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REVIEW

The regulation of zein biosynthesis in maize endosperm

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Abstract

Key message **We review the current knowledge regarding the regulation of zein storage proteins biosynthesis and protein body formation, which are crucial processes for the successful accumulation of nutrients in maize kernels.**

Abstract Storage proteins in the seeds of crops in the grass family (*Poaceae*) are a major source of dietary protein for humans. In maize (*Zea mays*), proteins are the second largest nutrient component in the kernels, accounting for ~ 10% of the kernel weight. Over half of the storage proteins in maize kernels are zeins, which lack two essential amino acids, lysine and tryptophan. This deficiency limits the use of maize proteins in the food and feed industries. Zeins are encoded by a large super-gene family. During endosperm development, zeins accumulate in protein bodies, which are derived from the rough endoplasmic reticulum. In recent years, our knowledge of the pathways of zein biosynthesis and their deposition within the endosperm has been greatly expanded. In this review, we summarize the current understanding of zeins, including the genes encoding these proteins, their expression patterns and transcriptional regulation, the process of protein body formation, and other biological processes affecting zein accumulation.

Introduction

Maize (*Zea mays*) is a major crop for the food and feed industries due to its high yields. In maize kernels, starch and proteins account for ~ 70% and ~ 10% of the kernel weight, respectively, and the nutritional quality of maize kernels largely depends on their protein content and composition (Flint-Garcia et al. 2009). About 70% of maize kernel proteins are storage proteins, of which 60% are prolamins, also known as zeins. The zeins lack lysine and tryptophan, two essential amino acids for humans, livestock, and poultry, meaning that maize kernels contain relatively low-quality proteins (Osborne et al. 1914). This poor quality means that despite its high yields, maize cannot be used to provide balanced nutrition without the addition of lysine and tryptophan (Bhan et al. 2003), limiting its use in the food and feed industries. Research into the regulation of the expression and accumulation of zeins could therefore help improve the nutritional quality of maize kernels.

Fifty years after pioneering work uncovered the nutritional deficiency of zeins (Osborne et al. 1914), a discovery in the 1960s revealed that a few maize mutants with opaque endosperms have higher protein nutritional qualities (Mertz et al. 1964; Nelson et al. 1965), kicking off the modern era of zein research. In the 1970s, researchers focused on the cellular translation and organellar deposition of the zeins, while in the 1980s, the spotlight shifted to the cloning and sequencing of zein-coding cDNAs. The cloning of *opaque2* in 1987 marked an important milestone for the study of zeins (Schmidt et al. 1987). This was one of the first plant transcription factors (TFs) to be cloned and was found to be a major transcriptional regulator of zein genes. By the 1990s, a significant progress had enhanced understanding of the transcriptional regulation of zein genes and the functions of their protein products. This was not limited to Opaque2 (O2), but also included the discovery of other TFs, including the opaque2 heterodimerizing proteins (OHPs) (Pysh et al. 1993) and prolamin-box-binding factor 1 (PBF1) (Vicente-Carbajosa et al. 1997).

In the twenty-first century, the field of genomics blossomed, enabling the elucidation of the complicated composition and genomic organization of zein genes. New

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technologies, such as RNA interference (RNAi), were applied to the study of zeins, leading to further important discoveries. The most significant progress made this century has been the cloning of a large number of genes found to regulate the expression of zein genes. This included the analysis of some important classic *opaque* endosperm mutants with altered zein expression or deposition and the identification of new TFs regulating zein genes. In this review, we focus mainly on recent studies (since 2000) to summarize our current understanding of zein genes and their regulation.

Zein genes

Seed storage proteins mainly serve as biological reserves of amino acids that can be mobilized and utilized for seedling growth. Several types of storage proteins have been discovered in maize kernels, including albumins, globulins, glutamines, and prolamins, which were classified by their dissolution in different solvents (Shewry et al. 1995; Wu and Messing 2014).

Zeins account for more than 60% of the total storage proteins in maize kernels and are encoded by a large superfamily of genes. They can be classified into α-, β-, γ-, and δ-types, based on their solubility and ability to form disulfide bonds (Coleman and Larkins 1999; Esen 1987; Holding and Larkins 2009). The β-, γ-, and δ-types of zeins are usually encoded by single-copy genes, including one 15-kD β-type zein (Pedersen et al. 1986), two δ-type zeins of 10 and 18 kD (Chui and Falco 1995; Kirihara et al. 1988), and three γ-type zeins of 16, 27, and 50 kD (Prat et al. 1987; Woo et al. 2001). The two members of δ-zeins, 10-kD and 18-kD, are originated from the diploidization of an ancient allotetraploidization event (Swigonova et al. 2004). In certain so-called quality protein maize (QPM) lines, the gene encoding the 27-kD γ-zein is duplicated (Liu et al. 2016).

The α-zein subfamily, the most abundant fraction of zeins, is much more complex. The α-zeins can be further divided into 19- and 22-kD subclasses based on their apparent molecular weights. Earlier studies based on protein or cDNA analyses indicated that the 19- and 22-kD zein subclasses are encoded by multiple gene families (Pedersen et al. 1982; Wienand et al. 1981; Wilson and Larkins 1984). Studies of the large genomic segments comprising these gene families (Song et al. 2001; Song and Messing 2002) indicated that they are organized as gene clusters, which are sometimes disrupted by transposons or other genes (Song et al. 2002; Song and Messing 2003). The sequencing of genes encoding the α-zeins revealed the existence of four multimember families; the 19-kD zeins are encoded by three gene families, *z1A*, *z1B*, and *z1D*, while the 22-kD zeins are encoded by a single gene family, *z1C* (Pedersen et al. 1982; Song et al. 2001; Song and Messing 2002; Wienand et al. 1981). These gene family members derive from a single ancestor, which underwent gene amplification and chromosome translocation during evolution (Xu and Messing 2008). In modern maize inbred lines, their constituent genes occupy seven chromosomal locations, which vary between the different inbred lines (Song et al. 2001; Song and Messing 2002; Xu and Messing 2008). The B73 inbred line has 41 α-zein genes, including 16 *z1C*, 12 *z1A*, 8 *z1B*, and 5 *z1D* family genes (Song and Messing 2002, 2003). In combination with the one, three, and two genes encoding β-, γ-, and δ-zeins discussed above, there are 47 zein genes in the maize B73 inbred line (Xu and Messing 2008). The number of genes and the genomic organization of the α-zein gene families are highly variable between the different maize inbred lines (Feng et al. 2009; Song and Messing 2003).

Zein gene expression

Zeins represent the most abundant proteins in the maize endosperm, and their expression levels are extremely high in this tissue. In one study, zein-specific sequences accounted for nearly 50% of the total cDNAs obtained from the developing maize endosperm (Woo et al. 2001). Among them, transcripts encoding α-zeins (19-kD and 22-kD) comprised about 30% of the total library, while those encoding β-zein (15-kD), γ-zeins (16-kD, 27-kD and 50-kD), and δ-zeins (10-kD and 18-kD) accounted for about 5, 10, and 1.5% of the library, respectively (Woo et al. 2001). In a more comprehensive transcriptome analysis of the developing endosperm and embryo (Chen et al. 2014), zein genes accounted for about 65% of the transcripts across a developmental period of 10 to 34 days after pollination (DAP), with α-zein genes (19-kD and 22-kD) alone accounting for nearly 50% of the total transcripts (42% encoded 19-kD zeins, while 8% encoded 22-kD zeins).

Even though they are encoded by multiple gene families, a unique feature of the expression of α-zein genes is that only select members are expressed from each gene family (Song et al. 2001; Song and Messing 2002). A detailed expression analysis of α-zein genes in maize inbred lines (Feng et al. 2009) indicated that only 18 of the 41 α-zein genes were expressed in the B73 inbred line (eight of the *z1A* genes, two *z1B*s, six *z1C*s, and two *z1D*s), all of which contained intact protein-coding sequences. These features of α-zein gene expression were also observed in the cDNA and transcriptome analyses (Chen et al. 2014; Woo et al. 2001). The *z1A* and *z1C* gene families contain relatively more members than the other families, and which *z1A* and *z1C* genes are expressed varies dramatically between the different inbred lines (Feng et al. 2009).

The functions of zeins in protein body formation

Zeins are biosynthesized on the rough endoplasmic reticulum (RER) and form an insoluble matrix within membrane-bound organelles called protein bodies (PBs), which are always attached to the RER (Burr and Burr 1976; Larkins and Hurkman 1978). Immunogold staining of the storage proteins in isolated PBs revealed that the PBs begin as small accretions containing mainly γ- and β-zeins, consistent with the slightly earlier onset of the expression of genes encoding these zeins (Lending and Larkins 1989). As the PBs expand, α- and δ-zeins enter the PB core, becoming encapsulated in a shell of γ-zeins. The arrangement of the two subfamilies of α-zeins is also ordered; the 19-kD α-zeins are concentrated in the center, whereas the 22-kD α-zeins are distributed as a discrete ring at the interface between the 19-kD zein core and the peripheral γ-zein shell (Holding et al. 2007).

The temporal and spatial expression patterns of zeins also agree with the observation of PB formation by immunogold staining. RNA run-on assay indicated zeins start to accumulate specifically in developing endosperm from ~ 10 days after pollination (DAP) (Kodrzycki et al. 1989). With in situ hybridization, temporal and spatial expressions of different zein gene families were analyzed. Each type of zein mRNA accumulates at a different level in different endosperm regions (Dolfini et al. 1992). γ-zeins are synthesized before α- and δ-zeins (Woo et al. 2001), indicating its substantial role for PB initiation.

The specific distribution of different types of zeins in the PBs suggested that they could have distinct functions in PB formation, and zein protein interactions have been found to be crucial for PB assembly. Strong interactions determined by yeast two hybrid assays occur among the 50-, 27-, and 16-kD γ-zeins and the 15-kD β-zein, consistent with their colocalization in the PBs and their known important roles in PB formation. Strong interactions were also detected between the α- and δ-zeins and the 16-kD γ-zein and the 15-kD β-zein (Kim et al. 2002), consistent with the role for the 16-kD γ-zein and the 15-kD β-zein in the protein body expansion.

Many zein gene families consist of multiple members, meaning that a mutation in a single gene is not sufficient to severely impact their accumulation. RNAi provides an effective method for suppressing the expression of a particular group of zein genes in a dominant way, enabling the elucidation of the redundant and non-redundant roles of different zeins. The *o2* mutants are known for their reduced accumulation of zeins, which elevated lysine contents and improved nutritional quality; however, when used as a breeding tool, the *o2* mutation works in a recessive fashion, and it can be difficult to maintain the homozygous mutant genotype because open pollination can easily introduce other alleles. RNAi of zein-coding genes was first used to generate a dominant mutation that suppressed the biosynthesis of the 22-kD zeins without interrupting the expression of *O2* (Segal et al. 2003). The RNAi approach practically solves the open pollination problem by producing a dominant suppression effect.

RNAi was also applied to other type of zeins. And with the observation of PB formations by TEM, the functions of different types of zeins in the process of PB formation were revealed (Guo et al. 2013; Wu and Messing 2010). RNAi of the *z1C* genes resulted in the substantial loss of the 22-kD α-zeins, resulting in PBs with unusual budding structures (Wu and Messing 2010); however, RNAi targeting the biosynthesis of both the 19- and 22-kD α-zeins led to the production of smaller PBs without suppressing their overall numbers (Guo et al. 2013). These results indicated that the 22-kD α-zeins may function in the regulation of PB morphology, while the 19-kD α-zeins are mainly responsible for PB filling. The α-zeins have no function in the initiation of PB formation.

The RNAi suppression of different subclasses of γ-zeins revealed their distinct roles in PB formation (Wu and Messing 2010; Guo et al. 2013). The RNAi of genes encoding the 27-kD γ-zeins did not decrease PB size, but did reduce their numbers, indicating that the 27-kD γ-zeins control the initiation of PB formation but are not involved in PB filling. PBs in the developing endosperm of plants subjected to simultaneous RNAi of the genes encoding the 16- and 50-kD γ-zeins and 15-kD β-zein were smaller than usual, but accumulated in normal numbers, suggesting that the non-27-kD γ-zein family members function more in PB expansion than in PB initiation (Guo et al. 2013).

The δ-zeins do not appear to be essential for PB formation. In the inbred line A654, which lacks both the 10- and 18-kD δ-zeins, the PBs are similar to those produced by other inbred lines (Wu and Messing 2010).

The localization of zeins from the ER to the PBs

The formation of PBs depends on the successful localization of zeins after their translation on the RER. Mutations in genes involved in zein localization can alter the proportion of zeins in the PBs, leading to abnormal PB formation and opaque endosperms, which indicates that proteins mediating the formation of PBs play a key role in proper zein accumulation (Fig. 1).

Opaque1 encodes a member of the plant myosin XI family (myosin XI-I; O1) that is associated with the RER and PBs in endosperm cells. In the *o1* mutant, the

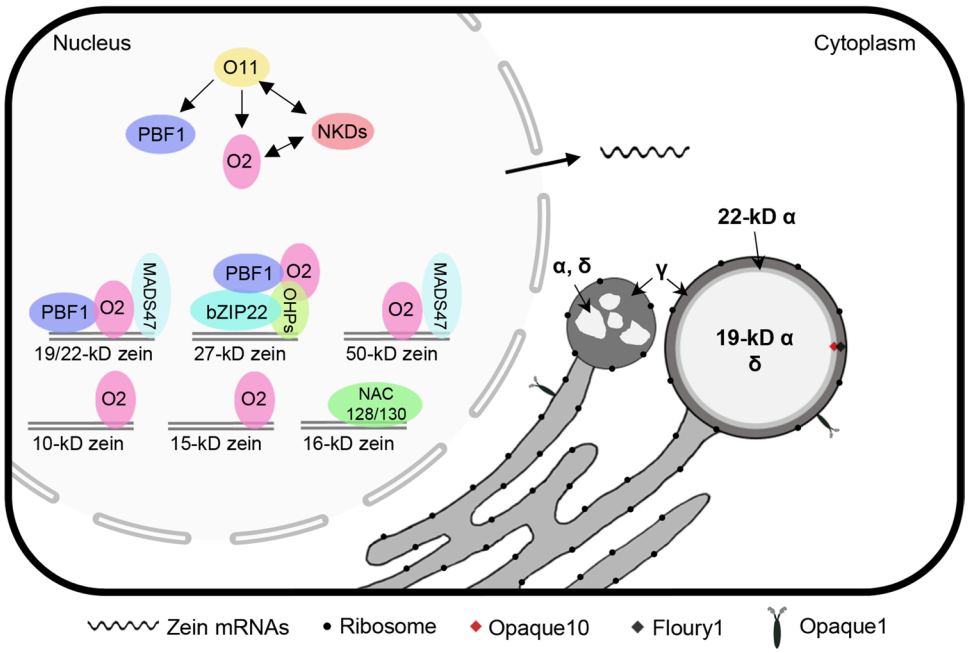

Fig. 1 A schematic diagram showing the transcriptional regulation of zein-coding genes and zein distribution in the protein bodies. In the nucleus, TFs work in combination to regulate the expression of the members in zein gene family. Some of these TFs, e.g., O2, NKDs, PBF1 and O11, also have mutual direct regulation. In the cytoplasm, zeins are translated in RER and then localized in PBs in an ordered manner with the aid of O1, Fl1, and O10

endosperm cells display dilated ER structures and have an increased number of misshapen PBs, as observed by transmission electron microscopy (Wang et al. 2012). This phenotype is consistent with the role of myosin XI proteins in endomembrane system and trafficking network. The abnormal synthesis of PBs is supposed to be the reason for the changed endosperm texture in *o1*.

Floury1 (*Fl1*) encodes a novel PB membrane protein with three predicted transmembrane domains and a C-terminal plant-specific domain of unknown function (DUF593) (Holding et al. 2007). Fl1 interacts with the 22-kD α-zein through its DUF593 domain and participates in PB formation by facilitating the localization of this zein. Mutations in *Fl1* result in the production of opaque endosperm and the accumulation of the 22-kD α-zeins in the γ-zein-rich periphery and center of the PB core, rather than their normal discrete location in a ring at outer edge of the core, while the distribution of 19-kD α-zein is not affected in the *fl1* mutant PBs. Despite these changes, the PBs in the *fl1* mutant are normal in size, shape, and abundance.

Opaque10 (*O10*) encodes an endosperm-specific protein that is also located in the PBs (Yao et al. 2016). Immunolocalization and yeast two hybrid assays indicated O10 colocalizes and interacts with 16-kD γ-zein and 22-kD α-zein, forming a ring-shaped structure at the interface between the α-zein-rich core and the γ-zein-rich peripheral region of the PB. Misshapen PBs were observed in the *o10* mutant. This change in PBs is believed to be caused by the disruption of the ring-shaped distribution of 22- and 16-kD zeins. Both Fl1 and O10 participate in the formation of PBs by facilitating the localization of the zein proteins.

Zein accumulation can also be altered by the retention of a zein peptide, including *fl2* (22-kD α-zeins), *DeB30* (19-kD α-zeins), and *fl4* (19-kD α-zeins) (Coleman et al. 1997; Kim et al. 2004; Wang et al. 2014a). Another zein mutation is *mucronate1* (*mc1*), which results in the production of a misfolded 16-kD γ-zein protein that causes the formation of irregular PBs. An opaque phenotype similar to the *Mc1* could be obtained by expressing the gene encoding the mutant 16-kD γ-zein using transgene in wild-type maize (Kim et al. 2006). All four of the zein mutations outlined above are dominant mutations, and the erroneously biosynthesized zeins in these mutants cause physiological stress in the ER and trigger the unfolded protein response, which in turn reduces zein biosynthesis and elevates the production of chaperones.

Although the zeins are the predominant component of the maize PBs, they also contain many non-zein proteins. Proteomic analysis of intact PBs identified 1756 proteins, which could be grouped into five major categories: metabolic pathways, response to stimulus, transport, development and growth, and regulation (Wang et al. 2016). How these non-zein proteins are transported into the PB and how they function once inside are still not clear.

TFs regulating zein gene expression

Although there are several pieces of evidence suggesting zeins are subjecting to posttranscriptional regulation (Chaudhuri and Messing 1994; Lai and Messing 2002), the transcription regulation mediated by TFs plays the predominant role in the final accumulation of zeins (Table 1). O2 was

Table 1 The zein gene family and their transcription regulators

Zein member	Family	TFs	(*cis* elements)	References
10-kD	δ	O2	–	Li et al. (2015)
15-kD	β	O2	TGACGTGG	Cord Neto et al. (1995)
16-kD	γ	ZmNAC128/130	ACGCAA	Zhang et al. (2019)
18-kD	δ	O2	–	Zhan et al. (2018)
19-kD	α	O2	ACGT for *z1A*, ACAT for *z1B* and *z1D*	Li et al. (2015); Yang et al. (2016)
		PBF1	TGTAAAG	Vicente-Carbajosa et al. (1997)
		ZmMADS47	CATGT	Qiao et al. (2016)
22-kD	α	O2	TCCACGTAG	Schmidt et al. (1992)
		PBF1	TGTAAAG	Vicente-Carbajosa et al. (1997)
		ZmMADS47	CATGT	Qiao et al. (2016)
27-kD	γ	O2	TTTACGTAG	Li et al. (2015)
		PBF1	TGTAAAG	Vicente-Carbajosa et al. (1997)
		OHPs	TTTACGTAG	Zhang et al. (2015)
		ZmbZIP22	ACAGCTCA	Li et al. (2018)
50-kD	γ	O2	TGACATGTAA	Qiao et al. (2016)
		ZmMADS47	CATGT	Qiao et al. (2016)

the first TF known to regulate the zein genes (Schmidt et al. 1987). The *o2* mutant has a well-known kernel phenotype, in which the biosynthesis of most zeins is reduced and the levels of non-zein storage proteins are increased (Mertz et al. 1964). This increases the relative abundance of the essential amino acids, lysine and tryptophan, thus significantly increasing the nutritional value of the kernels. The gene *O2* was cloned by transposon tagging and found to be specifically expressed in the developing endosperm (Schmidt et al. 1987). Studies using Chromatin Immunoprecipitation Sequencing (ChIP-seq) revealed that O2 directly activates almost all zein-coding genes, with the exception of the genes encoding 16-kD γ-zein; however, O2 has different levels of activation on the various zein gene promoters (Cord Neto et al. 1995; Li et al. 2015; Schmidt et al. 1990; Zhan et al. 2018).

The prolamin box is a highly conserved *cis* element (5′-TGTAAAG-3′) found in the promoters of many cereal seed storage protein genes, including most zein-encoding genes. Prolamin-box-binding factor 1 (PBF1), a DOF (DNA-binding one zinc finger)-type TF, is specifically expressed in the developing maize endosperm. It binds to the prolamin boxes of most zein genes in vitro and was therefore speculated to play a key role in their transcriptional regulation (Vicente-Carbajosa et al. 1997). Additionally, PBF1 interacts with O2, whose target site is 20 bp downstream of the prolamin box in the promoter of the gene encoding 22-kD zein. Surprisingly, the silencing of *PBF1* using RNAi resulted in a severe reduction in the abundance of 27-kD γ-zeins and a minor reduction of the 22-kD α-zeins (Wu and Messing 2012), indicating that the role of PBF1 in zein gene expression may be complex. Its specific role in endosperm development is largely unknown, however, due to the lack of available mutants.

OHP1 and OHP2 are two bZIP-type TFs first characterized as O2-heterodimerizing proteins (Pysh et al. 1993; Pysh and Schmidt 1996). Unlike O2, the OHPs are constitutively expressed in all maize tissues (Zhang et al. 2015). They bind to the O2-like box in the promoter of the gene encoding the 27-kD γ-zein, and their silencing leads to a significant reduction in the abundance of the 27-kD γ-zein proteins.

ZmbZIP22, another endosperm-specific bZIP-type TF, is a transcriptional activator of the 27-kD γ-zein-encoding gene and was identified using a DNA pull-down assay with the promoter of this gene followed by mass spectrometry (Li et al. 2018). ZmbZIP22 binds the ACAGCTCA box in the promoter of this gene, while in the *zmbzip22* mutant, the accumulation of 27-kD γ-zein is specifically reduced.

ZmMADS47 is a MADS-box-containing TF first characterized as an interacting partner of O2. ZmMADS47 controls the activation of the genes encoding α-zein and the 50-kD zeins, largely through its interaction with O2 (Qiao et al. 2016). Notwithstanding this interaction, ZmMADS47 shows a constitutive expression pattern rather than the endosperm-specific pattern of O2 expression, which is similar to that of the OHPs (Zhang et al. 2015).

The gene encoding the 16-kD γ-zein protein is unique because its promoter lacks both the prolamin box and the O2 box. The expression of this gene is not affected by O2 or PBF1. A recent study showed that two endosperm-specific NAC-type TFs, ZmNAC128 and ZmNAC130, could specifically activate the transcription of the 16-kD γ-zein-encoding

gene by directly binding to its promoter. These NAC TFs have been suggested to affect multiple processes, and their silencing by RNAi had a pleiotropic effect on the utilization of carbohydrates and amino acids in the endosperm. The accumulation of both zein and non-zein proteins was also affected in these RNAi kernels (Zhang et al. 2019).

Of the TFs regulating the zein genes, O2, PBF1, ZmbZIP22, ZmNAC128, and ZmNAC130 are all specifically expressed in the endosperm, while the OHPs and ZmMADS47 are constitutively expressed. This suggests that the endosperm-specific expression pattern of the zein-coding genes is regulated by other pathways in addition to the endosperm-specific TFs. The ectopic expression of O2 and PBF1 has been found to activate transgenic zein-encoding gene promoters, but not the endogenous zein-coding gene promoter (Wu and Messing 2012), an inconsistency that was assumed to be caused by the different methylation statuses of 22-kD α-zein-coding genes at their O2- and PBF1-binding sites. The endogenous 27-kD γ-zein promoter does not undergo methylation changes, but its regulators, the OHPs, are constitutively expressed and the 27-kD γ-zein gene is endosperm specifically transcribed, indicating the existence of other regulating pathways.

Coordinated regulation of zein expression by multiple TFs

TFs usually work coordinately by protein–protein interaction to intricately regulate transcription in space and time. For the TFs regulating the 27-kD γ-zein expression, most of them were found to be able to interact with others. For example, OHP1 and OHP2 are able to form homodimer or heterodimer with O2 when binding to the promoter of the 22-kD α-zein gene in gel mobility shift assays (Pysh et al. 1993). Pull-down assay indicated that PBF1 interacts with both O2 and OHPs (Vicente-Carbajosa et al. 1997; Zhang et al. 2015). ZmbZIP22 interacts with PBF1 and OHPs but not with O2, as determined by luciferase complementation image (LCI) assay (Li et al. 2018). Co-expression of these TFs in a luciferase activation assay showed that the absence of any one of these TFs did not significantly affect the activation of the gene encoding 27-kD γ-zein, suggesting that these TFs may form TF complex and function redundantly in their regulation (Li et al. 2018). The 27-kD γ-zein is not only a storage protein, but also plays a unique role in the formation of the PBs. This regulating model including five TFs is speculated to optimize the transcription of the 27-kD γ-zein-encoding gene in the face of diverse physiological or environmental challenges, promoting the successful formation of the PBs and the efficient accumulation of other zein storage proteins (Li et al. 2018).

Another example of a transcription complex regulating the expression of zein-coding genes is the interaction between ZmMADS47 and O2, as determined by Co-immunoprecipitation assay (Qiao et al. 2016), which enables ZmMADS47 to be recruited to its binding site on the zein promoters, next to the O2-binding site. This O2-ZmMADS47 protein–protein interaction most likely causes a conformational change in ZmMADS47, opening its C-terminal transactivation domain to enhance its transcriptional activity.

It is worth mentioning that *O2* and the *OHP* genes originated from an ancient duplication of a genome segment before the split of rice (*Oryza sativa*) and maize (Xu and Messing 2008), after which these genes diverged in both sequence and expression pattern. O2 is an endosperm-specific TF, while the OHP proteins are expressed in multiple tissues. These three bZIP TFs interact with each other (Zhang et al. 2015); however, their function in the transcriptional regulation of the zein genes also diverged during evolution. Both O2 and the OHPs regulate the α- and 27-kD γ-zein-encoding genes, but their activation ability also diverged; in the regulation of *α-zein* expression, O2 is the major TF, while for the regulation of the gene encoding the 27-kD γ-zeins, the OHPs are the major TFs (Yang et al. 2016).

The transcriptional regulation of the zein genes is part of the endosperm regulatory network

TFs have multiple targets, including genes encoding other TFs. In this fashion, they form transcription regulation networks to coordinate the various biological processes required for the successful accumulation of nutrients in the maize kernels. Zein content is an important part of the nutrient reservoir in the developing maize endosperm, and the regulatory pattern of zein genes involves a large TF network (Fig. 1).

O2 was first identified for its dominant role in the transcriptional regulation of zein-coding genes; however, a recent study revealed that O2 can function together with PBF1 to activate the promoters of *pyruvate orthophosphate dikinase 1* and *2* (the *PPDK* genes) and *starch synthase III* (*SSIII*), which encode critical components of the starch biosynthetic enzyme complex (Zhang et al. 2016). This suggests that O2 not only acts as a master regulator of the zein-coding genes, but also directly regulates carbon accumulation. An analysis of the transcriptomes of developing *o2* mutants combined with studies of the direct target genes of O2 using ChIP-seq indicated that O2 directly activates genes associated with a variety of nutrient storage processes and those encoding enzymes involved in the carbon and amino

acid metabolic pathways (Li et al. 2015; Zhan et al. 2018). Importantly, this TF also regulates the expression of genes encoding other TFs, such as G-box binding factor 1 (GBF1), Mybr13, bZIP17, and NAKED ENDOSPERM 2 (NKD2), which in turn regulate various other aspects of plant metabolism (Li et al. 2015; Zhan et al. 2018), indicating that the transcriptional regulation during endosperm development is coordinated in a TF cascade.

O2 itself works downstream of the other TFs. O11 is a central hub of the regulatory network for maize endosperm development and nutrient metabolism and also directly regulates the expression of genes encoding key TFs involved in endosperm development (NKD2 and ZmDOF3) and nutrient metabolism (O2 and PBF1) (Feng et al. 2018). NKD1 and NKD2 and both can directly activate the promoters of *O2* and *Viviparous1* and influence the expression of numerous genes. Of the 34,014 genes detected in aleurone cells, 2188 differentially expressed genes (DEGs) were identified in *nkd1 nkd2* mutant. For the starchy endosperm cells, the DEG number is 2193 out of 31,792 detected genes (Gontarek et al. 2016). Two redundant TFs ZmNAC128 and ZmNAC130 were also reported to have a pleiotropic effect on the utilization of carbohydrates and amino acids (Zhang et al. 2019). Knockdown of expression of the two TFs with RNAi caused shrunken kernel phenotype. Further analysis revealed that ZmNAC128/130 not only positively regulate the 16-kD γ-zein gene, but also participate in starch synthesis by regulating the expression of the gene encoding Bt2, a rate-limiting enzyme in starch synthesis. These TFs, O2, O11, and the NKDs regulate both endosperm development and nutrient metabolism, and their regulatory networks are linked via their mutual transcriptional regulation, suggesting that, during kernel maturation, endosperm development and nutrient accumulation are highly coordinated at the transcriptional level through a complex gene regulatory network.

PBF1 is involved in both protein and starch accumulation (Zhang et al. 2016) and represents the alleles typical of modern maize 4400 years ago (Jaenicke-Despres et al. 2003). The allelic diversity of *PBF1* in maize is lower than that in teosinte (*Zea mays ssp. parviglumis*), probably because of selection by early farmers. A better understanding of the gene regulatory network involving PBF1 may help us to understand the phenotypes associated with this protein in the future.

The translation of the zeins is affected by amino acid biosynthesis

Mutations in genes involved in amino acid biosynthesis can result in an altered zein accumulation and the production of an opaque endosperm. *Proline responding 1* (*Pro1*; also known as *Opaque6*) encodes a Δ^1-pyrroline-5-carboxylate synthetase that catalyzes the biosynthesis of proline from glutamic acid, a process that is significantly inhibited when *Pro1* function is lost (Wang et al. 2014b). This proline deficiency results in the increased accumulation of uncharged $tRNA^{pro\ AGG}$ and triggers the phosphorylation of eukaryotic initiation factor 2α (eIF2α) in the *pro1* mutant, leading to a general reduction in protein biosynthesis (Wang et al. 2014b). *Mutator-tagged opaque 140* (*mto140*) encodes an arogenate dehydrogenase (ZmAroDH-1), an enzyme involved in tyrosine and phenylalanine biosynthesis. There are four members of the maize arogenate dehydrogenase gene family, namely *ZmAroDH-1–4*, which share highly similar sequences. Both *mto140* and *zmarodh-3* mutant kernels contain an opaque endosperm (Holding et al. 2010). Like the *pro1* mutant kernels, which have inhibited proline biosynthesis, the *mto140* kernels also showed a general reduction in the accumulation of the zein storage proteins, rather than defects in the accumulation of specific groups of zeins. The characterization of the maize opaque endosperm mutants *pro1* and *mto140* suggested that amino acid(s) limitation directly represses zein protein biosynthesis.

Alterations in plant metabolism can also affect zein accumulation. The *opaque7* (*o7*) mutant was identified as a defective Acyl-CoA synthetase (Miclaus et al. 2011; Wang et al. 2011). Oxalyl-CoA decarboxylase1 (ZmOCD1), which functions downstream of O7, encodes an oxalyl-CoA decarboxylase, which catalyzes oxalyl-CoA, the product of O7, into formyl-CoA and CO_2 (Yang et al. 2018). The *zmocd1* mutant had an opaque endosperm, and both the *o7* and *zmocd1* mutations caused dramatic alterations in the endosperm metabolome, affecting endosperm development and the nutritional quality of the maize kernel. O7 and ZmOCD1 are involved in amino acid biosynthesis by affecting α-ketoglutaric acid and oxaloacetic acid, respectively. The corresponding deficiencies in amino acid biosynthesis in the *o7* and *zmocd1* mutants may be the reason for their decreased zein concentrations and, thus, their opaque kernel phenotypes.

Zeins and protein quality in maize kernel

The amino acid composition of zein proteins is unbalanced. In general, zeins are rich in glutamine and proline, but lacking in lysine and tryptophan. The α-zeins are severely lacking in three essential amino acids, lysine, tryptophan, and methionine (Osborne et al. 1914). Due to the leading expression level of α-zeins in maize kernel, the total lysine, tryptophan, and methionine levels in many maize cultivars are very low. The δ-zeins are also lacking in lysine and tryptophan, but rich in methionine (Wu et al. 2012). The methionine level can be elevated to a sufficient level in cultivars rich in δ-zeins (Messing and Fisher 1991; Wu et al. 2012).

Due to the lack of significant phenotype in the mutants of the two δ-zein genes, conventional breeding of maize accumulated many cultivars with low methionine (Wu et al. 2009). The 10-kD δ-zein gene is subject to posttranscriptional regulation (Chaudhuri and Messing 1994). A high level of 10-kD δ-zein can be obtained by the expression of a chimeric 10-kD δ-zein gene with substituted untranslated regions in its transcript (Lai and Messing 2002), indicating that the untranslated regions are the target of this regulation. Over-expression of the 10-kD δ-zein can indeed increase the methionine level, but with the expense of cysteines present in β- and γ-zeins, which is another sulfur-containing amino acid (Wu et al. 2012). Recent studies showed that higher accumulation of 10-kD δ-zein and total protein sulfur without reduction of other zeins can be achieved by either deregulated sulfate reduction capacity in the kernel (Planta et al. 2017), or enhanced S-assimilation in the leaf (Xiang et al. 2018).

The *opaque2* mutant is a promising candidate for maize protein quality improvement, especially for the increase in lysine and tryptophan levels. In this mutant, α-zeins decreased greatly while non-zein protein increased compensatorily, leading to the increase in lysine and tryptophan content and thus higher protein quality (Mertz et al. 1964). However, the soft opaque endosperm of *o2* kernels prevented its breeding promotion (Vasal et al. 1980). By accumulating quantitative trait loci (QTLs) in *o2* background, hard and vitreous *o2* varieties called Quality Protein Maize (QPM) were developed (Vasal et al. 1980; Gevers and Lake 1992). The QPM kernels kept the high lysine and tryptophan content as in *o2* mutant and also the hard and vitreous endosperm. The QPM has been introduced into many developing countries and improved the nutritional imbalance problem in these areas (Nelson 2001). Many efforts had been put to characterize the genetic basis of QPM. Zein content in QPM kernels was found different from that in either wild-type or *o2*. The 27-kD γ-zein accumulates much higher in QPM kernels (Geetha et al. 1991; Wallace et al. 1990). Further studies indicated that high content of 27-kD γ-zein has a strong correlation with kernel vitreousness in QPM (Lopes and Larkins 1991; Wu et al. 2010). Genetic analysis identified 7 *o2* modifier QTLs, which accounted for approximately 75% of the phenotypic variation. The *o2 modifier1* locating near the 27-kD γ-zein locus has a major effect on endosperm modification (Holding et al. 2008). A recent study using genome-wide association study analysis, linkage mapping analysis, and map-based cloning confirmed that the gene duplication of 27-kD γ-zein confers its enhanced expression and the endosperm modification in QPM (Liu et al. 2016).

The compensatory increase in non-zein protein in *opaque2* (Holding and Larkins 2009) kernels clearly indicates that proteome rebalancing occurs in maize. A similar phenomenon was also observed in dicotyledonous soybean (Schmidt et al. 2011). It seems that the seeds of plants, like maize and soybean, have the ability to possess compensatory mechanisms when the expression of some storage protein is interrupted, as reviewed by Wu and Messing (2014) and Herman (2014). The proteome analysis of *o2* mutant indicated that some specific proteins might participate in the rebalancing process (Jia et al. 2013). Among these proteins, eIF2 and GAPDH are rich in lysine. Their elevated translation was thought to make a substantial contribution to the overall lysine elevation in *o2* (Habben et al. 1993, 1995). Understanding the mechanism of proteome rebalancing may give clues on breaking the limit of protein ratio in the seeds.

Future perspectives

Advances in genomics, biochemistry, and high-resolution microscopy have dramatically expanded our knowledge of zein biosynthesis and PB formation since the turn of the millennium; however, several scientific questions are still unanswered.

Our knowledge of the regulation of zein gene expression could still be expanded. Although most zein genes are reported to be regulated by one or more TFs, mutants of the TF-encoding genes known to be involved in zein biosynthesis often show a reduced but not completely absent expression of their target zein-coding genes, suggesting the existence of uncharacterized TFs regulating the zein pathway. O2 and PBF1 are TFs both known to promote the biosynthesis of 19-kD α-zein; however, the *o2* and *pbf1-RNAi* mutant kernels still showed some expression of the 19-kD α-zein-encoding gene (Yang et al. 2016), indicating the existence of other TFs regulating this gene. Our recent characterization of the transcriptional machinery regulating the gene encoding the 27-kD γ-zein (Li et al. 2018) suggests that the transcriptional regulation of the zein genes is likely to be mediated by complexes of TFs, whose interactions and regulatory networks are also largely unclear. Future studies may focus on the identification of new TFs and the elucidation of their networks. Meanwhile, the tissue-specific methylation of the promoters of the zein-coding genes (Wu and Messing 2012) suggests that their expression involves other complex regulatory mechanisms. The role of DNA methylation in this regulation and whether other modifications, such as histone modifications and PcG complexes, are involved also warrant investigation.

Another important issue is how zein PBs are formed and how zeins are localized and packed into PBs. In maize endosperm, zeins are retained in the lumen of RER rather than forming a storage protein vacuole as is the case in other cereals (Herman and Larkins 1999). The cloning of *O1*, *Fl1*, and *O10* revealed important information about the regulation of the transportation of the zeins from the ER to the

PBs and their assembly within the PBs (Holding et al. 2007; Wang et al. 2012; Yao et al. 2016); however, there are still gaps in our understanding of these processes, and the formation of zein-containing PBs has yet to be fully elucidated. An analysis of the PB proteome revealed many non-zein proteins associated with these bodies (Wang et al. 2016). Characterization using high-resolution mass spectrometry and functional analyses of these non-zein proteins making up the protein body may help to further address these questions in the future.

With the recent advance in the regulation of zein expression, new information in the mechanism of QPM and proteome rebalancing, as well as new methods in molecular genetics, it becomes feasible to manipulate the kernel nutrient composition without losing its good agronomic traits. The expression of zein protein affects seed protein quality. By taking advantage of CRISPR-Cas9-based gene editing, zein expression can be selectively reduced by deleting some zein-coding genes. Comparing with *o2* mutant, this approach does not bring other pleiotropic effects to maize seed development. The high expression level of the 27-kD γ-zein contributes to the vitreous endosperm in QPM. It is possible to over-express this zein in other opaque endosperm mutants with high nutrient quality, to convert the opaque endosperm to vitreous one. The δ-zeins are important sulfur sink in maize seeds. In combining the over-expression of the δ-zein and modifying genes involved in S-assimilation, a high accumulation of methionine can be archived in the kernel. These approaches can be combined to produce a more nutritious variety for corn breeding.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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