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RESEARCH ARTICLE

Elucidation of zein isoforms associated with high protein quality traits for targeted improvement in maize-based nutrition

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Abstract

Background and Objectives: Zein proteins of maize endosperm are nutritionally poor, but important for vitreous kernel texture. The *Opaque-2* mutation enhances the protein quality but downregulates zein expression, distorting the kernel texture. Quality protein maize (QPM) is nutritionally improved, hard endosperm maize developed by introgression of endosperm modifiers under an *opaque-2* background. The present study aims to analyze the variability in zein expression patterns in normal, *opaque-2*, and QPM lines through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis analyses.

Findings: Results revealed that the total number of zein isoforms is almost similar in *opaque-2*, normal, and QPM lines. Overall, in this study, it is concluded that the *opaque-2* mutation specifically affects the expression of high-molecular-weight zeins, which are being counterbalanced by the expression of low-molecular-weight zein isoforms and nonzein proteins.

Conclusion: Genomic data retrieval studies revealed the possible presence of multiple 27 kDa isoforms with varied isoelectric points (pI), which is a subject for further investigation. The differential expression of 15 kDa zein in QPM emerged as a novel player in endosperm modification.

Significance and Novelty: The present study provides insight into the diversified expression of zeins in different maize types. The variable isoform expression has the potential to generate stable QPM lines by targeting effective isoforms, ensuring endosperm modification without hampering the nutritional quality.

KEYWORDS
2D-PAGE, nonzein, opaque-2, protein isoforms, QPM, zein

1 | INTRODUCTION

The introduction of quality protein maize (QPM), which boosted the maize quality by combining the dual benefits of nutritional quality and superior agronomic performance, revolutionized the maize breeding program. Maize grain consists of four major physical structures: the endosperm, the germ (embryo), the outer seed coat (pericarp), and the tip cap. The endosperm constitutes 80%–85%, followed by the germ, which constitutes 9%–10%, and the pericarp (5%–6%) of maize kernel. The endosperm is composed of 70% starch and 8%–10% protein and has a relatively low fat content (Li et al., 2014; Prasanna et al., 2001). Endosperm protein is composed of two major fractions: zeins (nutritionally poor) and nonzeins (nutritionally rich). Zeins contain a small number of essential amino acids such as threonine (3%), histidine, and cysteine (1%), (0.9%), and methionine lysine (0.1%), but are devoid of tryptophan (Trp), whereas nonzeins have a balanced proportion of essential amino acids, and so has superior protein quality (Wu & Messing, 2014).

Zein and nonzein fractions can be separated depending on solubility. Albumin (water-soluble) (3%), globulin (salt soluble) (3%), and glutelin (alkali-soluble) (34%) together represent the nonzein fraction, whereas prolamin and prolamin-like fractions (alcohol soluble) (60%) represent the zein fraction (Gibbon & Larkins, 2005). Zeins, the most abundant class of endosperm protein, are further classified into α- (22 and 19 kDa), β-(15 kDa), γ- (50, 27, and 16 kDa), and δ- (10 and 18 kDa) zeins (Coleman & Larkins, 1999; Wu et al., 2010). The categorization of zeins is based on differences in solubility, molecular weight, ability to form disulfide interactions, and the sequence of their coding genes (Coleman & Larkins, 1999; Holding & Messing, 2013; Wu et al., 2010). β-, γ-, and δ- zeins have a high proportion of sulfur-rich amino acids. δ-zeins are very rich in methionine, whereas γ-zeins are abundant in cysteine; β-zein has high percentages of cysteine and methionine, while α-zeins lack both (Wu et al., 2012). The most abundant zein is α-zein, which accounts for 70% of all zein proteins. Gene families *Z1A*, *Z1B*, and *Z1D* encode 19-kDa α-zeins, whereas the Z1C subfamily encodes 22-kDa α-zeins (Feng et al., 2009; Song et al., 2011). The *2β15* gene encodes the 15 kDa β-zeins. The 50, 27, and 16 kDa γ-zeins are encoded by *z2γ50*, *z2γ27*, and *z2γ16*, respectively. The *z2δ10* and *z2δ18* genes, respectively, encode 10 and 18-kDa δ-zeins (Xu & Messing, 2008). Several gene families in multicopy forms are involved in the synthesis of variable zein isoforms, and these gene families have undergone several duplication events during the course of evolution. The zein genes were reported to have syntenic alignments with gene sequences with other cereals like sorghum, rice, wheat, and barley, showing the co-linearity of cereal genomes at prolamin loci (Xu & Messing, 2008). Among all zeins, α- and γ-zein have been extensively amplified during the course of evolution, having multiple isoforms under different genetic backgrounds (Xu & Messing, 2008).

Zeins are the major endosperm storage proteins, synthesized within 10–45 days after pollination (DAP) (Li et al., 2014). Membrane-bound polyribosome is the site for the synthesis of zein proteins, which are further translocated to the lumen of the endoplasmic reticulum, where they assemble to form the protein bodies (Khoo & Wolf, 1970; Larkins & Hurkman, 1978). Among different zein isoforms, γ-zein initiates the formation of protein bodies, followed by β-zein (Lending & Larkins, 1989; Woo et al., 2001). In QPM, 27 kDa γ-zein facilitates protein-body formation, and its compaction with starch granules is essential for endosperm modification (Salazar-Salas et al., 2014). γ-zein forms a periphery in a mature protein body, with an intermittent layer of 22 kDa α-zeins and the most abundant 19 kDa α-zeins in the center (Kim et al., 2002; Lending & Larkins, 1989). Zein accumulation in the proper stoichiometric ratio is necessary for its proper functionality (Guo et al., 2013).

Zein synthesis is affected by several mutations that confer an opaque phenotype to maize kernel; of these, the most widely studied *opaque-2* mutation reduces 50%–70% of the total zein content as compared to normal maize lines. The *O2* gene is located on the short arm of chromosome 7; it encodes for the *bZIP* transcription factor, 47–48 kDa protein, which binds to the target sequence by its leucine zipper motif (Hartings et al., 1989; Holding, 2014). The direct effect of *opaque-2* on gene transcription was demonstrated on several zein isoforms (Huang et al., 2006; Schmidt et al., 1992). The mutant *opaque-2* lines are reported to have double the content of two essential amino acids, that is, lysine (Lys) and Trp than the wild type by decreasing the zein content and subsequently increasing nonzein counterparts (Jia et al., 2013). It is observed that in *opaque-2* mutants, the contents of 22 and 19 kDa α-zein decrease considerably, which disrupts the overall protein body shape and leads to a chalky endosperm appearance (Guo et al., 2013). Ultimately, zein determines the biophysical properties of mature endosperm tissue, such as kernel vitreousness (Holding, 2014), which is a key agronomic trait since it protects against damage during harvesting and marketing as well as insect and fungal damage. The improved *opaque-2* lines with vitreous kernel textures are known as QPM. The mechanism by which the conversion of a soft kernel type into a vitreous texture occurs is poorly understood. Some studies have demonstrated that 27 kDa

γ-zein is positively correlated with the hard kernel texture of QPM genotypes (Holding et al., 2007; Song & Messing, 2002). It is postulated that increased 27 kDa zeins lead to an increase in the number of protein bodies and their compaction with starch granules.

Numerous zein isoforms have been identified using two-dimensional (2D)-electrophoresis, indicating that zein isoform expression under varied genetic backgrounds of maize is highly heterogeneous (Consoli & Damerval, 2001). Detailed information on the expression of the proteins involved in endosperm modification is not available. Given this, the present study was carried out to analyze the variation in zein isoform expression among stable germplasms of normal, *opaque-2*, and QPM through 2D-polyacrylamide gel electrophoresis (PAGE) to precisely understand the variation in zein isoform expression among different maize types. The present study aimed to focus on different zein isoforms and to elucidate their probable role in protein body formation in mature maize kernels.

2 | MATERIALS AND METHODS

2.1 | Plant material

The normal maize inbred HKI 1105 (N), *opaque-2* endosperm mutant DQL 1001, and QPM line HKI 1105 (QPM) with a modified kernel texture were procured from ICAR-IARI, New Delhi. The endosperm mutant's genotype was converted into the HKI 1105 background through six backcrossing cycles, followed by several rounds of self-pollination in a controlled field experiment. The lines selected were phenotypically uniform and appeared genetically homogeneous. Experimental lines were grown in the fields of ICAR-IIMR, Ludhiana, during Kharif, 2017. It should be noted that all methods were carried out in accordance with relevant institutional, national, and international guidelines and legislation. At physiological maturity, a minimum of eight well-filled ears of each genotype were sampled. An equal number of dissected endosperms from eight ears was pooled and treated as one sample to reduce the effect of biological variations between ears on gene expression.

2.2 | Sample processing

Samples were screened under a light box to differentiate samples based on the degree of opaqueness varying from 0% to 100%, with modification scores of 1–5 (N. Tandzi et al, 2017). Maize kernels of normal genotype are transparent and show 0% opaqueness, that is, modification score 1, whereas *opaque-2* mutants show 100% opaqueness, with modification score 5. Endosperm modifiers expressed in QPM lines tend to reduce opaqueness from 100%, 75%, 50%, and 25% to 0% with modification scores 5, 4, 3, 2, and 1, respectively. Then, screening was performed, and specific gravity was estimated. Kernels in triplicate were soaked in distilled water, and the pericarp and the embryo were removed. Endosperm collected was dried at room temperature and ground through a 100-mesh, and flour was defatted with petroleum ether (40–60°C).

2.3 | Estimation of physiological and biochemical parameters

Various physiological parameters including hundred kernel weight, grain yield potential, and moisture content were recorded for three samples in triplicate for two subsequent years. Concomitantly, biochemical parameters including total protein, zein content, nonzein content, and prolamin-like content were also recorded for 2 years in three replicates. The procedure for all the physiological and biochemical parameters mentioned above was followed according to previous studies (Sethi, Kaur, et al., 2020; Sethi, Kumar, et al., 2020).

2.4 | Zein isolation

Zein fractions were isolated from the finely grounded defatted sample using the previously described method (Chen et al., 2014), with certain modifications. Processed samples were mixed with zein extraction buffer (70% ethanol + 2% β-mercaptoethanol) and incubated at room temperature with intermittent shaking for 8 h. Samples were centrifuged for 15 min at 12,000 rpm at room temperature. The supernatant was collected as a zein fraction and concentrated using the trichloroacetic acid (TCA)-acetone precipitation method. One volume of extracted sample was mixed with four volumes of ice-chilled TCA-acetone (10% TCA in acetone and 20 mM dithiothreitol [DTT]). Samples were properly mixed by inverting tubes and kept at −20°C overnight, followed by centrifugation at 8000 rpm for 20 min. The supernatant was discarded, and the pellet was washed two times using acetone and DTT mixture to remove TCA from the precipitated sample. The pellet was air-dried at room temperature. The pellet was dissolved in 200 μL of rehydration buffer. The rehydration buffer used for the present experiment was a strong chaotropic buffer with 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, and 0.2% BioLite (detergent). DTT and BioLite were added immediately before rehydration of pH strips.

2.5 | Sodium dodecyl (SDS)-PAGE analysis

Zeins were separated by SDS-PAGE before 2D-PAGE analysis. Isolated zeins were dried in a concentrator and then suspended in 200 μL of IPG solution buffer (8 mol/L urea, 2% CHAPS). Protein quantification was performed using Lowry's method (Lowry et al., 1951), and estimated protein was subjected to SDS-PAGE analysis using 0.15 mg/mL polyacrylamide gels (acrylamide:bisacrylamide 29:1, w/w) and stained with Coomassie Blue R250 as described by Walker (2009).

2.6 | Zein quantification

Zein proteins dissolved in the rehydration buffer are difficult to quantify due to the presence of detergent and reductant in the rehydration buffer. The 2D Quant kit was used for accurate quantification of protein samples prepared for 2D electrophoresis. For the present experiment, a 13 cm pH strip with a 3–10 pH range and passive mode of rehydration was used. Staining was performed with Coomassie Brilliant Blue; 250 μL of the sample with 150 μg of protein concentration was used. For rehydration, the sample was spread on a strip holder and the strip was placed gel side down and covered with the cover fluid or mineral oil. Rehydration was performed for 12–14 h.

2.7 | 2D-PAGE

Isoelectric Focusing is an electrophoretic method that separates proteins according to their isoelectric points (pI). Rehydrated IPG strips were placed on a strip holder along with small paper wicks at the terminal to absorb the proteins that are beyond the pH range of interest. Mineral oil or cover fluid was added on top to prevent drying out of the strip and crystallization of the urea/thiourea present. For improved sample separation, the voltage was increased step by step as per the program (100 V-1 h, 500 V-1 h, 1000 V-30 min, 3000 V-1 h, 8000 V gradient-30 kVh, 8000 V-12 kVh, and 300 V-12 h).

After isoelectric focusing, the strips were equilibrated first with a reductant (DTT) and then an alkylating agent (iodoacetamide). Equilibrated strips from the first dimension were subjected to the second dimension. For the second dimension, 15% SDS-PAGE gels were prepared. Along with IPG strips, the molecular marker was also subjected to SDS-PAGE on one terminal of a gel by forming a single well. The agarose sealing mixture, along with bromophenol blue (BPB), was added to the IPG to seal the IPG strip with PAGE gel, and BPB was the dye front. Agarose sealing mixture contained 1X TAE Buffer (50 mL), agarose (0.5 g), and 1% BPB. Proteins were separated at a constant voltage, that is, 120 V in the second dimension.

2.8 | Gel staining and image scanning

Coomassie Brilliant Blue was used for staining. Colloidal staining methods were recommended, because they show the highest sensitivity, down to 100 ng/protein spot. The gel was fixed by placing it in a solution of 10% acetic acid, 10% methanol, and 40% ethanol for 1 h, followed by staining-I (1% acetic acid and 10% ammonium sulfate) for 2 h, then staining-II (5% acetic acid, 45% methanol, and 0.1% CBB R-250) for 4–5 h, and the last step was destaining (5% acetic acid and 45% methanol) until the background of the gel was white. Images of destained gel were obtained using an Amersham imager 600, and results were evaluated for three independent replicates of each sample using Image Master™ 2D Platinum software, which gave detailed descriptions regarding several spots in a gel and density of spot representing the quantity of particular protein isoform in the sample.

2.9 | Statistical analysis

All experiments included three technical replicates, and data represent the average value of repeated-measures analysis of descriptive statistics. Analysis of variance (ANOVA) of different experimental genotypes was carried out using SPSS software. The data presented in Supporting Information: Table S1 are reported at a 5% significant level. Analysis of protein through 2D-PAGE in triplicate was carried out by background subtraction and spot detection. Spots were matched and normalized using the total density index in the gel image, and proteins with a change of 1.5 folds were considered differentially accumulated.

2.10 | Bioinformatics analysis

To determine the amino acid composition of each zein, information regarding each zein isoform reported in NCBI was collected, followed by a calculation of the percentage of each amino acid in a particular peptide sequence using the Expasy ProtParam tool. The peptide sequence of 15 kDa zein obtained from the Expasy ProtParam tool was fed to string-dbt.org to analyze the functional partners of 15 kDa zein.

2.11 | Data statement

All data generated and analyzed during this study are included in this published article and Supporting Information files.

3 | RESULTS

3.1 | Analysis of physiological and morphological traits

Samples HKI 1105 N, DQL1001, and HKI 1105, representing normal, *opaque-2*, and QPM lines, respectively, were processed and analyzed in terms of various physiological and biochemical parameters as shown in Supporting Information: Table S1. The modification score represents the degree of opaqueness, namely, 1 for normal lines (0% opaqueness), 5 (100%) for *opaque-2*, and 2 (25%) for QPM lines (Tandzi et al., 2017). Similarly, the moisture content varied from 12.5% to 26.7% among normal, *opaque-2*, and QPM lines, with the least moisture content in QPM and the maximum moisture content in *opaque-2* (Sethi, Kaur, et al., 2020). Specific gravity, 100 kernel weight, and yield potential were the highest in normal, intermediate in QPM, and the lowest in *opaque-2* lines.

The total protein content among normal, *opaque-2*, and QPM lines was 14.05%, 13.43%, and 12.42%, respectively (Sethi, Kumar, et al., 2020). Zein content was the highest in normal lines (51.31%), intermediate in QPM (28.08%), and the lowest in *opaque-2* (19.5%), whereas nonzein content was the highest in *opaque-2* (75.73%), intermediate in QPM (70.35%), and the lowest in normal lines (45.11%). As reported earlier, prolamin-like content is known to contribute to endosperm modification in QPM as the maximum prolamin-like content was accumulated in QPM (18.04%), intermediate in normal (11.14%), and the least in *opaque-2* (10.85%) (Sethi et al., 2021). On the basis of the biochemical and physiological parameters, the three lines under consideration show variability in terms of texture, zein content, and nonzein content, which was further examined by SDS-PAGE and 2D-PAGE.

3.2 | Variability in zein accumulation patterns among different maize types by SDS-PAGE

To analyze the difference between zein accumulation patterns in normal, *opaque-2*, and QPM lines, zein proteins were extracted from the total protein fraction and separated on SDS-PAGE, as shown in Supporting Information: Figure S1. Results revealed that the zein content was the highest in 19 and 22 kDa α-zeins in normal lines, whereas a low zein content was found for the *opaque-2* line. *Opaque-2* mutants were observed to have intense bands of low-molecular-weight zeins (16, 15, and 10 kDa) as compared with normal maize. QPM has a similar banding pattern of zein fraction as that of *opaque-2*, with the exception that 27 kDa γ-zeins are maximally expressed in QPM lines and are considered to play a role in retaining the vitreous endosperm.

3.3 | Difference in zein isoform expression among normal, *opaque-2*, and QPM lines

2D-PAGE analysis of zein protein extracted from normal, *opaque-2*, and QPM endosperm was performed to analyze the zein isoforms affected by the *opaque-2* mutation and endosperm modifiers. The analysis of zein proteins extracted from normal lines shows that most of the protein spots were in 19 and 22 kDa α-zein as shown in (Supporting Information: Figure S2A). The 3D image of the protein spots separated from zein extraction of the normal genotype is shown in Supporting Information: Figure S2B. Information on different zein isoforms present in normal lines along with protein percentage is presented in Supporting Information: Table S2, showing that two isoforms of 27 kDa γ-zein, five isoforms of 22 kDa α-zein, seven isoforms of 19 kDa α-zein, one isoform of 16 kDa γ-zein, one isoform of 15 kDa β-zein, and two isoforms of 10 kDa δ-zein were present in normal lines. The 2D-PAGE analysis of zein proteins extracted from the *opaque-2* genotype reveals that most of the protein spots were present in low-molecular-weight zein (Supporting Information: Figure S3A). In addition, a drastic decrease was observed in the 22 and 19 kDa α-zeins in *opaque-2* mutants. The 3D image of the protein spots separated from zein extraction of the *opaque-2* genotype is shown in Supporting Information: Figure S3B. The protein percentages of different zein isoforms expressed in *opaque-2* lines are presented in Supporting Information: Table S3, showing that the *opaque-2* line contains one isoform of 22 kDa α-zein, three isoforms of 19 kDa α-zein, four isoforms of 16 kDa γ-zein, five isoforms of 15 kDa β-zein, and four isoforms of 10 kDa δ-zein.

QPM expressed a similar pattern of zein expression as that of *opaque-2* lines, as shown in Supporting Information: Figure S4A,B. The expression pattern of different zein isoforms is presented in Supporting Information: Table S4, which shows that QPM has

two isoforms of 22 kDa α-zein, three isoforms of 19 kDa α-zein, four isoforms of 16 kDa γ-zein, six isoforms of 15 kDa β-zein, and two isoforms of 10 kDa δ-zein. The comparison of various zein isoforms in normal, *opaque-2*, and QPM lines is presented in Table 1 and Figure 1, which shows the overall percentage of zein expression in different maize types. Normal lines showed 57.5% of 19 kDa α-zein and 28.78% of 22 kDa α-zein, whereas low-molecular-weight zeins showed 2.21% of 16 kDa γ-zein, 1.19% of 15 kDa β-zein, and 1.05% of 10 kDa δ- zein. This indicates that out of the total zeins expressed in normal lines, almost 85% were α-zeins, whereas a small percentage of low-molecular-weight zeins were present in normal maize.

TABLE 1 Comparison of zein isoforms and their protein content in normal and *opaque-2* lines.

Zein isoforms (kDa)	Normal lines (zein isoform & protein percentage)	*Opaque-2* lines (zein isoform & protein percentage)	QPM (zein isoform & protein percentage)
27	2 (1.49)	0	0
22	5 (28.75%)	1 (4.99%)	2 (3.54%)
19	7 (57.5%)	3 (19%)	3 (14.18%)
16	1 (2.25%)	4 (48.55%)	4 (32.99%)
15	1 (1.19%)	5 (17.37%)	6 (31.16%)
10	2 (1.04%)	4 (13.97%)	2 (4.7%)

Abbreviation: QPM, quality protein maize.

Contrasting results for zein expression were observed in *opaque-2* and QPM lines compared with the normal line. In the *opaque-2* line, reduced expression of 22 kDa (4.99%) and 19 kDa (19%) α-zein was observed, whereas higher contents of low-molecular-weight zeins, that is, 16 kDa γ-zeins (43.85%), 15 kDa β-zeins (17.37%), and 10 kDa δ-zein (13.97%), were observed. Similarly, the QPM line had a low expression of 22 kDa (3.54%) and 19 kDa (13.55%) α-zeins and a high content of 16 kDa γ-zein (32.99%), 15 kDa β-zein (31.16%), and 10 kDa δ-zeins (4.7%), although 27 kDa isoforms were not separated in 2D-PAGE in the pH range 3–10, which resulted in an intense band on SDS-PAGE (Supporting Information: Figure S1) analysis for QPM. This indicates that 27 kDa γ-zeins have pI beyond pH 10 and, therefore, separation did not occur on 2D-PAGE. These results are contrary to those of a previous report, which revealed that a maximum of 27 kDa γ-zein isoforms fall in the range of pI 6–11 (Salazar-Salas et al., 2014).

3.4 | Genomic data mining for variability in 27 kDa expression in different genetic backgrounds

Details regarding the differential expression of 27 kDa γ-zein were extracted from the databases MaizeGDB and Gramene, and it was found that the *zp27* gene (Id:ZEAMMB73_Zm0000id020592) encodes the 27 kDa γ-zein protein. The polypeptide sequence of this gene codes for a proline-rich protein with approximately

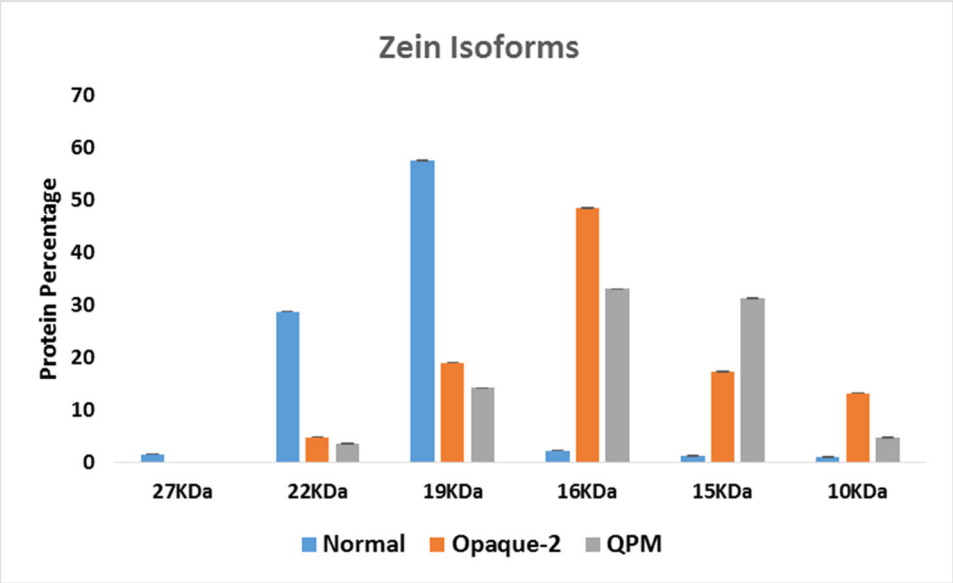

FIGURE 1 Zein isoform variation among normal, *opaque-2*, and quality protein maize (QPM) lines. [Color figure can be viewed at wileyonlinelibrary.com]

198 amino acids, pI 8.40, and 23688.67 Da molecular weight. The phenotypic outcome of the 27 kDa gene is associated with glassy and hard endosperm due to the accumulation of a high amount of 27 kDa zein protein. The expression pattern of 27 kDa γ-zein in terms of developmental stage and tissue suggests that this gene is highly expressed in the endosperm region of the gene, particularly showing high expression during 16–24 DAP. Allelic variations or polymorphism in terms of 27 kDa zein expression revealed from an analysis of gene clusters and QTLs associated with 27 kDa γ-zein showed that four gene clusters (A188-X56117, X53514, X56117, and X56118) are associated with 27 kDa γ-zein expression. It is reported that a tandem array of genes encoding these zeins is located on chromosome 7, which indicates that multiple isoforms may exist with differential pI expressed in different genetic backgrounds. In addition, a QTL qy27 is reported to be located on the long arm of chromosome 7 that is associated with enhanced expression of 27 kDa γ-zeins, which is closely related to the above-mentioned *zp27* cluster. It is also reported that this gene has one splice variant, two orthologues, and one paralogue. Therefore, this variability in terms of the 27 kDa γ-zein coding region suggests that zein isoforms and endosperm modifiers have a broad range, and their expression differs in different genetic backgrounds. Further studies are needed for in-depth analyses of isoforms involved in endosperm modification in the QPM line. In addition, on comparing *opaque-2* and QPM, it was observed that 15 kDa (β) zeins are expressed more in QPM, whereas 16 kDa (γ), and 10 kDa (δ) zeins are expressed more in *opaque-2* as compared with QPM lines (Consoli & Damerval, 2001). This indicates that 15 kDa β-zein contributes to endosperm modification of QPM lines along with 27 kDa γ-zein (Consoli & Damerval, 2001). Accumulation of 19 kDa (α) zein was more in *opaque-2* lines as compared to QPM lines, indicating that 19 kDa zein did not contribute to endosperm modification.

3.5 | Bioinformatic analysis for estimation of essential amino acid percentage in different zeins

The effect of changing zein isoform expressions among normal, *opaque-2*, and QPM lines was revealed by mining the peptide sequence of each zein. Results revealed that (Table 2) the high-molecular-weight zeins (19, 22 kDa) have a low percentage of Lys (0.4), Trp (0.09), and Met (1.23) as compared to low-molecular-weight zeins, including 10 (δ), 15 (β), and 16 kDa (γ) zeins, which have a higher percentage of Lys (0.8), Trp (0.24), and Met (11.03), whereas other essential amino acids are present more in high-molecular-weight zeins as compared to low-molecular-weight zeins. A higher percentage of leucine and isoleucine in high-molecular-weight zeins negatively affects Trp accumulation, which decreases the overall quality of high-molecular-weight zeins (19, 22 kDa). In terms of conditionally essential amino acids, except for glutamine (Gln), all are present at a higher percentage in low-molecular-weight zeins as compared to high-molecular-weight zeins. Also, all nonessential amino acids except for glutamic acid (Glu) are reported to be found at a higher percentage in high-molecular-weight zeins as compared to low-molecular-weight zeins. Therefore, the overall amino acid composition of different reported zein isoforms shows that low-molecular-weight zeins accumulate nutritionally favorable amino acids as compared to high-molecular-weight zeins. Hence, the accumulation of high-molecular-weight zeins in normal maize decreases its nutritional quality in comparison to low-molecular-weight zeins, which are expressed more under *opaque-2* and QPM backgrounds.

3.6 | Proteomic analysis for identification of functionally associated proteins with 15 kDa zeins

As 15 kDa zein emerged as one of the novel players in endosperm modification, its interacting proteins were elucidated computationally using STRING program. 15 kDa zein has functional associations with 10 other proteins expressed in maize endosperm (Figure 2). Among these 10 proteins, six are zeins, including 18, 16, 50, 19, and 22 kDa zeins. All these zeins contribute toward protein body formation and retaining the vitreous endosperm texture. In addition, four other proteins, including trypsin inhibitor protein, granule-bound starch synthase-1, Hsp interacting protein, and *opaque-1* myosin motor protein, showed functional interaction with 15 kDa zeins. *Opaque-1* myosin motor protein is essential for endoplasmic reticulum and protein-body interactions. *Opaque-1* maize mutants have an opaque endosperm texture, but the zein content is equivalent to that of normal maize. Electron microscopy studies revealed that *opaque-1* mutants have a dilated endoplasmic reticulum along with smaller irregular protein bodies (Wang et al., 2012). There is an association between O1 protein and endoplasmic reticulum through heat shock interacting protein, which plays a major role in protein body biogenesis (Wang et al., 2012). *Opaque-1* and 15 kDa protein have a 0.68 homology score established by text mining. Also, Hsp interacting protein is associated with 15 kDa zein as established by text mining (Homology Score 0.68). Hsp interacting protein acts as a co-chaperone for Hsp 90, which is required for folding of the myosin motor

TABLE 2 Average amino acid percentage in low-molecular-weight zeins (10, 15, and 16 kDa) and high-molecular-weight zeins (19 and 22 kDa).

S. No.	Categories of amino acids	Amino acids	Average % amino acid in 10, 15, and 16 kDa zeins isoforms	Average % amino acids in 19, 22 kDazeins isoforms
1	Essential amino acids	His (H)	0.66	0.93
2		Ile (I)	1.46	4.47
3		Leu (L)	11.36	19.00
4		Lys (K)	0.80	0.40
5		Trp (W)	0.24	0.09
6		Met (M)	11.03	1.23
7		Phe (F)	2.23	5.27
8		The (T)	3.04	3.19
9		Val (V)	3.83	4.71
10	Conditionally essential amino acids	Arg (R)	1.77	1.16
11		Gly (G)	6.60	1.70
12		Pro (P)	11.09	8.68
13		Tyr (Y)	4.83	3.33
14		Cys (C)	4.87	0.82
15		Gln (Q)	13.84	18.39
16	Nonessential amino acids	Ala (A)	12.61	14.42
17		Asn (N)	1.46	4.67
18		Asp (D)	0.46	0.29
19		Glu (E)	1.30	0.46
20		Ser (S)	6.20	6.57

domain. These myosin motor proteins are further required for the biogenesis of storage proteins in maize endosperm (Morton et al., 2016).

The Trypsin/factor XIIA inhibitor is co-expressed with 15 kDa zein with a homology score of 0.78. Trypsin inhibitors restrict the proteolytic action of several proteases, including mammalian trypsin, serine protease coagulation factor XIIA, and proteases acting on different protein fractions, including albumin and zeins. Trypsin inhibitor also inhibits the action of alpha-amylase in maize (Reed & Penner, 1978). Trypsin proteases act on the storage protein and lead to its breakdown, whereas trypsin inhibitors inhibit the proteases' action and help in retaining storage protein and starch matrix essential for vitreous maize kernel formation. Trypsin inhibitor co-expressed with 15 kDa plays a potential role in retaining endosperm texture.

Granule-bound starch synthase-1 (GBSS-1) is co-expressed with 15 kDa protein with a homology score of 0.77. GBSS-1 is required for the synthesis of amylose in the endosperm. Proteomic studies revealed that the GBSS-1 level is increased in QPM lines as compared to normal lines (Figure 2). GBSS-1 plays a role in restoring vitreous kernel texture by increasing starch granule accumulation and its cohesion to form a regular matrix (Nalukenge et al., 2013). 15 kDa functional association with other proteins reveals that these proteins are essentially involved in vitreous kernel formation, implicating their potential role as endosperm modifiers.

4 | DISCUSSION

Physiological traits of normal, *opaque-2*, and QPM lines were studied, and it was reported that normal lines have high yield potential, 100 kernel weight, and specific gravity with 0% opaqueness, whereas the *opaque-2* mutation leads to 100% opaqueness, which distorts the starch protein matrix and subsequently increases the moisture content (Sethi et al., 2020); however, 100 kernel

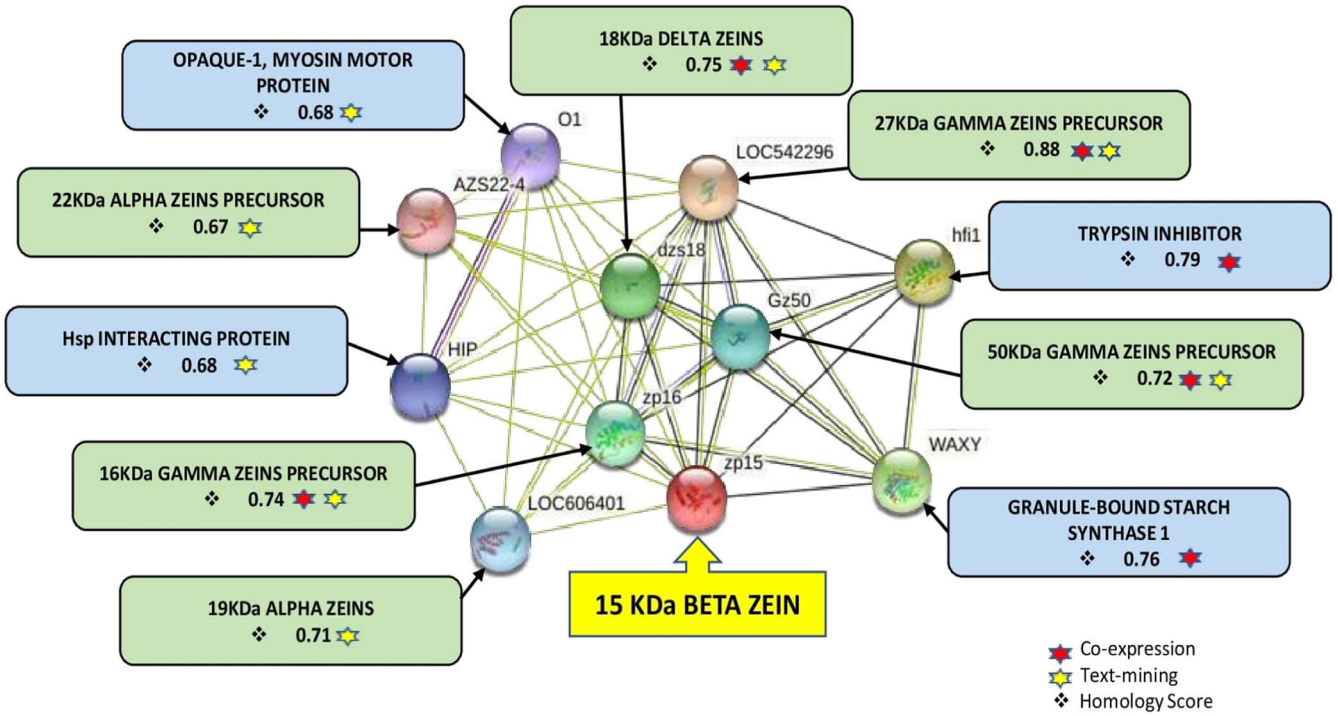

FIGURE 2 Functional partners of 15 kDa zein and their role in endosperm modification. [Color figure can be viewed at wileyonlinelibrary.com]

weight and specific gravity decrease in *opaque-2* lines and the *opaque-2* mutation is also known to negatively influence the yield potential. In QPM, introgression of endosperm modifiers leads to retention of the vitreous kernel texture and moisture content decreases, whereas 100 kernel weight, specific gravity, and yield potential are higher than those of *opaque-2*. Retention of less moisture content by QPM lines will lead to tolerance to stored grain insect pests. Also, three lines, including normal (HKI-1105N), *opaque-2* (DQI-1001), and QPM (HKI-1105) showing maximum variability for protein quality were selected for 2D-PAGE analysis (Sethi et al., 2020, 2021). Before 2D-PAGE analysis, the total zeins isolated were separated by SDS-PAGE.

Overall, normal lines expressed intense bands for 19, 22 kDa zeins and *opaque-2* mutants were observed to have intense bands of low-molecular-weight zeins (16, 15, and 10 kDa) as compared to normal maize, which indicates that the *opaque-2* mutation specifically affects α-zein accumulation (Schmidt et al., 1990). The QPM line showed a similar banding pattern as that of the *opaque-2* line, with comparatively high expression of 27 kDa γ-zeins, which is expected to contribute to retaining the vitreous endosperm in QPM lines. Similar results were reported previously, which proved that QPM endosperm accumulates 27 kDa γ-zein at two- to threefold higher levels than in the wild type and *o2* (Geetha et al., 1991). Lopes and Larkins (1991) reported that the degree of QPM endosperm vitreousness is closely correlated with the level of 27 kDa γ-zein protein. It was reported that the 27 kDa γ-zein gene was mapped to be the most significant QTL for endosperm modification in QPM located on chromosome 7 (Holding et al., 2008). It has also been reported previously that three QTLs are associated with endosperm modification at bin 1.06, 7.02, and 9.03, which mainly affect 19, 27 kDa zein accumulation and starch biosynthesis (Salazar-Salas et al., 2014).

In the present study, variations in zein expression among normal, *opaque-2*, and QPM lines were further refined by 2D-PAGE. A total of 59 different zein isoforms were reported by Consoli and Damerval (2001), out of which each maize genotype contains approximately 15–20 zein isoforms, which shows that the genetic background affects zein isoform accumulation irrespective of the *opaque-2* mutation. In the present study, it is found that the normal line shows a high content of α-zeins. Similar results were reported in the comprehensive transcriptomic analysis, showing that 65% of transcripts in the normal maize are associated with α-zeins during 10–35 DAP. Results from the present study showed that the *opaque-2* mutation affects the expression of 19, 22 kDa zeins, with a subsequent increase in low-molecular-weight zeins. Similar results

were reported by Consoli and Damerval (2001), who reported an increase in low-molecular-weight zein isoforms (10 kDa δ-zein and 16 kDa γ-zein) in *opaque-2* mutants. Out of the total zeins expressed in normal lines, almost 85% were α-zeins, whereas low-molecular-weight zeins formed a small fraction in normal maize. Contrasting results for zein expression were observed in *opaque-2* and QPM lines compared to the normal line. Similar results were reported earlier, showing that the *opaque-2* mutation mainly affects the accumulation of 22 and 19 kDa α-zeins (Hartings et al., 2011; Motto et al., 1989; Yuan et al., 2014). In addition, molecular data retrieval studies for 27 kDa zeins revealed that multiple isoforms for 27 kDa zeins are reported with a wide range of pI and they play a major role in endosperm modification in QPM lines. There is large genetic variability in terms of different zein isoform expressions concerning the genetic background, indicating that along with *opaque-2*, zein accumulation in developing kernels is governed by multiple regulatory genes (Consoli & Damerval, 2001; Yuan et al., 2014).

Similar results were reported in a study by Pandey et al. (2016), who reported that a diverse range of modifiers play a role in a diverse panel of well-established QPM inbreds. One of the critical results of the present study was that 15 kDa β-zein also plays a positive role in endosperm modification of QPM lines along with the previously reported 27 kDa γ-zein. Similarly, a comparison of 19 kDa zein expression among *opaque-2* and QPM suggests that 19 kDa zein plays no role in endosperm modification as the accumulation of 19 kDa zeins was more in *opaque-2* lines as compared to the QPM line. These results are in contrast to previous reports suggesting a cooperative role of 19 kDa zeins with 27 kDa to retain endosperm modification (Salazar-Salas et al., 2014). Thus, the current findings indicate that 27 and 15 kDa zeins play a substantial role in endosperm modification, while 19 kDa zeins seem to have no positive effect. Several proteins are functionally associated with 15 kDa and involved in endosperm modification. Therefore, the 15 kDa β zein is a novel player in endosperm modification along with the already reported 27 kDa zein. The detailed elucidation of the amino acid composition of different zein isoforms shows that low-molecular-weight zeins (10, 15, and 16 kDa) contribute to the nutritional quality of *opaque-2* and QPM lines mainly due to the high percentage of Lys, Trp, and Met and a lower percentage of leucine (Leu) and isoleucine (Ile) in comparison to normal maize. As reported earlier, the normal maize-consuming population develops pellagra (niacin/Trp deficiency disease) as maize protein is devoid of Trp and is enriched in Ile and Leu. It is reported that the high content of Leu and Ile negatively influences the Trp accumulation in humans by decreasing Trp absorption in the small intestine and increasing Trp oxidation in the bloodstream. Therefore, pellagra is caused by maize consumption due to the high amount of zein, especially 19, 22 kDa zeins, which have lower amounts of Trp and high amounts of Leu and Ile (Badawy et al., 2014). Therefore, the overall nutritional quality of *opaque-2* and QPM lines is contributed by both higher expressions of nonzeins (Sethi et al., 2021) and higher accumulation of low-molecular-weight zeins in comparison to normal maize.

In conclusion, the present study revealed that the overall number of zein isoforms in normal, *opaque-2*, and QPM lines remains the same/uniform. The reduction in zein is counter-balanced by an increase in the nonzein fraction, as observed in earlier investigations. It was also found that the expression of low-molecular-weight zeins (16, 15, and 10 kDa zeins) compensates for the decreased amount of α-zein due to the *opaque-2* mutation. Additionally, these expressed zein lines also contribute to protein quality along with the high content of nonzeins in *opaque-2* and QPM lines.

Furthermore, there is a lot of variation in terms of 27 kDa isoforms, and they are expressed differently in different genetic backgrounds. It has also been suggested that along with 27 kDa zeins, 15 kDa zeins play a significant role in endosperm modification, whereas 19 kDa zeins play no role in re-establishing QPM kernel texture. Although the present study provides information about novel isoforms in endosperm modification, there is a need to further validate these isoforms for their consistent role in diverse genetic backgrounds. Overall, the present study provides an avenue to further analyze the targeted endosperm modifiers under different genetic backgrounds, which will yield detailed information on the contribution of specific endosperm modifiers in retaining the vitreous kernel texture of QPM. The information obtained in the present study will aid in achieving targeted improvement in maize nutrition by enabling the introgression of specific, favorable zein isoforms. This specificity in trait introgression is expected to yield high protein quality with minimal pleiotropic effects.

AUTHOR CONTRIBUTIONS

Mehak Sethi conducted the experiment, analyzed the data, interpreted the results, and wrote the manuscript. Alla Singh envisaged the concept, analyzed the data, interpreted the results, and revised the manuscript. Mamta Gupta envisaged the concept, analyzed the data, and interpreted the results. Venkatesh Chunduri conducted the experiments. Parminder Kumar performed standardization of the experiment. Veena Devi contributed to

bioinformatics analysis. Firoz Hossain provided the material and helped in conducting field trials and data interpretation. Ramesh K. Phagna contributed to field trials, data collection, and interpretation. Mamta Gupta contributed to data analysis and manuscript writing. Dharam P. Chaudhary envisaged the concept, analyzed the data, interpreted the results, and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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