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Nitrogen Storage in Crops: Case Study of Zeins in Maize

Marija Duvnjak, Kristina Kljak and Darko Grbeša

Abstract

Crop grains accumulate significant amounts of nitrogen in the form of storage proteins. Grain storage proteins are not only important in the aspects of germination but also, storage proteins are a valuable food source in human and animal nutrition. This chapter will give insight into genotype and growing conditions influencing the quantity and quality of storage proteins, primarily maize storage proteins the leading cereal by world production. Main storage proteins in cereals are prolamins, and in maize prolamins are called zeins located within the endosperm in protein agglomerations called protein bodies. Four main classes of zein proteins are: alpha, beta, gamma and delta zein. Each of four zein classes has a distinctive position and role within protein bodies. Prolamin proteins define nutritional value of maize grain not only via amino acid quality but also via starch availability. Starch, the most important energy component of maize grain, is located within starch-protein matrix. Within this matrix, starch granules are surrounded by protein bodies that limit starch availability. In this chapter, we will describe how zein proteins influence characteristics of maize grain and nutritional value of maize.

Keywords: maize grain, zein proteins, starch, amino acid quality, starch digestibility, maize nutritional value, animal nutrition

1. Introduction

The protein content in cereal grain can vary greatly, from less than 6% to more than 20% in dry matter (DM), and this content depends on several factors such as the type of cereal, variety, agrotechnical conditions and others. These factors can be divided into two major groups, genotype and environment. Today, producers manipulate these factors to obtain grain of good quality and high protein content. For example, it has been found that the rate of maize yield gain is significantly higher after application of 220 kg N ha^{-1} ($0.12 \text{ Mg ha}^{-1} \text{ year}^{-1}$) than without fertilization ($0.05 \text{ Mg ha}^{-1} \text{ year}^{-1}$) [1].

In terms of their functions, there are three types of cereal grain proteins: structural proteins (as membrane proteins), metabolic proteins (as enzymes and enzyme inhibitors) and storage proteins, the largest fraction occurring primarily in starchy endosperm. Storage proteins account for 70–80% of the total protein content in grain and have a unique structure. The primary function of storage proteins is to supply grain embryo with nitrogen and amino acids during germination. However, these proteins are also a valuable food and feed source in human and animal nutrition.

The major storage proteins in cereal grains are called prolamins; the name is derived from their high content of the amino acids proline (Pro) and glutamine (Gln). However, the exact name of prolamins differs in different cereals – in maize prolamins are called zeins, in wheat gliadins, in oat avenins, in barley hordeins, in rye secalins, and in sorghum kafirins.

In human and animal nutrition, cereal crops are an important diet ingredient, and maize is the most commonly used cereal crop. Maize grain is palatable, highly digestible both by humans and animals, and it is an excellent source of metabolizable energy (ME). Although maize grain is low in protein content, the amount used in livestock production makes it an important source of protein in animal diets [2].

2. Maize storage proteins - zeins

Several types of storage proteins are classified in maize depending on their extraction in different solvents. About 70% of maize grain proteins are storage proteins, of which more than 60% are zein (prolamin) proteins. Except zeins, other types of storage proteins include albumins, globulins, and glutelins [3].

First reports describing zeins date back to 1821 when J. Gorham named proteins isolated from maize ‘zeine’. T.B. Osborne, the founder of seed storage protein research, classified zeins as prolamins and developed first extraction methods based on their hydrophobic nature at the beginning of 20 century [4].

Maize zein proteins are located in endosperm within protein aggregates called protein bodies (PBs) consisting of four distinctive zein proteins: alpha (α), beta (β), delta (δ) and gamma (γ) zein (**Figure 1**). The PBs are part of starch-protein matrix, where starch granules are surrounded with abundant zein proteins in PBs embedded in a matrix of glutelin proteins [7].

As shown in **Figure 1**, the location of each distinctive zein differs within PB. Alpha zeins are the most abundant group of zein proteins located in the central part of PB together with delta zeins. They account for up to 70% of total zeins. Based on apparent migration rates on SDS-page, two distinctive alpha zeins are defined, namely relative molecular mass (Mr) 19-kDa and Mr 22-kDa alpha zeins [8].

Figure 1. Schematic presentation of zein proteins location within protein bodies. Adapted data from the [5, 6].

Gamma zeins, the second most abundant zeins (up to 20% in the total zein fraction), are considered to be the most important zeins. This zein class is located on the surface of PB and in small spots within where they stabilize alpha zein core together with beta zein. Gamma zeins are responsible for number and features of PBs and consist of three distinctive proteins: 16-kDa gamma zein, 27-kDa gamma zein and 50-kDa gamma zein [8, 9]. The 27-kDa gamma zein is the most abundant gamma zein, followed by 16-kDa gamma zein and 50-kDa gamma zein, with the latter being in low abundance and for long misidentified as a dimer of the 27-kDa [10]. Based on the apparent migration rates on SDS-page, two distinctive delta zeins (10-kDa and 18-kDa) and one beta zein (15-kDa) are defined [8, 9].

Beta zein and delta zeins are expressed in much lower amounts in maize endosperm. Transcripts encoding alpha zeins account for about 30%, for gamma zeins about 10% whereas for beta and delta zeins 5% and 1.5% of total transcripts, respectively. In the same study, zein specific sequences accounted for almost 50% of the total cDNAs obtained from development of the maize endosperm [10] demonstrating the importance of zein proteins during nitrogen accumulation in maize grain.

2.1 Zein genes

Zein proteins are encoded by a large superfamily of genes. Gamma, beta and delta zeins are encoded by single-copy genes (3 for gamma, 1 for beta, 2 for delta). With some exceptions, two members of delta zeins originate from diploidization, and in quality protein maize (QPM) the genes encoding for 27-kDa gamma zein are duplicated [5]. Alpha zeins are different from the other zein proteins; this group of zein proteins are encoded by four multimember gene families, three for 19-kDa alpha zeins and one for 22-kDa alpha zeins [5]. Studies show large gene clusters that are sometimes disrupted by transposons or other genes [11, 12]. Both 19-kDa and 22-kDa alpha zeins share a common ancestor [12] which underwent amplification and chromosome translocation during evolution [13]. Alpha zeins gene families are located at seven chromosomal sites, but the exact location, and number and organization of genes varies greatly between different inbred lines. Furthermore, not all alpha zein genes are expressed, but only selected members of each alpha zein family [5]. For example, a detailed expression analysis of inbred line of B73 maize showed that only 18 of the 41 alpha zein genes were expressed [14].

2.2 Zein amino acid structure

Zein proteins, and prolamin proteins in general, are rich in amino acids Pro and Gln. However, zeins are devoided of essential amino acids lysine (Lys) and tryptophane (Trp). Thus, a great deal of research has been done to increase the amount of essential amino acids in zein proteins. Besides, the nutritional properties, solubility and chemical structure of zeins are influenced by amino acid characteristics. Zein proteins contain only a few charged amino acids and as a consequence have a hydrophobic nature and thus are insoluble in water. Solubility and chemical structure are important because they define and influence processing and manufacturing of food and feed [15].

Alpha zeins are encoded by large multigene families, and besides Pro and Gln, contain a high proportion of hydrophobic amino acids alanine (Ala) and leucine (Leu). Their structure is largely defined by a series of tandemly repeated peptides of 20 amino acids with nine repeats in the 19-kDa and ten in the 22-kDa alpha zeins. Each repeat is flanked by clusters of Gln residues [6]. Due to their structure, alpha zeins can be extracted with aqueous alcohol [4].

In addition to Pro and Gln, gamma zeins are rich in the cysteine (Cys) which has strong disulfide bonds and thus influencing stability and extractability of zeins [16]. Six highly conserved Cys-rich domains are found in gamma zein proteins [6]. All zein proteins, with the exception of alpha zein proteins, have a high content of sulfur-rich amino acids, which can vary in expression levels among maize cultivars. As mentioned above, gamma zeins are rich in Cys, delta zeins in methionine (Met), and beta zein in both Cys and Met [3].

The amino acid compositions in the most abundant zein proteins are shown in Figure 2.

2.3 The formation of protein bodies

Zein proteins form PBs, insoluble protein aggregates located in the starch-protein matrix. The expression of distinctive zein proteins controls the initiation and development of PBs. Immunogold staining showed that PBs start to aggregate approximately 9 days after pollination as small accretions mainly of gamma and beta zeins. During the PBs growth, alpha zeins and delta zeins enter the PB core and are responsible for the growth and the expansion of the PBs [6]. Each zein protein has a proposed distinctive role in initiation, formation and growth of PBs. The RNA interference (RNAi) technique was used to reduce the expression of a specific zein gene, and it showed that reduction of 22-kDa alpha zein led to PBs with an unusual budding structure [16]. RNAi suppression of both 19-kDa and 22-kDa alpha zein resulted in smaller PBs and with their typical number [17], indicating that 22-kDa can function in PBs morphology and 19-kDa alpha zein can function in PBs growth [5]. RNAi suppression of 27-kDa gamma zein resulted in fewer PBs,

Figure 2. Amino acid composition in most abundant zein proteins. Adapted data from the [6].

while suppression of 16-kDa and 50-kDa gamma zein resulted in smaller PBs but with their normal numbers. Results indicate a significant role of 27-kDa gamma zein in PBs initiation whereas 16-kDa and 50-kDa gamma zeins have a function in PBs expansion [17]. As the inbred line A654, deprived of both delta zeins, has PBs similar to other inbred lines with normal delta zein quantities [16], Li and Song assumed that delta zein has no essential role in the formation of PBs [5]. A study using a yeast two-hybrid system showed strong protein–protein interactions between all gamma zeins and beta zein, weak within alpha zeins, although they both interact strongly with the 10-kDa delta zein, 16-kDa gamma zein and beta zein whereas interacting poorly 27-kDa and 50-kDa gamma zeins [18]. The affinities shown here are consistent with the proposed location and role of each zein protein: 27-kDa gamma zein is responsible for the initiation and is located at the surface of PBs and 16-kDa gamma and beta zein are located towards the inner parts and stabilize alpha/delta zeins of PBs core (**Figure 1**).

2.4 Analysis of zein proteins

Biologically, zein proteins make a mixture of proteins varying in molecular size, solubility and charge [4]. Solubility of most zeins is good in aqueous-alcohol solutions, 60% isopropanol, 70% ethanol, 95% methanol. However, due to the high disulphide bonds in gamma zeins, this type of zein protein is only extractable when a strong reducing agent, such as 2-mercaptoethanol, is added [19].

When analyzing zein proteins, different extraction procedures are applied: wide range of aqueous ethanol solutions [20–22] or aqueous isopropanol solutions [23, 24], highly concentrated alkali solutions (pH 11 or above) [25], highly concentrated aqueous urea solutions (8 M) [26], or anionic detergent-containing solutions [20, 26, 27]. The extractions are performed on wide range of extraction temperatures, ranging from 25 to 130°C [20, 21, 26], with or without addition of reducing agents as 2% 2-mercaptoethanol [27] or 10 mM DTT in 25 mM ammonium hydroxide [22]. Solubility-enhancing ingredients, such as 0.0125 M sodium borate [27], 0.5% sodium hydroxide [28], 0.5% sodium bisulfite [24] are also often added.

As zein proteins are a divergent group of proteins, extraction procedures vary significantly. Hence, analysis of zein extracts is complex and include the application of various techniques often used in protein separations. Combination of electrophoresis methods, such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which only provides the molecular weights (MW) [22, 27, 29, 30], or the two-dimensional gel electrophoresis (2DE), which provides insight into MW and charge [22], with or without the mass spectrometry (MS) for protein identification [22, 31], are used in zein analysis. Capillary electrophoresis (CE), the electrophoretic method also used for zein separation, has enabled the identification of more fractions by MS [32]. Other methods of zein separation include chromatographic methods such as reversed-phase HPLC method on C18 column with a gradient of 45 to 75% acetonitrile-ultrapure water, both containing 0.01% trifluoroacetic acid [33] or with acetonitrile-trifluoroacetic acid gradient [34]. Liquid chromatography-mass spectrometry (LC–MS), a method widely used in proteome analysis due to its high sensitivity, was for the first time adopted for zein evaluation in 2020; the authors coupled multi-enzyme digestion with nano-LC–MS/MS [35]. Separation techniques mentioned here are used for the analysis of protein compositions in biological samples of high complexity [22]. Regardless of numerous approaches in zein analysis, the most suitable method will be selected based on desired trait of maize zein proteins.

3. Conditions influencing maize zein content

Factors affecting zein proteins in maize grain can be divided into two main groups – genotype and environment. The genotype is regarded as a primary factor influencing zein properties, while environment (nitrogen fertilization, irrigation, high temperatures, etc.) affect to a lesser extent causing small variability within the same variety.

3.1 Genotype

Maize is usually subdefined according to the kernel characteristic determined by grain vitreousness. Grain vitreousness is an important agronomic trait that influences hardness and post-harvest resistance to insects and fungi, rate of starch digestibility, and semolina yield for food production [36]. It is defined as a ratio of vitreous to floury endosperm and is strongly affected by the type and quantity of zeins within the starch-protein matrix in maize endosperm. The endosperm of flint maize consists mainly of vitreous while floury maize contains almost exclusively floury endosperm [37]. Dent maize hybrids, which are derivatives of flint-floury classes, differ in their ratio of vitreous to floury endosperm.

Zein proteins surround starch granules in the starch-protein matrix in both types of endosperm but with different interactions. In vitreous endosperm, smaller starch granules are tightly packed in multiple and well developed PBs. In contrast, in the floury endosperm, starch granules are larger, and the protein layer is thinner with lesser PBs and with numerous air-filled spaces (**Figure 3**). The texture differences between vitreous and floury endosperm lead to differences in physical properties of maize samples varying in vitreousness [38]. Kljak and coauthors showed, in a study of 22 maize samples varying in kernel vitreousness (50.23% – 76.41%) and zein content (53.86–86.37 g/kg endosperm DM), that zein proteins, acting through starch-protein interactions, have the most important influence on the maize virtuousness in comparison to the other characteristics of endosperm as amylose content and starch granule size and shape [39].

Zein proteins, as the largest protein fraction in grain, define the quality of maize protein. As mentioned above, zein proteins are devoided of essential amino acids

Figure 3. Scanning electron micrographs of ground samples of maize hybrids varying in endosperm texture. Larger starch granules with thinner protein layer in the floury endosperm (a) opposite to smaller starch granules with well-developed protein layer in the vitreous endosperm (b). The maize grain endosperm morphological features (starch-protein interactions) were examined visually on 1 mm ground samples using a scanning electron microscope (SEM) (FE-SEM/Mira, Tescan, Brno, Czech Republic) with magnification 5000x.

Lys and Trp, and to some extent Met due to the low Met content in alpha zeins. Therefore, despite high yields and sufficient production quantities, amino acid composition limits the usage of maize in nutrition of both humans and monogastric animals. Maize alone cannot provide a balanced meal, and it must be supplemented with sources of essential amino acids, as environmentally questionable soya or other amino acid supplements in the diet of monogastric animals. As a result, maize deficiency in amino acids is increasing costs of food and feed supply worldwide. In order to counteract this, researchers in the last century have focused on regulating the expression and accumulation of zeins in maize.

High Lys and Trp maize mutants have been developed. In the *opaque2* (*o2*) mutant regulating zein O2 transcription factor (TF), alpha zeins have been reduced with compensatory higher amount of non-zein maize proteins. However, those mutants have undesirably soft, opaque and brittle endosperm and their use is limited due to the poor agronomic performance [40]. On the other hand, later developed quality protein maize (QPM) with higher Lys and Trp regained vitreous endosperm and agronomic performances of normal maize [41]. The QPM has zein content different from wild-type or other maize mutants. This maize has a low content of alpha zeins and accumulates a high amount of 27-kDa gamma zeins, which confirms that gamma zeins are essential in PB functionality and preserved hardness in QPM [29]. Liu and coauthors, in their study using genome-wide association study analysis, linkage mapping analysis, and map-based cloning, showed that duplication of gene encoding 27-kDa gamma zein resulted in overexpression of this class of gamma zein protein [30].

The expression of most zein proteins is regulated by one or more TFs, however, maize mutations involving zein proteins may include mutations that can alter the accumulation of zeins in PBs, resulting in abnormal PBs and opaque endosperms or mutations in genes involved in amino acid biosynthesis [5]. For example, affecting myosin XI proteins in *opaque1* (*O1*) leads to an increased number of misshapen PBs [42]. The *floury1* (*Fl1*) mutation leads to a disrupted accumulation of 22-kDa alpha zein in the outer gamma zein region and 19-kDa alpha zein core rather than in the proposed discrete ring at the outer edge of PBs core. However, without changing the PBs size, shape or abundance [43]. Mutant *O10* with mutation in *opaque10* leads to misshapen PBs, probably caused by disruption of the discrete ring-shaped outer core of PBs containing 22-kDa alpha and 16-kDa gamma zeins [44]. Mutations that lead to altered retention of zeins in PBs include *fl2*, *DeB30*, *fl4*, *mucronate1* (*mc1*) mutation affecting 22-kDa alpha zein, 19-kDa alpha zein, 19-kDa alpha zein and 16-kDa gamma zein, respectively [5].

Mutations affecting amino acid biosynthesis include the *Pro1* mutant with inhibited Pro biosynthesis resulting in a lower amount of Pro while the *mtol40* mutant inhibits the Tyr and Phe biosynthesis. However, both mutations lead to a general reduction of the accumulation of zein proteins and not to a specific zein reduction. The characterization of this type of maize opaque endosperm mutants suggests that amino acid limitations repress zein protein biosynthesis [5]. Other mutations affecting the expression and accumulation of zein proteins on the translation level are *opaque 7* (*o7*) mutant with defective Acyl-CoA synthetase [45] and *zmocd1* mutant affecting oxalyl-CoA decarboxylase [46]. Both *o7* and *zmocd1* have a major impact on amino acid biosynthesis by affecting α -ketoglutaric acid and oxaloacetic acid leading to altered endosperm metabolome and opaque endosperm with reduced zein content [5].

3.2 Environment

Duvick compared the yield of American hybrids grown from 1934 to 2004 and showed that the average annual increase of maize grain yield is 115 kg/ha. The

comparison also showed that genotype contributes 50–60% to variability of hybrids (hybrids more resistant to abiotic and biotic stresses) while remaining 40–50% is affected by agricultural technology (fertilizers application, control of diseases and pests, etc.), which demonstrates the importance of not only genetic maize characteristics but also agronomic technology improvements [47].

Agronomic improvements in maize yield have been extensively evaluated; major factors are water management (irrigation) and the application of nitrogen fertilizer depending on environmental conditions. In the United States, for example, the importance of irrigation practices and plant population in the low rainfall Western region of the U.S. corn-belt has been emphasized. In contrast, nitrogen fertilizer use and plant population have been emphasized in the high rainfall Central and Eastern regions [48]. Nitrogen fertilizers are important in modern maize production; the rate of maize yield gain is much higher when 220 kgN ha⁻¹ is applied compared to when no fertilizer is applied (0.12 vs. 0.05 Mg ha⁻¹ year⁻¹, respectively) [1].

Nitrogen fertilizers are required to maintain maize production and soil fertility, however, negative effects of their use on the environment are a global concern, and thus, nitrogen use efficiency (NUE) is an extremely important issue. Nitrogen use efficiency is defined as the amount of grain produced per unit N accumulated above what is provided by soil N mineralization [1]. In modern maize hybrids, the increase in grain yield is often accompanied by a reduction in grain protein concentration [49]. However, Mueller and coauthors in their analyses of NUE in maize hybrids showed that grain yields increased faster than grain N concentration decreased. The same authors concluded that although previous research indicated that maize grain yields and NUE gains over time were primarily due to greater total N accumulation and dilution of grain N accumulation, changes in N accumulation within the plant itself are important to achieve efficient N conversion into grain yield. Key plant factors are increased stem N remobilization and retention of leaf N during reproductive growth [1].

Precipitations and temperature are two key environmental factors affecting maize grain yields and N accumulation. The warm climate will accelerate the phenological development (e.g. leaf appearance), however, drought and high temperatures at pollination as well as during the grain-filling period will reduce yields [50]. In controlled environmental studies when temperature exceeds normal temperatures by only 3°C, maize grain yields were reduced by half [51]. In field studies, rise in 6°C during grain filling period resulted in 13–88% reduction of maize grain yields and yield loss was much larger under fertilization (authors compared application of 200, 100 and 0 kg N ha⁻¹) [52]. Some studies show that protein concentration is positively correlated with high temperature during cereal grain growth [53]. However, Monjardino and coauthors concluded that heat stress during early stages of endosperm development reduces zein accumulation at synthesis level while later in development had no significant effect on zein quantity. Later during kernel development, the reduction in zeins was mainly result of protein degradation, which appears to be a part of the natural progression of kernel development [54].

4. Zein proteins influence on maize nutritional value

In maize, starch and proteins account for approximately 70% and 10% of grain DM, respectively. Depending the cultivar, starch has the potential for complete digestion in livestock digestive tract [2], and thus, it is the most important energy component in animal diets. Opposite to high energy potential of starch, zein proteins are devoided of essential amino acids Lys and Trp, with the exception for

50-kDa gamma zein which contains 2.7% of Lys. The lack of essential amino acids, Lys, Trp, and to some extent of Met is important characteristic of zein proteins which limits the use of maize protein in food and feed industries. As mentioned earlier, new maize mutants, with different amino acids ratios and protein quality have been developed by maize breeders' worldwide. The change in amino acid ratios is mostly due to the change of zein proteins compared to other grain proteins [5]. Of maize mutants, QPM, shows excellent characteristics due to the higher Lys and Trp contents and agronomic performances of normal corn. With the use of QPM or high-lysine maize, the need for Lys supplements (soybean meal, synthetic amino acid supplements) is reduced. Although QPM was primarily intended for human nutrition in developing countries, it is nowadays implemented in swine and poultry nutrition as well [2, 41].

In standard diet of monogastric animal, maize grain and soybean meal complement each other. Zein proteins have adequate quantities of sulfur amino acids, Cys and Met (depending on the quantities of alpha zeins) and low Lys and Trp whereas soya has adequate quantities of Lys and Trp and is a relatively poor source of sulfur amino acids [2]. However, it should be noted that higher protein content in maize is often connected with higher zein content, resulting in even lower quantities of Lys and Trp compared to normal maize. Thus, when using maize in monogastric animal nutrition, the amount of essential amino acids relative to energy and total feed intake is more important than the quantity of total protein [55].

The second important aspect of zein influence on maize nutritional value is related to starch rather than to the proteins. As starch granules are embedded in protein layer with PBs, zein poses a physical barrier that can limit the starch availability to digestive enzymes and rumen microorganisms [56]. As a result, starch digestibility in maize varieties with higher zein content will be slower. Maize grain with higher vitreousness has lower digestibility than grains of lower vitreousness containing less zein [7, 56–58]. Kljak and co-authors in their study on eight yellow high-yield maize hybrids varying in zein content (from 70.3 to 88.7 g kg⁻¹ of total starch) showed that fractional starch digestion rate (kd) in *in vitro* poultry digestibility experiment correlated negatively to zein content (-0.36 , $P < 0.05$). The authors concluded that when starch granules are embedded in a complex protein matrix, zein limits their accessibility to enzymes and affects the starch digestibility rate to a greater extent than starch properties [59].

Furthermore, when maize grain is subjected to different processing methods as silage production [33, 60] or steam flaking [61], reduction in zein content and destruction of the starch-protein matrix will occur [33]. As a result, starch granules will become easily accessible to digestive enzymes and amylolytic bacteria [61], starch digestibility increases, and starch will be digested in a higher rate [62–65].

The influence of zein proteins on starch digestibility is becoming a key factor when determining starch efficiency in poultry and swine. Since starch is almost completely digested in their digestive tract, the rate of starch digestibility is the key determinant of animal performance. Weurding showed the importance of evaluation of starch digestion rate for poultry [66]. Higher content of slowly digestible starch appears to support faster, more efficient poultry growth, and the feeds with starch digestibility rates closer to 1.26 h⁻¹ seem to be the most efficient [59]. The results of studies showed that lower digestibility rates are related to a higher amount of zein proteins, and thus, maize hybrids with a higher content of slowly digestible starch are desired. Consequently, when selecting the appropriate maize hybrid for production, in particular for use in animal nutrition, it would not be efficient to know only the quantity of starch but also the quantity of the zein proteins surrounding the starch, since the zein proteins determine the availability of starch and the efficiency of starch digestion.

5. Concluding remarks

Zein proteins, maize storage proteins located in starch-protein matrix of endosperm are paramount for maize nutritional value. They not only define amino acid characteristics of maize grain but are a primary factor affecting starch availability and digestibility. Therefore, genotype and environment effects on zein protein composition and content in grain present a basis to regulate maize nutritional value.

Conflict of interest

The authors declare that there is no conflict of interest.

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