# The ZmbZIP22 Transcription Factor Regulates 27-kD γ-Zein Gene Transcription during Maize Endosperm Development

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**Review timeline:**

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|---|---|---|
| **TPC2018-00422-RA** | Submission received: | May 31, 2018 |
| | 1st Decision: | July 15, 2018 *revision requested* |
| **TPC2018-00422-RAR1** | 1st Revision received: | Aug. 13, 2018 |
| | 2nd Decision: | Sept. 2, 2018 *accept with minor revision* |
| **TPC2018-00422-RAR2** | 2nd Revision received: | Sept. 5, 2018 |
| | 3rd Decision: | Sept. 12, 2018 *acceptance pending, sent to science editor* |
| | Final acceptance: | Sept. 19, 2018 |
| | Advance publication: | Sept. 21, 2018 |

**REPORT:** (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

**TPC2018-00422-RA 1st Editorial decision – *revision requested* July 15, 2018**

Given the importance of y-zein in developing high lysine maize, the reviewers collectively agree that more data needs to be provided with respect to the following (Reviewer comments and **author responses)**:

Point 1. Is the bZIP22 mutant opaque?

**RESPONSE: We apologize for the omission. The *zmbzip22* mature kernels showed vitreous endosperm and cannot be distinguished from wild-type kernels by visual inspection. For phenotype analysis, wild type and *zmbzip22* mature kernels were taken from the same F2 ear. An image of mature *zmbzip22* and wild-type kernels over a light box was provided in this revision as Figure 4D. Images of transverse and sagittal sections of the mature kernels were also provided in Figure 4E.**

Point 2. What is the influence on protein bodies? several reviewers felt that the TEM image provided was not sufficient to support your conclusions and specifically recommend providing images over a light box, as well as transverse and sagittal sections of mature kernels.

**RESPONSE: Thanks for the suggestion. The previous TEM images were replaced by new images with higher resolution. More details of the protein bodies could be observed with the additional enlarged sections (Figure 5D). We also calculated the number and size of the protein bodies in the cells of the fourth layer from the aleurone layer (Figure 5E-F). The results were added to this revision. These new data indicated that the *zmbzip22* mutation affected PB structure and PB number, but not PB size. Please see our response to reviewer #1 and reviewer #2 for details.**

**The images over a light box, and transverse and sagittal sections of mature kernels, were provided as Figure 4D-E..**

Point 3. A more precise statistically relevant measurement of starch content, particularly given the influence on sh2 and bt2 expression, and inclusion of the raw data.

**RESPONSE: Thanks for the suggestion. For starch quantification, endosperm of 20 mature kernels for each of the wild type and *zmbzip22* from the same segregating ear was pooled. Amylose was also quantified according to a previously described method (Wang et al. 2011). Three biological replicates were made with the quantification of three different segregating ears. The significance was calculated using paired t test.**

**A description of the quantification of the total starch and amylose was added to this revision as follows:**

***"Starch and amylose content was also quantified in mature zmbzip22-mu9 endosperm. The total starch in the mutant endosperm decreased slightly. The amylose ratio in total starch showed a slight increase in zmbzip22-mu9 (Figure 6B, Supplemental Dataset 1). "***
**Please see our response to reviewer #3's comment 4 for details.**

Point 4. Consider experiments regarding this transcription factor's influence on lysine and tryptophan content in the kernel.

**RESPONSE: Thanks for the suggestion. Zein proteins lack lysine, tryptophan and methionine (Mertz et al., 1964). The altered proportion of zein and non-zein proteins in *zmbzip22* may affect the amino acid content and quality in the endosperm. According to this analysis, lysine levels increased ~8%, tryptophan increased ~4% and methionine increased 3% in *zmbzip22-mu9* endosperm, respectively (Figure 6C). Please see the response to reviewer #1's comment 1.**

Point 5. Additionally, both reviewers #1 and #3 provided specific comments with respect to how your findings will lead towards an advances in altering kernel amino acid content and quality. Discussion of the combinatorial nature of this factor, along with others in regulating the really exceptional magnitude of expression of this gene (y-zein), should also be considered as you prepare your revision.

**RESPONSE: Thanks for the suggestion. The increase of lysine and tryptophan levels in *zmbzip22* endosperm may improve its protein quality. Meanwhile, the kernel of *zmbzip22* is hard and vitreous, making it a potential quality protein resource. However, the rise of lysine and tryptophan content in *zmbzip22* was limited, not enough to reach the protein quality goal via a single gene mutation. However, in combination with other mutants with similar effects (slightly increased protein quality without causing opaque endosperm), this could be a promising route towards optimization of amino acid balance in maize endosperm storage proteins. Possible candidates for such combination could be OHPs and ZmMADS47, whose mutation decreased zein content but without visible changes to kernel texture (Zhang et al. 2015; Qiao et al, 2016). Discussion about the changes in amino acid content and quality was added. Meanwhile, discussion about the nature of TFs, including ZmbZIP22, PBF1, OHPs and O2, co-regulating the vast expression of 27-kD γ-zein was re-written according to the reviewers' advice. Please see our response to Reviewer #1 and #3.**

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

**TPC2018-00422-RAR1 1st Revision received Aug. 13, 2018**

Reviewer comments and **author responses**:

Reviewer #1:

The manuscript by Li et al. characterized a new transcription factor, ZmbZIP22, in maize endosperm that regulates the 27-kD gamma-zein gene besides the already known O2, PBF1, OHP1 and OHP2. Li et al. identified the motif recognized by ZmbZIP in the promoter and demonstrated the transactivation by this TF. They created null mutants by CRISPR/Cas9, in which the accumulation of the 27-kD gamma-zein protein was mildly but apparently reduced. They showed that ZmbZIP22 was able to interact with PBF1, OHP1 and OHP2, but not O2. Through the RNA-Seq and Chip-Seq technology, they characterized a few genes that were directly regulated and bound by ZmbZIP. These findings are interesting, but the novelty is not very strong.

Point 1. Although ZmbZIP was identified as a new TF, the mutants had only a limited effect on 27-kD gamma-zein gene expression. It was unclear whether it affected the total zein protein level. The protein-protein interactions of ZmbZIP with PBF1, OHP1 and OHP1 were not unexpected. It was an incremental rather than novel discovery about the current mechanisms of zein gene regulation.

**RESPONSE: Thanks for the suggestion. In this revision, quantification results for the total protein, zein and non-zein content in *zmbzip22* endosperm were added. The results indicated that zein protein levels in *zmbzip22-mu9* endosperm had a noticeable decrease, while non-zein protein levels increased compared with wild type, resulting in the unchanged content of total protein. Description of the protein quantification was added in this revision as follows: *"Protein content was quantified in mature zmbzip22-mu9 and wild type endosperm from the same segregating ears to determine if decreased PB number caused quantitative changes in zein proteins and non-zein proteins. The results indicated that zein proteins in zmbzip22-mu9 endosperm decreased significantly while non-zein proteins increased compared with wild type, resulting in the unchanged content of total proteins (Figure 6A). "***

**Zein proteins lack lysine, tryptophan and methionine (Mertz et al., 1964). The altered proportion of zein and non-zein proteins in *zmbzip22* may affect the amino acid content and quality in the endosperm. In this revision, total amino acid (TAA) content was quantified using a method described previously (Wang et al. 2011), and total tryptophan was determined using a previously described protocol (Hernández H, 1969). According to this analysis, lysine levels increased ~8%, tryptophan increased ~4% and methionine increased 3% in *zmbzip22-mu9* endosperm compared to wild type.**

**Our work not only discovered a new TF for a major class of zeins, but it also proved that the regulation of zein coding genes is complex. The multi-factor regulatory mechanism of zein genes may secure steady nutrient accumulation. Loss of function of any factor will only cause a mild change to its gene expression. In lines with mutations in a combination of multiple factors, a more significant and desirable change might be obtained. The mutation of *zmbzip22* slightly changed the amino acid quality without causing opaque endosperm. If mutations in OHPs or ZmMADS47 were combined with *zmbzip22*, a more significant and desirable change could be expected.**

**In the Discussion section, we added paragraph about nutrient quality: *"Zein, accounting for 70% of the storage protein in maize endosperm, are lacking of lysine and tryptophan. Reduction of zein content in the endosperm can increase the lysine and tryptophan, but usually causes irregular PBs and opaque endosperm (Wu and Messing, 2010b; Guo et al., 2013). In zmbzip22 mutant kernels, zein content had an apparent decrease while non-zein protein increased, resulting in unchanged total protein content (Figure 6A). The reduction in zein content was mainly due to the decreased accumulation of 27-kD γ-zein (Figure 5A). Of the amino acids making up 27-kD γ-zein protein, there are no lysine and tryptophan, and methionine accounts for only 0.9% in frequency. Mutation of zmbzip22 elevated the ratio of non-zein in storage protein, thus increased the total content of lysine (~8%), tryptophan (~4%) and methionine (~3%) (Figure 6C). Meanwhile, in zmbzip22, the specific reduction of 27-kD γ-zein did not cause opaque endosperm. The vitreous phenotype of zmbzip22 is consistent with previous studies that sole loss of 27-kD γ-zein was insufficient to cause opacity (Wu and Messing, 2010a; Wu and Messing, 2010b; Zhang et al., 2015). Although the rise of lysine and tryptophan content in zmbzip22 was limited, not enough to reach protein quality goal by a single gene mutation. However, in combination with other mutants with similar effects (slightly increase protein quality without causing opaque endosperm), this could still be a promising route towards optimization of amino acid balance in maize endosperm storage proteins. Possible candidates for such combination could be OHPs and ZmMADS47, whose mutation decreased zein content but without visible change to kernel texture (Zhang et al. 2015; Qiao et al, 2016). "***

Point 2. ZmbZIP was shown to regulate *Bt2* and *Sh2*. Were there any phenotypic abnormalities in endosperm development? The value of a gene is mainly reflected by its new mechanisms and mutant phenotypes. Mutations in *bt2* and *sh2* caused a dramatic reduction in starch synthesis and severe phenotypes in the endosperm. In this work, the expression of the two genes exhibited a significant reduction in *zmbzip* mutants, but the total starch content decreased only slightly. Apparently, ZmbZIP is not a critical TF for maize endosperm development.

**RESPONSE: Thanks for the suggestions and comments! No phenotypic abnormality was observed in *zmbzip22* during endosperm development. The transcription of *Bt2* and *Sh2* showed an 18% and 12% reduction in 15 DAP *zmbzip22* endosperm. However the change in total starch content in the mutant was not as expected. An AGPase complex composed of Bt2 and Sh2 could be activated by 3-phosphoglyceric acid (3-PGA) and inhibited by phosphate (Dickinson and Preiss, 1969; Hannah, 1997). The effect of the reduction at transcription level could be**

**compensated by the regulation at the post-translational level.**

**In the Discussion section, we added the following comments: *"In zmbzip22, we observed 4 DEGs encoding enzymes in the starch biosynthetic process. The two major AGPase subunits in endosperm, Bt2 and Sh2 showed 18% and 12% reduction at transcription level in 15 DAP zmbzip22 endosperm. However the change of total starch in mutant was not as expected (Figure 6B left panel). AGPase complex composing of Bt2 and Sh2 is reported to be activated by 3-phosphoglyceric acid (3-PGA) and inhibited by phosphate (Dickinson and Preiss, 1969; Hannah, 1997). The effect of the reduction at transcription level could be compensated by the regulation at the post-translation level. "***

**In this study, ZmbZIP22 was proven to be one of the components of the transcriptional activation machinery for the 27-kD γ-zein gene. Mutation of *ZmbZIP22* affected protein body initiation and altered amino acid quality without causing opaque endosperm (Figure 5D-F, Figure 6C). Although the rise in lysine and tryptophan content in *zmbzip22* was limited, this may be a route towards optimization of amino acid balance in maize endosperm storage proteins.**

Point 3. Li et al. conducted RNA-Seq and Chip-Seq to investigate the gene network regulated by ZmbZIP. They identified 1,765 DGEs by RNA-Seq and 514 ZmbZIP binding genes. However, only four genes including the 27-kD gamma-zein gene were eventually verified to be directly bound and modulated by ZmbZIP. I am very concerned about the reliability of this approach.

**RESPONSE: We fully understand you concern. By comparing the 1,765 DEGs and 514 ZmbZIP22 binding genes, 33 genes were both among the DEGs and bound by ZmbZIP22 at genic regions. These 33 genes were credible candidate target genes. However, due to the limitation of methods for functional validation, we could only examine the genes with down-regulated expression and with binding sites falling in the promoter or 5'UTR regions. To satisfy such criteria, only five genes (including the 27-kD γ-zein gene) were qualified for further functional validation. Among them, four genes were confirmed to be activated by ZmbZIP22 using the described assay in this study. The candidate genes with ZmbZIP22 bound at other regions of the gene body may also be directly regulated by ZmbZIP22. However, so far, there's no effective method to test them.**

Point 4. Lines 195-197, can formation of a homo-dimer exclude the possibility that ZmBZIP may not require other proteins for DNA binding and transactivation?

**RESPONSE: Sorry for the confusion. The formation of a homo-dimer does not exclude the possibility that ZmbZIP22 may or may not require other proteins for DNA binding and transactivation. TFs of the bZIP type function as homo- or hetero-dimers to bind DNA (Deppmann et al., 2006). By saying "additional proteins may not be required for ZmbZIP22 to bind DNA and activate downstream genes", we were trying to state that when ZmbZIP22 was expressed solely, it had the ability to form homo-dimer and bind to target DNA. The sentence has been replaced with a more accurate description as follows: *"As a bZIP protein, ZmbZIP22 was predicted to form a homo-dimer (Deppmann et al., 2006). This was confirmed by a luciferase complementation image (LCI) and Y2H assay (Figure 3B-C). Consequently, when expressed solely, ZmbZIP22 may be able to bind DNA and activate downstream genes by forming homo-dimer."***

Point 5. In Figure 4G, quantitative RT-PCR is required to show that the mRNA levels are reduced in *zmbzip*.

**RESPONSE: Thanks for the suggestion! Quantitative RT-PCR detecting the expression of 27-kD γ-zein gene was performed. The result was added to the main manuscript as Figure 5C and Supplemental Figure 6. We added the following sentences to the main text of the revised manuscript: *"To determine if the decrease in 27-kD γ-zein gene transcription caused the reduction in 27-kD γ-zein accumulation, 27-kD γ-zein gene transcription was quantified in 15 DAP wild type and zmbzip22-mu9/10 endosperm by qPCR. The 27-kD γ-zein transcription reduced to ~70%, indicating the reduction in accumulation was caused by reduced transcription of the 27-kD γ-zein gene (Figure 5C, Supplemental Figure 6). "***

Point 6. Lines 543-545, zein proteins are storage proteins. Is there any reference paper reporting that the zein transcription needs to be fine-tuned to an optimal level? In other words, is the fine difference in zein contents important for maize endosperm development?

**RESPONSE: Thanks for the question. According to previous studies, an optimal level of zein gene transcription is crucial for maize endosperm development. Zeins are not randomly distributed in the endosperm cell but are**

**efficiently packaged as protein bodies (PBs) through protein-protein interactions (Hurkman et al., 1981). Moreover, PBs play a central role in the formation of vitreous endosperm (Holding, 2014). Suppression of particular types of zeins by RNAi resulted in abnormal PB and opaque endosperm, indicating that the proper level of each type of zein is critical to develop normal PBs and vitreous endosperm. During endosperm development, the transcription of α-zein showed "up-and-down" oscillating expression patterns, clearly indicating that α-zein gene expression is precisely regulated during endosperm development (Feng et al. 2009). Meanwhile, the activity of O2, a master TF of most zein genes, is regulated by diurnal phosphorylation modification, indicating that zein transcription is regulated diurnally (Ciceri et al., 1997). All of the evidence indicates that zeins are subjected to fine-tuned regulation, and the fine difference in zein content should be important for maize endosperm development.**

**In our revised manuscript, we added the following paragraph: *"There is evidence indicating that zeins are subjected to fine-tuned regulation, and the fine difference in zein content would be important for maize endosperm development. In endosperm, zeins are efficiently packaged as protein bodies (PBs) through protein-protein interaction (Hurkman et al., 1981). Moreover, PBs play a central role in the formation of vitreous endosperm (Holding, 2014). Suppression particular type of zeins by RNAi resulted in abnormal PB and opaque endosperm, indicating the proper level of each type of zeins is critical to develop normal PB and vitreous endosperm. During endosperm development, the transcription of α-zein showed "up-and-down" oscillating expression patterns, clearly indicating that α-zein gene expression was precisely regulated during endosperm development (Feng et al. 2009). Meanwhile, O2 as a master TF of most zein genes, its activity was regulated by diurnal phosphorylation modification, indicating zein transcription is regulated diurnally (Ciceri et al., 1997). ZmbZIP22, O2 and the OHPs are all transcriptional activators, and they act together to maintain a high expression level of the 27-kD γ-zein gene. Negative regulator might be needed to modulate these activators to balance the gene expression. PBF1's repression property may be important to fine-tune the zein transcription to an optimal level. "***

Reviewer #2:

27-kD γ-Zein is the most abundant protein encoded by a single gene in maize endosperm. Along with alpha zeins, it makes up a large proportion of storage protein capacity in maize. Aside from its storage capacity, the protein in known to have distinct roles in initiating protein body formation and thus, in the generation of the vitreousness of the mature endosperm. Even greater accumulation of the protein is known to be an essential part of *opaque-2* modification in maize protein quality. The massive strength of the 27-kD γ-Zein promoter makes it the most widely used promoter in endosperm transgenic experiments. Despite the fact that four different transcription factors (PBF, OHP1 and 2 and Opaque2) are known to act synergistically and semi redundantly in 27-kD γ-Zein transcription, transcription is not completely abolished by their absence. This prompted the authors to hypothesize the existence of additional transcription factors and methodically go about screening them and thoroughly functionally characterizing a resulting factor: ZmbZIP22. This transcription factor increases 27-kD γ-Zein expression and interacts with PBF and OHP but not Opaque-2. The paper is complete and well written and the subject matter is of broad enough significance to a general plant biology audience, as it demonstrates the evolution of massive level gene expression from a single gene. Furthermore, it is a good exercise in utilizing a multi-pronged suite of gene discovery and promoter bashing experimentation while optimizing them and incorporating genomics tools.

Point 1. I invite the authors to consider if coordinating is an appropriate word in the title. It implies that this transcription factor has a more important or overarching role than the other known transcription factors. I don't this is the intention or that such a more significant role has been demonstrated. Suggest saying 'contributing to'

**RESPONSE: Thanks for the comment and suggestion. The title has been revised accordingly as: *"ZmbZIP22 is a Transcription Factor for 27-kD γ-Zein Gene Transcription during Maize Endosperm Development"*.**

Point 2. Line 50. The 'Holding and Larkins' citation is one of several appropriate reviews to cite. Suggest adding more and stating that these are reviews e.g. Holding and Messing 2013, Seed Genomics and maybe one other.

**RESPONSE: Thanks for the suggestion. Citations were added to this revision accordingly as follows: *"The complexity of the zein gene family has been well described in several reviews (Thompson and Larkins, 1994; Holding and Larkins, 2009; Holding and Messing, 2013)."***

Point 3. Line 56. Could insert "an exception to this is the duplication of the 27-kD γ-Zein gene in Quality Protein Maize which has even higher levels of the protein" and cite Yongrui Wu's paper.

**RESPONSE: Thanks for the suggestion. The manuscript has been revised accordingly as "*The β-, γ- and δ-types of zeins are usually coded by a single gene. An exception to this is the duplication of the 27-kD γ-zein gene in Quality Protein Maize (QPM) which has even higher levels of the protein (Liu et al., 2016).*"**

Point 4. Line 121. Please explain how the transcription start site was determined. Was this previously experimentally determined or is it assumed from the cDNA length?

**RESPONSE: We apologize for the omission. The determination of the transcription start site was based on the S1 nuclease mapping result reported by Ueda and Messing (Ueda and Messing, 1991). We inserted the following sentence to the text: *"The TSS was determined according to the S1 nuclease mapping result reported previously (Ueda and Messing, 1991)."***

Point 5. Line 185. Expression is high until at least 33 DAP, not 24 as stated. Do the authors believe the slight dip in expression at 24 DAP is real?

**RESPONSE: Thanks for the comment. This part of the manuscript was re-written accordingly as follows: *"The results indicated ZmbZIP22 is endosperm specifically expressed, begins to accumulate at 9 DAP, and maintains a high level of expression from 15 ~ 33 DAP."***

Reviewer #3:

This study discovers and analyzes the function of a bZIP transcription factor in maize, ZmbZIP22, that regulates expression of the gene encoding 27-kD γ-zein. To my knowledge the results are novel, in the regard that ZMbZIP22 has not been previously described, and the fact that 27-kD γ-zein is regulated by this TF in addition four previously known TFs active at this promoter is presented here for the first time.

The results are comprehensive, including 1) identification of the target binding site in the 27-kD γ -zein gene promoter, 2) purification of the protein ZmbZIP22 that binds to the target, 3) verification of protein binding to the target DNA sequence using recombinant protein, 4) characterization of the tissue-specific, temporal, and subcellular expression pattern of ZmbZIP22 (it is endosperm specific and present during grain fill), 5) demonstration of homodimerization, 6) demonstration of TF function in yeast, 7) demonstration of TF activity towards 27-kD γ -zein in heterologous plant cells, 8) generation and confirmation of mutant maize lacking ZmbZIP22, 9) characterization of the effects of the mutation on 27-kD γ-zein expression (very clear demonstration of decrease but not complete loss) and PB and starch formation (see comments below), 9) RNA-Seq on the ZmbZIP22 mutant, identifying DEGs, 10) ChIP-Seq on ZmBZIZP22-bound genomic DNA, finding some 30 genes that are both DEG and bound by the TF, 11) more trans-activation assays in tobacco of other target genes besides 27-kD γ-zein, and 12) binding of ZmbZIP22 to the other known TFs active at the 27-kD γ-zein gene promoter, and 13) combinatorial transactivation assays with the other TFs (O2, PBF, OHP). So, an impressively comprehensive study, most but not all of it very convincing. There's too much here for me to review every aspect of the technical details, but in general it looks good. I quibble with two of the findings in the comments below.

Point 1. A major question - is the mutant opaque? This must be discussed whether it is or it isn't, either way. The reason to do this study is to learn about protein and amino acid balance in maize grain (although this fact is not well addressed, see minor comment below). Loss of 27-kD γ-zein improves amino acid balance but makes the grain opaque, soft and not useful in the large scale, which is why so much work has been done on the expression of this gene. The paper essentially ignores this aspect of the significance of the work, and the omission is obvious. My guess is there is no kernel phenotype and this is why the question is not addressed. But this is not a fatal flaw, in fact we learn that 27-kD γ-zein levels can be partially reduced without major effects on PBs. Maybe this could be a positive feature towards optimization of amino acid balance?

**RESPONSE: Thanks for the question and comment. We apologize for the omission. The *zmbzip22* mature kernels showed vitreous endosperm and cannot be distinguished from the wild type kernels by visual inspection. Please see our response to reviewer 2's comment 16 for a discussion of kernel phenotype.**

**Quantification results for the total protein, zein and non-zein content in *zmbzip22* endosperm were added. The results indicated that zein proteins in *zmbzip22-mu9* endosperm showed a noticeable decrease while non-zein proteins increased compared with wild type, resulting in the unchanged content of total protein. Total amino acid (TAA) content was quantified. Lysine levels increased ~8%, tryptophan increased ~4% and methionine increased 3% in *zmbzip22-mu9* endosperm, respectively. Please see our response to reviewer 1's comment 1 for details.**

**The mutation of *zmbzip22* slightly changed the amino acid quality without causing opaque endosperm. In a combination of multiple factor mutations, a more significant and desirable change might be obtained. If mutations in OHPs or ZmMADS47 were combined with *zmbzip22*, a more significant and desirable change could be expected.**

**In the Discussion section, we added a paragraph about nutrient quality: *"Zein, accounting for 70% of the storage protein in maize endosperm, are lacking of lysine and tryptophan. Reduction of zein content in the endosperm can increase the lysine and tryptophan, but usually causes irregular PBs and opaque endosperm (Wu and Messing, 2010b; Guo et al., 2013). In zmbzip22 mutant kernels, zein content had an apparent decreased while non-zein protein increased, resulting in unchanged total protein content (Figure 6A). The reduction in zein content was mainly due to the decreased accumulation of 27-kD γ-zein (Figure 5A). Of the amino acids making up 27-kD γ-zein protein, there are no lysine and tryptophan, and methionine accounts for only 0.9% in frequency. Mutation of zmbzip22 elevated the ratio of non-zein in storage protein, thus increased the total content of lysine (~8%), tryptophan (~4%) and methionine (~3%) (Figure 6C). Meanwhile, in zmbzip22, the specific reduction of 27-kD γ-zein did not cause opaque endosperm. The vitreous phenotype of zmbzip22 is consistent with previous studies that sole loss of 27-kD γ-zein was insufficient to cause opacity (Wu and Messing, 2010a; Wu and Messing, 2010b; Zhang et al., 2015). Although the rise of lysine and tryptophan content in zmbzip22 was limited, not enough to reach protein quality goal by a single gene mutation. However, in combination with other mutants with similar effects (slightly increase protein quality without causing opaque endosperm), this could still be a promising route towards optimization of amino acid balance in maize endosperm storage proteins. Possible candidates for such combination could be OHPs and ZmMADS47, whose mutation decreased zein content but without visible change to kernel texture (Zhang et al. 2015; Qiao et al, 2016). "***

Point 2. The comments below point out that I feel discussion of DEGs without specific experiments to back up the points are entirely unwarranted. There is so much data here that a very comprehensive study can be presented without undue speculation. So in my view, some major paring down of the manuscript is necessary. Fig. 3D,E, and discussion of ZmbZIP22 structure: Further information is needed regarding the structure of the protein. I am not an expert in this subject, so I would have liked to learn where is the dimerization domain and where is the DNA binding domain. This is pertinent to Fig. 3D, E that implies residues 1-332 are entirely sufficient to activate transcription. Does the blue box in Fig. 3D indicate the specific residues of the leucine zipper? If not, what does the blue box represent? And if so, what is the implication that residues 1-332, lacking the leucine zipper, can activate the reporter? Further detail of the domains in Fig. 3D would be useful to address these questions.

**RESPONSE: We apologize for the confusion. More details of ZmbZIP22 structure have been added to the revised manuscript. The protein sequence of ZmbZIP22 was subjected to domain annotation by CDD/SPARCLE (Marchler-Bauer et al., 2017). The basic leucine zipper (bZIP) domain was 332-387 a.a. The basic region of the bZIP domain was 332-355 a.a. and the leucine zipper region was 354-387 a.a., as shown in Figure 3D upper panel. For bZIP protein, the basic region is responsible for the DNA binding activity while the leucine zipper region is responsible for the dimerization (Jakoby et al., 2002).**

**For the yeast activation assay in Figure 3E, residues 1-332 were fused with DNA binding domain of the GAL4 transcription factor (GAL4-BD). The GAL4-BD was originally built in the effecting vector pGBK-T7. If 1-332 had activation activity, the fusion protein would bind to the reporting promoter with the aid of GAL4-BD and activate the reporter gene.**

**We re-wrote the corresponding paragraph as follows: *"The protein sequence of ZmbZIP22 was subjected to domain annotation by CDD/SPARCLE (Marchler-Bauer et al., 2017). The basic leucine zipper (bZIP) domain was from 332 ~***

***387 a.a. The basic region of the bZIP domain was from 332 ~ 355 a.a. and the leucine zipper region was from 354 ~ 387 a.a., as shown in Figure 3D upper panel. The basic region is responsible for the DNA binding activity while the leucine zipper region is responsible for the dimerization (Jakoby et al., 2002). To investigate if ZmbZIP22 has transactivation activity, a yeast transactivation assay was performed (Ye et al., 2004; Li et al., 2006). The full-length ZmbZIP22 open reading frame (ORF) was cloned into the pGBK-T7 vector. Meanwhile, to further locate the activation domain of ZmbZIP22, the protein was truncated as shown in Figure 3D. The truncated coding sequences were also cloned into the pGBK-T7. The pGBK-T7 vector contains the DNA binding domain of GAL4 transcription factor (GAL4-BD), which was fused at the C-terminal of the protein inserted. The vectors were separately co-transformed into the EGY48 yeast reporter strain with the pG221 vector. pG221 contains a β-galactosidase reporter gene with a minimum promoter that can be bound by the DNA binding domain of GAL4 (GAL4-BD) expressed from the pGBK-T7 vector. "***

Point 3. Fig. 4G and lines 268-271: I disagree that there is a significant effect on PBs. The wild-type image is more darkly stained than the mutant. So the conclusion that the PB periphery is thinner is not justified. The statement that "PBs were uneven" is non-specific, i.e., has no meaning. The shape and size of the PBs do not appear to differ between wild type and mutant. Importantly, is the endosperm opaque, as occurs in RNAi-induced loss of 27-kD γ-zein? This is not stated but is a critical point.

**RESPONSE: Thanks for the comment. The previous TEM images were replaced by new images with higher resolution. More details of the protein bodies could be observed with the additional enlarged sections (Figure 5D). We also calculated the number and the size of the protein bodies in the cells of the fourth layer from the aleurone layer (Figure 5E-F). The results were added to this revision. We believe the high resolution TEM images and the analysis of PB number and size in this revision should support our statement about the protein bodies in this study. Please see our responses to reviewer #1 and reviewer #2 for details. Images of *zmbzip22* mature kernels over a light box and transverse and sagittal sections of mature kernels were provided as Figure 4D-F.**

**In this study, the 27-kD γ-zein gene was proven to be the only zein gene regulated by ZmbZIP22. Therefore, only the level of 27-kD γ-zein showed an apparent decrease in *zmbzip22*. The vitreous phenotype of *zmbzip22* is consistent with previous observations that the sole reduction of 27-kD γ-zein was insufficient to cause opacity (Wu and Messing, 2010a; Wu and Messing, 2010b; Zhang et al., 2015). Discussion of the *zmbzip22* phenotype has been provided in this revision. Please see our response to reviewer #3's major question.**

Point 4. Supplemental Fig. 6: I do not believe that such a small decrease in starch content has ever been shown to be statistically significant in many hundreds of papers making such measurements. With n=3, I simply do not believe these results. Dozens of biological replicates would be required to even approach statistical significance. If the authors wish this to be accepted, the raw data would need to be included in the supplemental data.

**RESPONSE: Thanks for the suggestion. For starch quantification, endosperm of 20 mature kernels for each of the wild type and *zmbzip22* from the same segregating ear was pooled. Amylose was also quantified according to a previously described method (Wang et al. 2011). Three biological replicates were made with the quantification of three different segregating ears. The significance was calculated using paired t test. Description of the total starch and amylose content in *zmbzip22* was re-written in this revision as follows:**

***"Starch and amylose content was also quantified in mature zmbzip22-mu9 endosperm. The total starch in the mutant endosperm decreased slightly. The amylose ratio in total starch showed a slight increase in zmbzip22-mu9 (Figure 6B, Supplemental Dataset 1). "***

Point 5. Lines 673-680: In what inbred background were the mutants analyzed? How many backcrosses after the Hill transformation? This is essential information: why was it not specified in the Methods section? Were the DEG data obtained from congenic lines? If not, they probably mean nothing.

**RESPONSE: We apologize for the omission. After the Hill transformation, the *zmbzip22* was backcrossed for more than 5 generations to W22 genetic background. A description of the inbred background has been added to the Method section accordingly as follows: *"zmbzip22-mu9 and zmbzip22-mu10 were backcrossed to W22 genetic background for at least five generations. The segregating F2 ears were used in this study. "***

**For RNA-Seq, immature endosperm tissue from individual kernels (15 DAP) were harvested from the segregating F2 ears as described above. In the Method section, we added the following sentences: *"For RNA-Seq, immature***

***endosperm tissue from individual kernels (15 DAP) were harvested from the same F2 ear. The embryo from the same kernel was subjected to DNA extraction and genotyping to determine the wild type or zmbzip22 kernels. The endosperm from 20 kernels of wild type or zmbzip22 were pooled for RNA isolation. Replicates were made using endosperm from three F2 ears. "***

Point 6. Lines 273-319: This discussion is far too speculative to merit publication in Plant Cell. Any of the DEGs may be significant or not and there is no information to support any of the speculation. There are some starch genes affected and some minor (if any) effect on starch, but there is no support for any functional effect in these genes or any others. This should all be left to a table of observations; all the discussion is meaningless without further experimentation. The discussion should center on those genes that are DEGs in the mutant and also promoter-bound by the TF. There should be a table of these genes published in the paper, not in the supplemental data set. The discussion on lines 364-366 is confusing. There are 33 genes that meet both criteria (DEG and promoter-bound) and four of these show reduced transcription. Does this mean the other 29 have increased transcription? Please do not make the reader dig into a supplemental data set to understand what is being discussed; make it clear in the text of the paper.

**RESPONSE: Thanks for the comment and suggestion. The part of discussion about DEGs has been pared down or re-written. Please see the Discussion section in our revised manuscript. The table of the 33 candidate target genes has been moved to the main manuscript as Table 2. The criteria for candidate targets was that DEGs bound by ZmbZIP22 at the genic region (including promoter, UTR, intron, exon and terminator). Of the 33 candidate target genes, ten genes were bound by ZmbZIP22 at promoter or 5'UTR regions. Five of them showed reduced transcription levels and the five other genes showed increased transcription level in *zmbzip22*. The manuscript has been revised accordingly with a more straightforward description as follows:**

***"RNA-Seq revealed 1,756 DEGs between wild type and zmbzip22 endosperm, and ChIP-Seq identified 33 of these as putative targets bound at their genic region by ZmbZIP22 (Table 2). Of the thirty-three candidate target genes, ten genes were bound by ZmbZIP22 at promoter or 5'UTR region. Five of them showed reduced transcription level and the other five genes showed increased transcription level in zmbzip22. In addition to the 27-kD γ-zein gene, the other four genes bound by ZmbZIP22 at promoter or 5'UTR region and showed reduced transcription level, ZmGRP1 (GRMZM2G080603), a remorin coding gene (GRMZM2G137352), a C2H2 RING domain containing protein gene (GRMZM2G144645) and a RRM domain containing protein gene (GRMZM2G141386), were used for further validation".***

Point 7. Lines 636-643: Since the starch biosynthesis genes are not direct targets of ZmbZIP22, they are indirectly regulated, and this could be also for the reason that ZmbZIP22 regulates other TFs that regulate the downstream genes. I again suggest that this paper drastically overspectulates from the DEG data and it all should be essentially eliminated. There are other deficiencies in the discussion of starch biosynthesis. For one thing, GBSS is not ADP glucose glucose transferase, because it only transfers to existing glucan chains, not glucose. For another, since GBSS affects only amylose, and it's relatively easy to test for amylose, if the authors are trying to build a case for GBSS and Sbe3 transcriptional control by ZmbZIP22 being significant, then they should check for amylose content in the starch. For another, *sbe3* mutants have no effect on endosperm starch.

**RESPONSE: Thanks for the suggestion. The over-speculated discussion from the DEG data has been eliminated in this revision.**

**We apologize for the incorrect definition of GBSS. The transcription of both GBSSI (Granule-bound starch synthase I) and SBEIIa (starch branching enzyme IIa) increased ~10% in *zmbzip22*. The amylose/total starch ratio increased slightly (Figure 6B right panel). Please see our response to reviewer #3's comment 4 for details of amylose content quantification.**

**In maize endosperm, SBEI (GRMZM2G088753) predominates, expressing at a level 50 times more than SBEIIa (Gao et al., 1997). Increased expression of SBEIIa is probably not sufficient for increasing the amylopectin ratio. On the other hand, as GBSSI is the dominate enzyme in amylose synthesis, the elevated amylose ratio may be caused by increased expression of GBSSI. In the Discussion section, we re-wrote the corresponding sentences as follows: *"The transcription of both GBSSI (Granule-bound starch synthase I) and SBEIIa (starch branching enzyme) increased 10% in zmbzip22. The amylose/total starch ratio increased slightly (Figure 6B right panel). In maize***

***endosperm, SBEI (GRMZM2G088753) predominates, expressing 50 times more than SBEIIa (Gao et al., 1997). Increased expression of SBEIIa is probably not sufficient for increasing the amylopectin ratio. On the other hand, GBSSI is dominate enzyme in amylose synthesis. The slightly elevated amylose ratio may be caused by the increased expression of GBSSI."***

**TPC2018-00422-RAR1 2nd Editorial decision – *accept with minor revision* Sept. 2, 2018**

We have received reviews of your manuscript entitled "ZmbZIP22 is a Transcription Factor for 27-kD γ-Zein Gene Transcription during Maize Endosperm Development." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following:

Please highlight all changes and include a detailed annotation to changes to the text, with line numbers, and noting your responses to the comments.

**TPC2018-00422-RAR2 2nd Revision received Sept. 5, 2018**

Reviewer comments and **author responses**:

Reviewer #1:

My concerns have been fully addressed.

Reviewer #2:

The authors have satisfactorily addressed all my comments and supplied additional data to support their conclusions. I have also reviewed the responses to the other reviewers' comments, some of which were similar to my own, and which I was familiar with from reviewer discussions. These responses appear to be comprehensive and convincing.

Reviewer #3:

Most of my major criticisms have been satisfactorily addressed. The paper now is more informative about the functional role of 27α on protein body formation and determination of total amino acid content, which expands the general relevance of the study. PB characterization is now convincing from the new data that has been added. The genetic background has now been clarified, and the five backcrosses to standard are specified. Extensive, overspeculative discussion of DEGs has been reduced, but is still a bit overstated in my opinion; however, this should not prevent publication, as this viewpoint is subjective and the authors prerogative should have precedent. The minor points that were confusing to this reviewer have been satisfactorily addressed.

There are still problems with the discussion of starch biosynthesis. The majority of AGPase is cytosolic, not all of it, there is some in the plastid also, so the manuscript is not fully accurate (line 373). ADPGlc is used to make starch, not sucrose (line 375).

Point 1. Importantly in my opinion, the proper conclusion from Fig. 6A is that starch content as a percentage of dry weight is essentially unchanged by the mutation (Line 332). The reported starch contents are higher than are typically reported in the literature, which normally top at about 70%, so a conclusion that there is a significant reduction from 78.9% to 77.2% in the mutant is questionable.

**RESPONSE: Thanks for the comment. We agree that the reduction of total starch content in *zmbzip22* is very mild. The description of Figure 6B has been revised as follows: *"The total starch in the mutant endosperm is essentially unchanged. The amylose ratio in total starch showed a slight increase in zmbzip22-mu9 (Figure 6B, Supplemental Table 1)."***

Point 2. PPDK knockouts do not affect starch content (Lappe et al., PNAS, 2018), so the statement in line 704 is misleading.

**RESPONSE: Thanks for pointing out the mistake. The statement in line 704 has been revised as follows: *"It was reported that PBF1 directly influences starch synthesis (Zhang et al., 2016)."***

Point 3. Regarding branching enzymes, in fact the most highly expressed isoform is SBEIIb (GRMZM2G032628), not SBEI (line 697). Also, mutation of neither *sbe1* nor the *sbeIIa* gene affects endosperm starch structure (Blauth et al., 2001, 2002), so why these two genes are discussed with relation to amylopectin biosynthesis is not clear (lines 696-698). Also, the discussion presumes that increased transcript levels must imply increased function, and this is not the case for many enzymes (as the paper points out elsewhere with regard to zein gene expression). Again, this is overinterpretation of the DEG results. Of all of this, the only part that seems logical to me is that amylose content is increased (this seems well supported) and that GBSS transcript levels are elevated (although whether 10% more transcript would cause increased function is unknown and not addressed).

**RESPONSE: Thanks for the comment. The over-interpreted discussion about SBEs has been removed. The corresponding sentences have been re-written as follows: *" The starch showed no major changes while amylose / total starch ratio increased slightly in zmbzip22 (Figure 6B). Granule-bound starch synthase (GBSS) mediates the synthesis of amylose from ADP-glucose and GBSSI is the dominate enzyme in amylose synthesis in endosperm (Hannah and James, 2008). The transcription of GBSSI (Granule-bound starch synthase I) increased 10% in zmbzip22. The slightly elevated amylose ratio may be caused by the increased expression of GBSSI."***

Point 4. More about starch content: Lines 686-696 take the view that it is expected that increased AGPase transcript level by 18% and 12% should cause an increase in starch content. There are problems with this. Again, it is not necessary that increased transcript level implies increased protein level. Also, it is not clear that starch content will respond to such slight increases in AGPase activity. Also, the regulation of endosperm AGPase may be different from that of other AGPases, so it should be checked carefully that the endosperm enzyme is regulated as stated in the manuscript (I don't recall the nuances of AGPase regulation).

Taken together, I don't understand why the details about starch content are included here. Regulation of the genes is indirect, not direct, by ZIP22 binding to the promoter. Changes are minor if any. The relationship between gene expression and starch content is not known. It's all highly speculative and there is only a minor effect at best. What's the point of all this, in a paper that has an enormous amount of other data unrelated to starch biosynthesis? I think the inference that ZIP22 functions to regulate starch biosynthesis is much weaker than the rest of the paper. If a paper were to focus on the relationship between ZIP22 and starch, a lot more would be required, especially measurement of activity levels of the proposed affected enzymes. My advice is to leave the starch part out of the paper other than to state there were no major changes in total content and a possible amylose content increase, and leave all that for a more detailed study if the authors wish to pursue those questions.

**RESPONSE: Thank for the comments and suggestion! The discussion about starch and amylose was re-written as suggested: *"In endosperm, chloroplasts are specialized as amyloplasts, organelles dedicated to starch synthesis. The starch showed no major changes while amylose / total starch ratio increased slightly in zmbzip22 (Figure 6B). Granule-bound starch synthase (GBSS) mediates the synthesis of amylose from ADP-glucose and GBSSI is the dominate enzyme in amylose synthesis in endosperm. (Hannah and James, 2008). The transcription of GBSSI (Granule-bound starch synthase I) increased 10% in zmbzip22. The slightly elevated amylose ratio may be caused by the increased expression of GBSSI."***

Point 5. Regarding the title: Perhaps change "for" to "that regulates"? Using "for" could imply ZIP22 is exclusive to 27$\alpha$, whereas the data show it regulates many genes directly in addition. This is a minor comment for the authors' consideration, not a major point.

**RESPONSE: Thanks for the suggestion. The word "for" in the title has been replaced by the word "that regulates" as follows: "ZmbZIP22 is a Transcription Factor that Regulates 27-kD γ-Zein Gene Transcription during Maize Endosperm Development".**

**TPC2018-00422-RAR2 3nd Editorial decision – *acceptance pending*** **Sept. 12, 2018**

We are pleased to inform you that your paper entitled "ZmbZIP22 is a Transcription Factor that Regulates 27-kD γ-Zein Gene Transcription during Maize Endosperm Development" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

**Final acceptance from Science Editor** **Sept. 19, 2018**