16                  | 337.18± 9.69 | na                                      |
| CML533 × QCL3024      | O2O2/O16O16                  | 312.25± 30.24| 0.197<sup>ns</sup>                      |
|                       | O2O2/o16o16                  | 378.34± 41.43| na                                      |
| CML537 × QCL3024      | O2O2/O16O16                  | 372.98± 30.59| 0.787<sup>ns</sup>                      |
|                       | O2O2/o16o16                  | 423.12± 32.14| na                                      |
| CML543 (Normal)       | O2O2/O16O16                  | 426.45± 21.56| na                                      |
| MGUQ-102 (Full opaque)| o2o2/O16O16                  | 188.19 ± 13.33| na                                      |
| HKI193-1 (QPM)        | o2o2/O16O16                  | 301.06± 19.04| na                                      |
| **SE**                |                              | 21.18        | na                                      |

s: significant; ns: non-significant

Grain hardness analyses of F₃ seeds derived from the F₂ plants genotyped as mentioned in the middle column were carried out with the Texture Analyser. The force (N) required to break each grain were recorded. The last column indicates the mean force required to break seeds of the respective genotypes for each crosses.

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based umc1141 and umc1149SSR markers were successfully used in genotyping the present study’s F₂ populations, and in classifying the individual plants into different genotypic classes for further physico-biochemical studies.

Phenotypic screening of individual seed opacity under light box is the most convenient and efficient strategy for studying the endosperm modification. The significant degree of opacity in F₂ seeds of populations where both o₂ and o₁₆ were segregating and the non-significant in populations, where o₁₆ was segregating alone suggested that o₁₆ did not influence endosperm modification significantly as opposed to o₂ which induces various degree of endosperm opaqueness. The average opacity in the two o₂ and o₁₆ segregating F₂ populations (26.09% and 28.98%) is expected if o₂ alone is affecting the modification and segregating in the ratio of 3 vitreous/translucent: 1 opaque [24](Table 2). This was further confirmed through F₃ seed analyses where the F₂-derived o₁₆o₁₆ showed a negligible opacity and F₂-derived o₂o₂/o₁₆o₁₆

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**Fig 5. Microscopic view of protein bodies and starch granules arrangement under SEM.** (A) O₂O₂ genotype normal line, CML543 (B) o₂o₂-soft and opaque line, MGUQ-102 (C) o₂o₂-modified QPM, HK1193-1 (D) o₁₆o₁₆ genotype (opaque₁₆ line) (E) o₂o₂/o₁₆o₁₆ genotype (double mutant) (Yellow arrow: proteinaceous matrix spreading over the round starch granules).

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**Fig 6. SDS-PAGE analysis of components of zein proteins in o₂o₂-soft and opaque line, MGUQ-102 (1 & 2 lane); o₂o₂/o₁₆o₁₆ (3 & 4 lane); o₂o₂-modified QPM, HK1193-1 (5 & 6 lane); O₂O₂ genotype normal line, CML543 (7 & 8 lane) and different o₁₆o₁₆ lines (9–14 lane).** The profiling had been done with two replications for each genotype.

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showed full opacity of endosperm. Therefore, o16 alone possesses negligible effects (0.35–4.30% opaqueness) on inducing opaqueness. In contrast, Yang et al. [25] reported o16-based inbreds viz., QCL3024 and QCL3021 having opaque phenotype in endosperm, however, the extent of opaqueness has not been mentioned. Grain hardness corresponds the kernel density and determines the resistivity towards storage pests infestation and fungal infection [26, 27]. Similar hardness observed in O2O2/o16o16 and O2O2/O16O16 genotypes derived F3 seeds with wild type inbred (CML543) and more hardness than the o2o2(MGUQ-102) and o2o2/o16o16 segregants as well clearly demonstrated that o16 alone did not induce softness in the endosperm. However, the degree of softness in o2 genetic background is determined by the presence of modifier loci. In the case of o2o2/o16o16 and o2o2/O16O16, grains were almost entirely soft; much favourable modifiers may be absent in the genetic background. However, grains of QPM were much harder due to the presence of favourable modifier loci [8, 22]. The o16 therefore, did not have any negative impact on the endosperm hardness unlike o2 which generally inflicts softness in the kernel. This was also evident from the proportion of hard- (orange or yellow translucent portion) and soft- (white portion) endosperm in the grains of o2o2-soft, QPM, normal (O2O2) and o16o16 genotypes (Fig 4).

During desiccation of seeds, rough endoplasmic reticulum membranes break down exposing the zeins protein mixing with the other content of the cytoplasm. It acts as cementing glue thereby providing an airtight interaction with starch granules in normal vitreous seed endosperm in wild maize endosperm [13, 28]. Angular polygonal shape starch granules with surrounding proteinaceous matrix making them a tightly packed structure with no air space, similar to the normal maize endosperm, o16o16 exhibited a vitreous texture of endosperm. This also explained the similarity observed in the grain hardness of o16o16 genotypes with normal line, CML543. The compact protein bodies and its interaction with starch granules through amorphous, non-crystalline amylpectin molecules at the surface links starch granules together, and makes the packaging more compact and grain appearance as vitreous [12, 28]. In the case of soft and opaque endosperm line, MGUQ-102, the protein matrix was scanty owing to weak interaction with the starch granules, followed by the large intergranular space making the endosperm loosely packed. The opacity is due to the diffraction of light caused by the air spaces left due to loose packaging of protein and starch granules in the endosperm [13]. QPM seeds showed more vitreous and hard due to accumulation of o2 modifiers in the genetic background (Table 4) [28] and with more of proteinaceous matrix as compared to MGUQ-102. The compact packaging of starch and protein bodies in o16o16 thus conferred vitreous kernels, while the air space left due to weak interaction made the kernels of o2o2 and o2o2/o16o16 as soft and opaque.

SDS-PAGE was used to compare qualitatively and to some extent quantitatively as well for prolamin fraction in the lines [29]. Similar profile of o16o16 genotypes with the normal line further strengthens the finding of o16 having similar grain hardness and vitreous grain endosperm with the wild normal maize line, CML543. However, it showed a completely different pattern from o2o2-soft line with higher level of expression in 19- and 22-kDa α-zein, but similar expression of 27-kDa γ-zein. The zein profile of o2o2/o16o16 was unique with intermediate levels of 19- and 22-kDa α-zein as compared to o2o2-soft and o16o16. Considerable reduction in both 19- and 22- kDa α-zein in o2o2 individual had been observed in earlier studies [30]. Two-fold increase in the expression of 16-, 27- and 50-kDa γ-zein in modified-o2o2 has been identified as the major factor in endosperm modification [28]. Several studies demonstrated a positive relationship between the content of 27-kDa γ-zein and endosperm vitreousness [31]. Segal et al. [32] induced a full opaque kernel phenotype by silencing the 22-kDa α-zeins by RNAi, while the overproduction of 27-kDa γ-zein enhanced protein body number resulting with more vitreous phenotype in QPM [33]. The disulfide bonds of cystein residues in γ-zein
helps in extensive cross-linking and covalent linkage between protein bodies could provide a mechanism for cementing protein bodies around starch grains [34].

The findings here thus establish that the mechanism of higher synthesis of lysine and tryptophan in o16 mutant is entirely different from the o2. The higher accumulation of lysine and tryptophan might be due to regulation of genes operating in amino acid biosynthesis pathway, or other unknown mechanisms. O2 located on chromosome 7 codes for a DNA binding protein belonging to basic leucine zipper class of transcriptional factors, and acts as transcriptional activator of 19- and 22-kDa α-zein genes [35, 36]. The mutant o2-based protein induces an overall reduction of 50–70% in zein protein which increases non-zein proteins proportionally, resulting in an increase of lysine content twice than that in normal maize [37]. The mechanism behind the enhanced nutritional value of o16 needs further investigation since zein profile of o16o16 differs considerably from o2o2. It is worth mentioning that among the various discovered high lysine mutants, only o2, fl2 and Def-B30 affect different aspects of storage protein synthesis and alter zein content and compositions [38]. The other mutants such as o5, o15, fl1, Mc do not induce significant changes in zein content and composition suggesting that additional factors are also important in determining the kernel texture [39]. The o15 mutation exerts its effect primarily on the 27-kDa γ-zeins [40]. The fl1 mutation is rather resulted due to abnormal placement of α-zeins within the protein bodies. Fl1 encodes a transmembrane protein that is located in the protein body ER membrane [41]. Similarly, o5 mutant phenotype is caused by a reduction in the galactolipid content of the maize endosperm, with no change in zein proteins [42].

The novel high lysine and tryptophan mutant o16 thus possessed no adverse effect on the endosperm modification. The recessive o16 alone improves the nutritional quality of maize and can be utilized as effectively as o2 [15]. Thus, it holds a significant promise in quality breeding programme. QPM breeding programme has traditionally used o2 coupled with modifier for enhancement of lysine and tryptophan. However, the challenge remains in accumulation of favourable modifiers in o2 genetic background to impart kernel hardness [8, 22]. Since the o16o16 genotypes possessed vitreous endosperm and equivalent grain hardness to normal line, the mutant provides a tremendous advantage to the breeders as accumulation of modifiers in the genetic background need not be looked into while breeding for high lysine and tryptophan. The pyramided genotype o2o2/o16o16 has higher lysine and tryptophan over o2o2 alone [14]. So in this case of double mutant combination, accumulation of modifier loci would remain the challenge during the line development. However, several QTLs for these modifiers have recently been identified and diverse set of QPM inbreds have been characterized using SSRs linked those loci. Availability of SSRs associated with o2, o16 and QTLs linked to modifier loci provide great opportunity to undertake marker-assisted selection to develop high lysine and tryptophan maize with hard endosperm; it can be further used to fine map the o16 locus, and through chromosome walking the sequence of o16 can be derived. Besides, gene silencing approach may also lead to the cloning and characterization of the o16 locus. Though in the present study, o16 was not characterized at sequence and transcript/polypeptide level, the information generated here on its effect on kernel attributes are of paramount importance in QPM breeding programme. This is first ever study reported on the effect of o16 on kernel hardness, zein protein profiles and microscopic arrangement of starch granules with proteinaceous matrix.

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