

Round 1: Reviewers' Comments and Authors' Responses

Reviewer 1:

In this manuscript, the authors apply spatial transcriptomics to reciprocal maize hybrids to investigate the cellular heterogeneity of genomic imprinting in developing kernels. The study provides a spatially resolved view of allele-specific expression (ASE) and imprinted genes across distinct endosperm domains.

Overall, this work addresses a limitation of previous maize imprinting studies that used bulk sequencing of the endosperm, thereby ignoring cell-type specific expression changes. As a proof-of-concept study this work is valuable; however, due to the lack of biological replicates the statistical power to identify imprinted genes is low, limiting the value of this study. This limitation and the resulting consequences need to be discussed.

Another major concern is the framing of this study. From the early sections of the manuscript, the work is presented as a construction of a spatial transcriptome map of reciprocal hybrid kernels. Then, as the manuscript progresses, it becomes clear that the authors themselves view the study as constrained by important technical and experimental limitations. The manuscript would clearly benefit from greater coherence between its initial framing and its actual contribution. If the authors wish to present the study as a definitive and exhaustive characterization of spatial imprinting in maize endosperm, addressing the aforementioned statistical limitations through additional experimentation would be necessary. Alternatively, the manuscript could be framed more explicitly as a proof-of-concept and methodological demonstration of the power of spatial transcriptomics for studying genomic imprinting that future studies can expand.

Response: We sincerely appreciate your supportive and thoughtful comments on our work. Regarding to the issue of biological replicates, we fully agree that increasing biological replicates would increase the accuracy of imprinted gene identification. However, at present, both 10X Visum and Stereo-seq library preparation and sequencing suffer from relatively high failure rates and are quite expensive (30,000-50,000 RMB per sample). Therefore, as a proof-of-concept study, we did not generate biological replicates. On the other hand, the identification

of imprinted genes relies on cross-validation between two reciprocal crosses (BM and MB in this study), which itself functions as a form of “biological replicates”. Moreover, in the revised manuscript, we have further applied the Pearson’s chi-squared test with Yate’s continuity correction (as suggested by reviewer2) to validate the confidence of imprinted gene calls. Finally, we have discussed the strength and limitation with/without biological replicates in the Discussion section (see Lines 384-387).

---“Going forward, advances in spatial transcriptomics will make it feasible and necessary to include 2-3 biological replicates per reciprocal cross, in order to mitigate the influence of developmental stage differences and sampling errors on the accuracy of imprinted gene calling.”

Concerning the overall framing of the manuscript, our original intention was to present this work as a proof-of-concept study. As pointed out by you and other reviewers, the lack of biological replicates, lack of additional data from other developmental stages (reviewer2), and the inability of Visum to achieve true single-cell resolution may all affect the comprehensiveness and accuracy of imprinted gene identification. Therefore, we have deliberately avoided claiming our work as “a definitive and exhaustive characterization of imprinted genes in the maize endosperm”. This is reflected in the throughout modifications we have made, such as revising the title to “Spatial transcriptomics reveals the complexity of genomic imprinting in maize”, incorporating statistical tests to evaluate the confidence of imprinting status, validating the spatial transcriptomics-based imprinted genes using independent long-read and bulk RNA-seq data, and revising the wordings where appropriate. However, considering the timeliness of the study and the feasibility of many further analyses, we have not undertaken extensive restructuring of our manuscript’s framework.

Other comments:

1. Line 169: “Notably, a consistently higher number of ASE genes favouring B73 expression was found for all cell types, with only one exception of AL in MB.” Is the same bias observed

in the bulk RNA seq data? Could this be a consequence of B73 being the reference genome for read alignment? A brief discussion of reference bias and potential alternative strategies would strengthen the manuscript. For example, Anderson et al 2021 use a non-SNP-based approach by mapping reads to concatenated parental genome assemblies and then quantifying parent-of-origin bias based on where the reads uniquely align, rather than on informative SNPs (<https://doi.org/10.1371/journal.pgen.1009491>).

Response: Thank you for raising the question regarding the bias of ASE genes. First, to address allele mapping bias during the alignment process, we did not simply use the standard B73 reference genome. Instead, we constructed an N-masked version (the SNPs between B73 and Mo17 were masked by N) of the B73 reference genome for read mapping, which was reported to be effective in mitigating this bias¹. We then mapped the bulk short-read RNA-seq data to this N-masked reference genome. The results showed that in both the BM and MB hybrid crosses, the number of genes with B73 ASE detected in both embryo and endosperm tissues was higher than that of genes with Mo17-ASE (Fig. R1a). Additionally, we generated deep long-read cDNA sequencing data from embryo and endosperm of BM and MB hybrids. Long-read cDNA sequencing data were expected to have less mapping bias compared with short-read data. When aligned to the B73 reference (Fig. R1b), B73-ASE genes accounted for approximately 66–70% of the total, whereas alignment to the Mo17 reference (Fig. R1c) showed that B73-ASE genes still represented about 60–64%. Therefore, we conclude that the preferential expression of the B73 allele in both BM and MB hybrids is likely to be a genuine biological phenomenon. We added a new supplementary figure (Fig. S11) in the revised manuscript to further strength this observation.

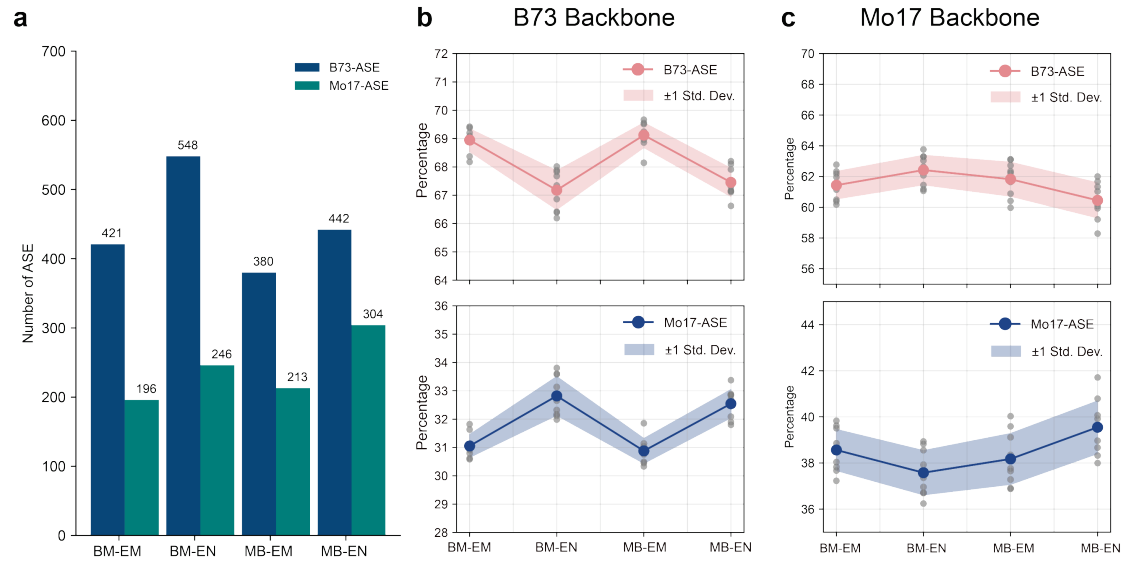


Fig. R1 Analysis of allele-specific expression (ASE) genes. **a** Bar plot depicting the number of B73-ASE and Mo17-ASE genes identified from bulk RNA-seq across four samples. **b, c** Line plots showing the proportion of B73-ASE and Mo17-ASE genes detected when using **b** (the B73 reference genome) and **c** (the Mo17 reference genome) for read alignment. The gray dots represent ratios obtained by applying different filtering thresholds (ranging from 20 to 60 valid reads). EM, embryo manually dissected; EN, endosperm manually dissected.

The method for ASE identification independent of SNPs, as proposed by Anderson et al. (2021), represents a significant attempt in the field. We also tried this approach. Overall, in the VE where we identified the largest number of imprinted genes, the positive rates for MEG and PEG were 80.77% and 79.17%, respectively. In the AL, the positive rates for MEG and PEG were 88.89% and 83.33%, respectively (Fig. R2 a, b). However, Anderson’s method detected tens of times more imprinted genes than SNP-based methods (Fig. R2 c, d), which may lead to a higher false positive rate—especially considering the extensive presence-absence variation (PAV) genes, paralogous genes, and structural variations between B73 and Mo17 genomes. In contrast, the SNPs identified by the SNP-based method are located in more conserved orthologous regions, thus likely yielding a lower false positive rate. In the Discussion section, we have added texts regarding potential biases introduced by using SNP-based methods and a single reference genome, and explored potential solutions (Lines 391-398).

---“Addressing mapping bias remains an urgent challenge in the study of genomic imprinting. Deep long-read cDNA sequencing represents a potential solution to this issue. Additionally, the application of non-SNP-based mapping methods and development of new SNP-sensitive

mapping approaches also holds promise for mitigating mapping bias. However, several technical obstacles await to be overcome in this process, including differences in genome quality between parental lines, presence/absence variation (PAV) genes, copy number variations, and structural variations within gene regions”

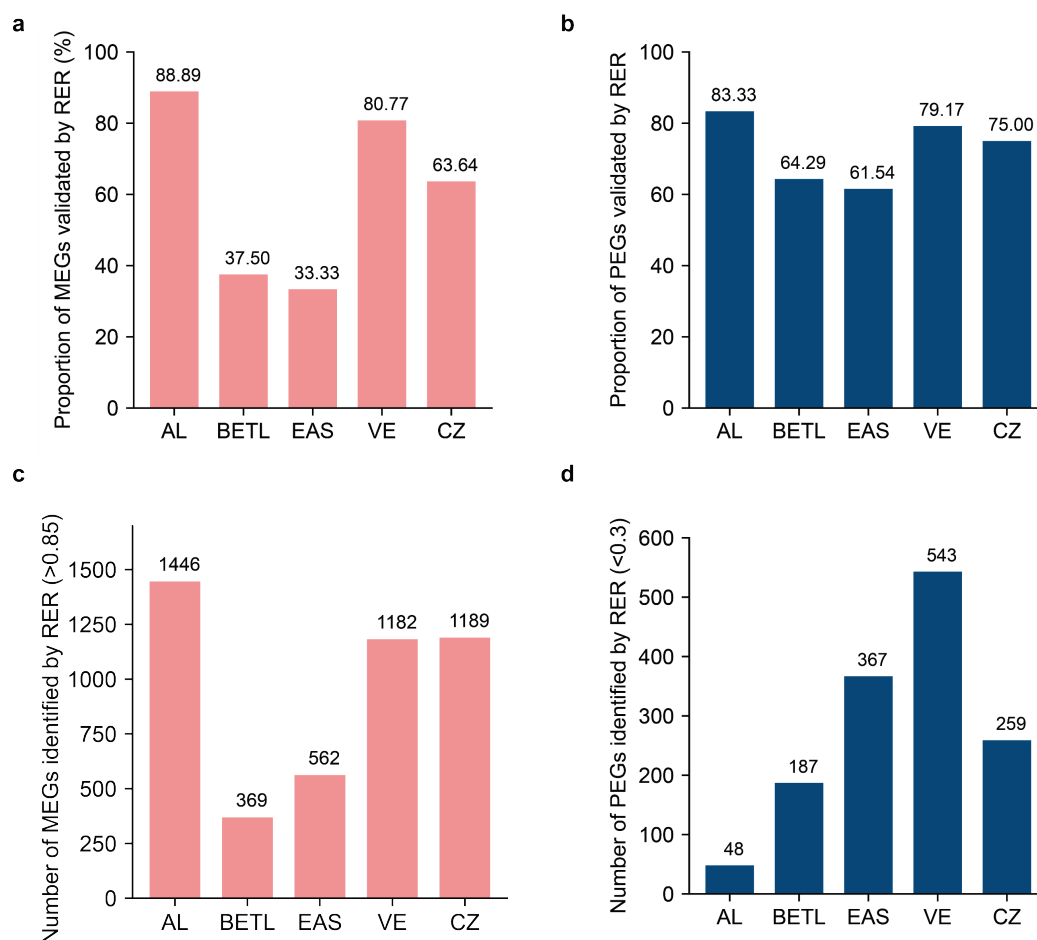


Fig. R2 **a, b** Bar plots showing the proportion of MEGs (a) and PEGs (b) that could be validated by the RER-based method in different cell types. **c, d** Bar plots displaying the number of MEGs (c) and PEGs (d) identified by the REP-based method across cell types.

2. An important methodological detail that is currently missing concerns the thickness of the tissue sections used for spatial transcriptomics, which is not specified, and how this relates to cell size and spot diameter. Is it possible that a Visium spot encompasses several different cells? Given the pronounced differences in cell size and tissue architecture across maize kernel compartments, section thickness will strongly influence the number and identity of cells contributing to each spot and the extent of vertical (z-axis) mixing. The authors should report

section thickness and discuss how section thickness, cell size, and Visium spot size together define the effective spatial resolution of their analysis.

Responses: Thanks! Tissue sections in this study were prepared at a thickness of 10 μm . In addition, the effective capture area of the 10x Genomics Visium slide is a $6.5 \times 6.5 \text{ mm}^2$ square containing 4,992 spots, each with a diameter of 55 μm . It was reported that maize endosperm cells, at 14 DAP, had an average diameter of approximately 60 μm , whereas embryo cells range from 10 to 30 μm in diameter². Consequently, a single spot may capture RNA molecules originating from one or a few cells. This multi-cellular mixing effect is expected to be more pronounced in the embryo, where cells are more densely packed, resulting in expression profiles that reflect a composite transcriptional signature of multiple neighboring cells. We have added these above information into relevant text in Method section (Lines 444-460).

3. Line 137ff: The authors argue that there may be developmental and anatomical alterations in the reciprocal crosses. Have the authors tested whether the development of the kernels is comparable in the reciprocal crosses? This should be analyzed to support this argumentation.

Response: Thanks! In the BM and MB hybrid seeds, distinct differences in kernel morphology were observed at 14 days after pollination, resulting in notable variations in endosperm development and grain filling (Fig. R3). Specifically, grain filling in BM occurred more uniformly from the apex to the base of the kernel, whereas in MB, filling and storage substance accumulation were largely concentrated at the apex. This explains the observed translucent endosperm at the basal region of MB kernels (Fig. R3). Such structural differences may lead to changes in cell type composition and proportions between BM and MB. We have updated the relevant text accordingly (Lines 136-139) and added the kernel phenotype as a Fig. S5 in revised manuscript.

---“*This difference may be attributed to developmental or anatomical variations between reciprocal crosses (Fig. S5) and/or unavoidable spatial discrepancies during tissue sectioning.*”

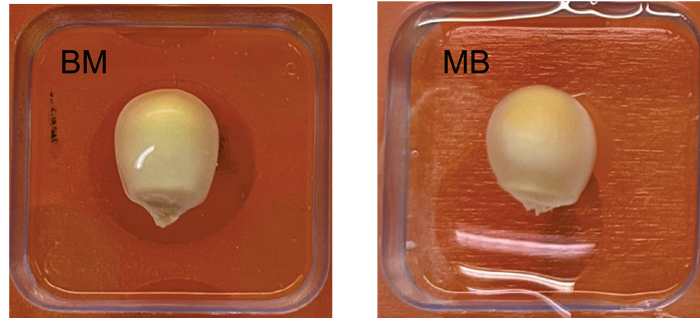


Fig. R3 The 14 DAP kernel phenotypes of BM and MB hybrids that used for Visum Spatial transcriptome sequencing.

4. How well do the bisulfite data explain the imprinting status of MEGs?

Response: Thank you for your question regarding the relationship between DNA methylation and the imprinting of MEGs. Overall, we consistently observed higher paternal CG and CHG methylation compared to maternal methylation near the transcription start sites (TSS) of MEGs in both reciprocal crosses (Fig. R4).

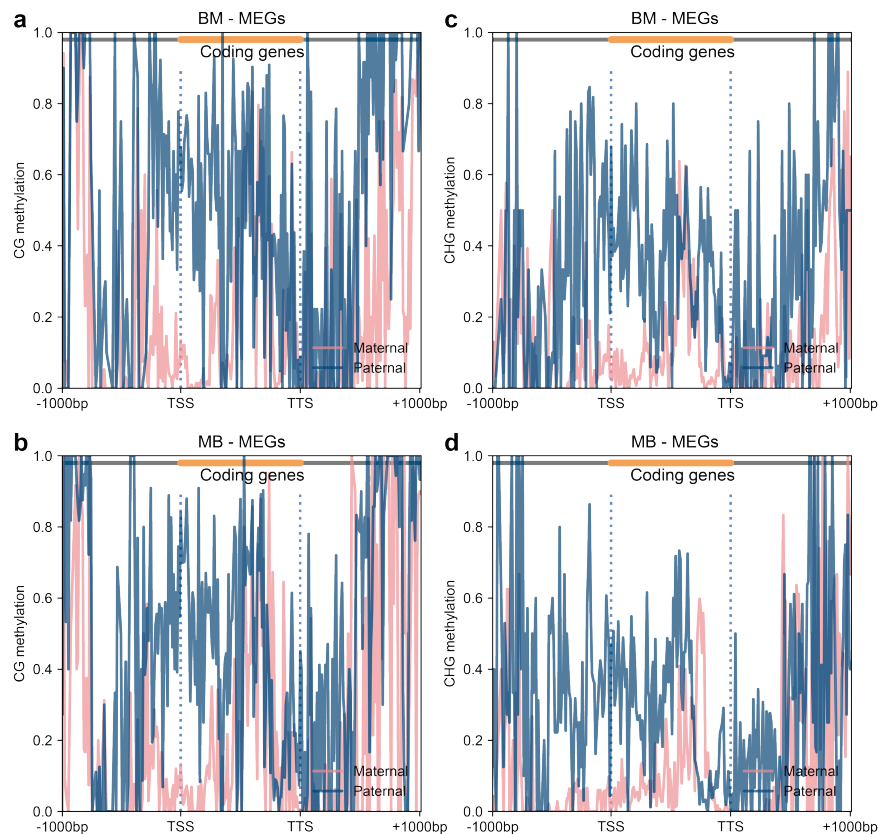


Fig. R4 **a, b** Metagene plot showing the CG methylation profiles for MEGs in BM and MB. **c, d** Metagene plot showing the CHG methylation profiles for MEGs in BM and MB.

5. Line 139ff: Which criteria were used to identify differentially expressed genes?

Response: Thanks. We have added a detailed description of the identification of differentially expressed genes (DEGs). Please see in detail under the small title of “Identification of DEGs between cell types” in the Methods section (Lines 505-512).

6. All abbreviations used in the manuscript should be defined (SE, VE missing) and a figure should be included showing the domains of the endosperm together with the abbreviations.

Response: Done. The initial definitions of SE and VE are provided in the Abstract (Line 31 and 35). We also revised Fig. 1e, f to define all abbreviations.

7. Line 272: Sequence-dependent (sdASEs) genes should be defined.

Response: We appreciate your helpful suggestion. In revised manuscript, we added explicit definitions of sdASEs in Lines 172-173:

---“*sdASEs, which refer to genes that consistently favor the same allele in both reciprocal crosses*”.

8. A revision of the manuscript for English grammar would improve the readability of the manuscript.

Response: Thanks. We have carefully revised our manuscript and improved the grammar and wording for better clarity and readability.

Reviewer 2:

The manuscript entitled “Spatial and cellular heterogeneity of gene imprinting in maize” characterized genomic imprinting patterns across distinct differentiated cell types within the maize endosperm. This is a very interesting study and represents a highly creative and technically innovative attempt. However, I don’t think current data can fully support this conclusion, genomic imprinting was heterogenous across the different types of endosperm. The followings are my major concerns on this study.

Response: We really appreciate your thoughtful comments and kind suggestions on our work. Please find our detailed responses below.

Firstly, I am confused about how the authors define “heterogeneity”. In my view, the term genomic imprinting heterogeneity is only appropriate when a given gene is imprinted in one cell type but shows no imprinting in the rest cell types. However, in the manuscript authors confused the notion of heterogeneity and uniqueness. A part of genes can be identified as a PEG in only one type of endosperm. For instance, for *Trps8*, *Nrg1*, *Thx41*, and *Sbp3*, the authors claim that these are PEGs identified for the first time in this study. However, they do not clarify why these genes could not be detected in whole-tissue analyses—whether this is due to genuine heterogeneity of imprinting, or because their expression is restricted to specific clusters of endosperm cells and therefore falls below the detection limit of whole-tissue RNA-seq.

Response: We sincerely thank the reviewer for raising this insightful question regarding the “heterogeneity” of genomic imprinting and for the valuable suggestions provided. In response, we have made efforts throughout the revised manuscript to replace the term “heterogeneity” with “cell-type specific imprinting” where appropriate, in order to avoid potential ambiguity in the definition of heterogeneity.

Regarding the question of why some imprinted genes are only imprinted in specific cell types, two scenarios can be distinguished: First, a gene may be expressed in multiple cell types but imprinted in only one of them (i.e., its imprinting status can be assessed across several cell types, yet imprinting is observed in only one). Second, a gene may be expressed exclusively in one cell type and imprinted specifically in that same expressing cell type. Among the 34 cell-type specific PEGs we identified, 24 genes fall into the first category, and 10 genes belong to the second. Accordingly, the 24 genes in the first category can be regarded as reflecting genuine heterogeneity of imprinting. In the previous version of the manuscript, we had already analyzed the expression patterns of these cell-specific imprinted genes. In the revised version, we have further clarified the related descriptions. Please see Lines 250-256 and Fig. S20 in the revised manuscript for details.

The differences between the imprinted genes identified by whole-tissue RNA-seq and those identified by Visium spatial transcriptomics likely arise from two main factors. First, whole-tissue RNA-seq represents the entire endosperm, whereas spatial transcriptomics profiles only a thin layer of cells within the endosperm (discussed in Lines 378-381). Second, cell-type-specific genomic imprinting—where imprinting strength or even direction differs across cell types—may cause certain imprinted signals to be undetectable at the whole-tissue level. To test the second hypothesis, we aggregated data from all endosperm spots into a pseudo-bulk dataset and performed imprinting calling. The resulting set of imprinted genes was then compared with the gene sets identified at the individual cell-type level. Our analysis revealed that 22 PEGs, including *Nrg1*, *Thx41*, and *Sbp3*, along with 19 MEGs, were not detectable in the pseudo-bulk data (Fig. R5 a, b). Further examination of the spatial expression patterns and parent-of-origin expression ratio (PMR) plots for *Nrg1*, *Thx41*, and *Sbp3* clearly demonstrated strong heterogeneity: certain spatial spots showed maternal expression bias while others showed paternal bias. This heterogeneity leads to a neutralizing effect on the imprinting signal when evaluated at the bulk level (Fig. R5 c-e).

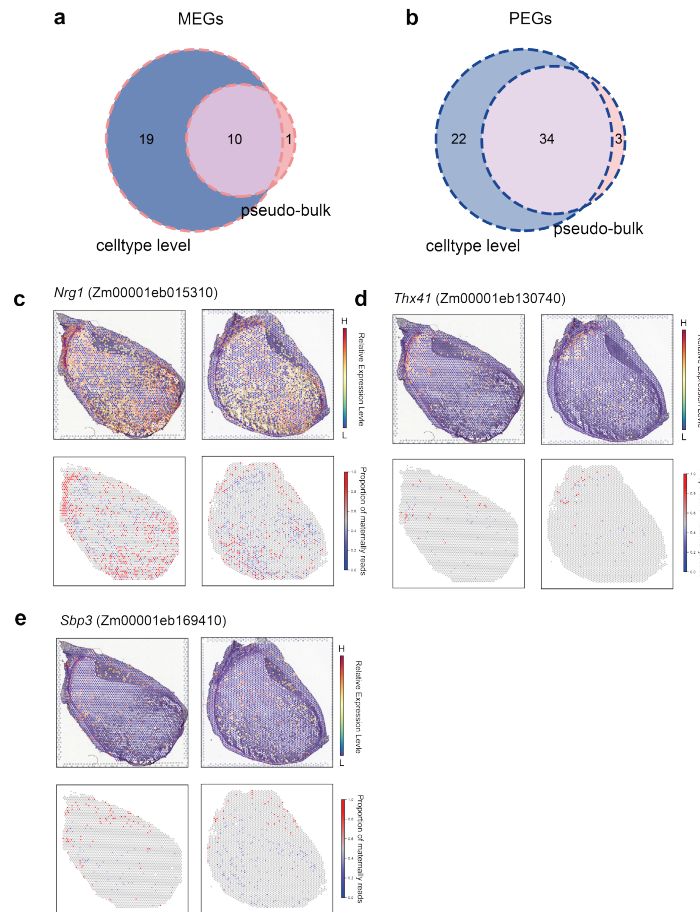


Fig. R5 **a, b** The overlap of MEGs (a) and PEGs (b) identified in cell-type level and those of pseudo-bulk level. **c-e** The spatial expression profiles and PMRs of *Nrg1* (c), *Sbp3* (d) and *Thx41* (e).

Secondly, I have serious concerns about the so-called heterogeneous imprinted genes identified in this manuscript. I do not understand the rationale for the criteria used to define imprinted genes, namely a 10% of maternal allelic ratio, especially given that no statistical support (chi-square test) is provided. In the endosperm, the expected proportion of paternal transcripts for a PEG is 67%. In Fig. S18, the authors list all imprinted genes and claim that the majority of PEGs lose imprinting in certain endosperm cell types. However, after checking several of these genes in the supplementary tables, I found that most of them still show clear paternal bias. In my view, these genes should therefore still be classified as PEGs across all endosperm cell types. For instance, Zm00001eb212040, authors claimed it showed non-imprinting in the EAS cell, but in the reciprocal crosses of B73 and Mo17, the maternal allelic ratios are 0.107 and 0.050, respectively. Similarly, I found that the maximum maternal ratio for most of the other PEGs is

also below 25%. I do not think that such subtle variation in maternal allelic ratio is sufficient to conclude that these PEGs are heterogeneous. Furthermore, for those real heterogeneous PEGs (if existed), why were these genes heterogeneous, how did these genes expression differ across different types of cells?

Response: Thank you for your question regarding the criteria for imprinting identification. The threshold of 10% maternal allelic ratio has been widely adopted in maize to identify high-confidence imprinted genes (e.g., Waters et al. defined genes with >90% maternal transcripts as strong MEGs³. Similarly, Mingming Xin et al. classified genes with >90% maternal reads at informative SNPs as MEGs⁴. In maize endosperm at 14 DAP, Waters et al. further defined high-confidence MEGs as genes showing $\geq 90\%$ maternal expression in both reciprocal crosses⁵). We fully agree that incorporating statistical testing can further improve the accuracy of imprinting calls. Accordingly, we have now applied Pearson's chi-squared test with Yates's continuity correction to all candidate imprinted genes and have updated both the Methods (Lines 514-557) and Supplementary table 4 accordingly. Because we employed the stringent 10% cutoff, all genes identified as imprinted show adjusted *P*-values ≤ 0.05 .

Regarding the observation that some genes exhibit a clear paternal allelic bias yet fall just below the 10%/90% threshold and thus are not called as imprinted, we acknowledge that the definition of imprinting itself is based on an arbitrary cutoff—standards of 10%, 15%, or 20% allelic ratio have all been used in the literature. To ensure high confidence in our set of imprinted genes, we chose the 10% threshold. Consequently, all subsequent analyses and comparisons must adhere to this cutoff. Even if a gene's allelic ratio falls short by only 1-2%, we cannot classify it as imprinted; otherwise, every expressed gene in each cell type would require manual inspection, and the criteria for defining imprinting would become arbitrary.

To evaluate how the choice of threshold affects the identification of PEGs, MEGs, and their cell-type specificity, we further compared results using the 10% and 20% cutoffs. The proportion of cell-type-specific imprinted genes shifted slightly: MEGs increased modestly from 41.38% to 43.59%, while PEGs decreased slightly from 60.71% to 53.40% (Fig. R6). However, the overall conclusion that PEGs show higher cell-type specificity than MEGs remains unchanged.

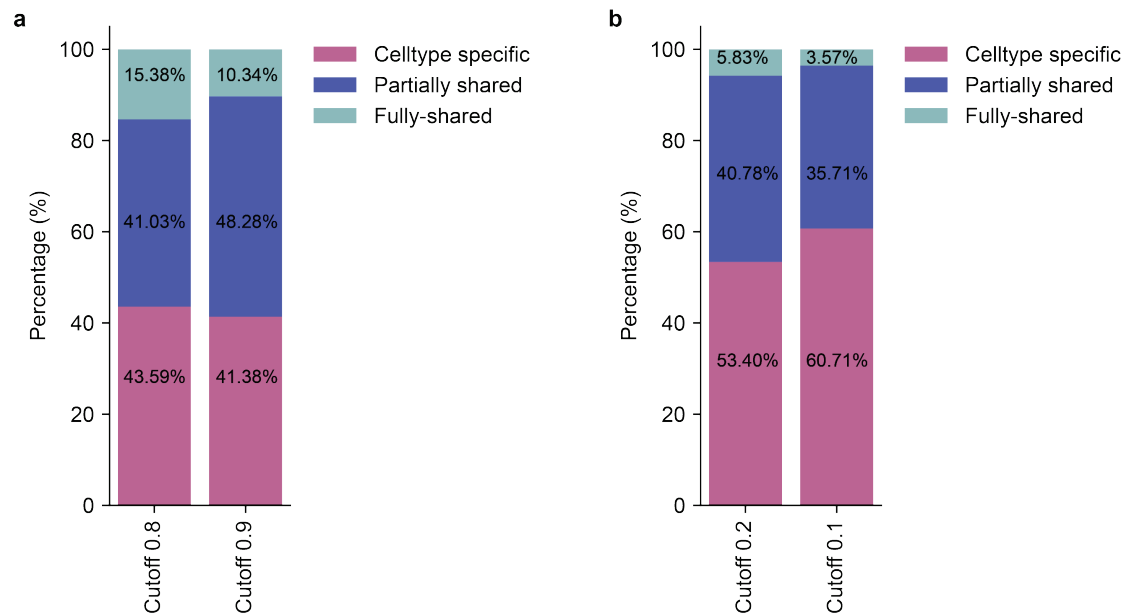


Fig. R6 The proportion of cell-type specific MEGs and PEGs using the imprinting threshold of 10% and 20%.

Concerning the heterogeneity of imprinting across different endosperm cells, an excellent review by Daniel Zilberman et al. in *Genes & Development* (2015) notes that “genomic imprinting can be influenced by both primary imprints established prior to fertilization and secondary imprints established during cellular differentiation post-fertilization”. During endosperm differentiation and proliferation, distinct cells or cell types acquire different epigenetic modifications. In some cell types, parent-of-origin differences in epigenetic marks emerge (potentially leading to ASE and imprinting), whereas in others, a balanced epigenetic state is maintained between the parental alleles. We have cited this relevant background knowledge in the Introduction section; please see Lines 64-68 for details.

Moreover, I also recommended that the authors perform additional sequencing of the endosperm at multiple developmental stages. Previous studies have already proven that PEG abundance in the endosperm peaks at 7 DAP, whereas MEG abundance peaks at 10 DAP (Mingming Xin, 2013). It is also possible to distinguish different endosperm cell types at the same developmental stage (Junpeng Zhan, 2015).

Response: We appreciate your insightful point regarding the variation in genomic imprinting across different developmental stages and hybrid combinations in maize (e.g., Mingming Xin,

2013; Junpeng Zhan, 2015)^{4,6}. However, the primary goal of this study was to establish a proof-of-concept for cell-type-specific imprinting within maize endosperm. For this purpose, we selected the classic B73 and Mo17 hybrid cross and the widely studied 14 days after pollination time point. On the other hand, at present, both 10X Visum and Stereo-seq library preparation and sequencing suffer from relatively high failure rates and are quite expensive (30,000-50,000 RMB per sample). Conducting such experiments across multiple time points and reciprocal hybrids would entail substantial financial investment and could not be completed within a short timeframe. In our revised manuscript, we have added a sentence in the Discussion (Lines 413-417) to outline future directions for investigating imprinting dynamics across developmental stages.

Last, I also have some concerns regarding several aspects of the methods used in this study. I believe the authors should provide a more detailed description of the methodology used for the identification of imprinted genes. I found that the genes exhibited an expression bias towards B73 allele. Hence, I would like to know whether the authors constructed a Mo17 pseudo-genome (by substituting the B73 reference sequence with SNPs identified in Mo17) and re-aligned the previously unmapped reads to this pseudo-genome. Otherwise, I would also recommend applying the RER method, which can provide more accurate calculation of parent-of-origin expression (Sarah N. Anderson, 2021). Moreover, in the manuscript authors claimed that SE cell exhibited pronounced parental asymmetry and MB endosperm showed lack of SE cells. I believe this is likely due to differences in genomic background. In the manuscript, the authors used the B73_v5 genome sequence as the reference. However, it is possible that SE cells preferentially express genes from the maternal genome, and that, in the MB endosperm, many maternal transcripts were not captured when using B73 as the sole reference. Therefore, I strongly recommend reanalyzing these data with the RER method and/or using the Mo17 genome as an additional reference.

Response: We appreciate your insightful suggestions regarding the methods in this study. First, we have updated the Methods section in detail to further clarify and improve their readability. Regarding to the reference genome issue (also raised by reviewer1), we did not simply use the

standard B73 reference genome or pseudo-genome. Instead, we constructed an N-masked version (the SNPs between B73 and Mo17 were masked by N) of the B73 reference genome for read mapping, which was reported to be effective in mitigating this bias¹. We then mapped the bulk short-read RNA-seq data to this N-masked reference genome. The results showed that in both the BM and MB hybrid crosses, the number of genes with B73 ASE detected in both embryo and endosperm tissues was higher than that of genes with Mo17-ASE (Fig. R1a). Additionally, we generated deep long-read cDNA sequencing data from embryo and endosperm of BM and MB hybrids. When aligned to the B73 reference (Fig. R1b), B73-ASE genes accounted for approximately 66–70% of the total, whereas alignment to the Mo17 reference (Fig. R1c) showed that B73-ASE genes still represented about 60–64%. Therefore, we conclude that the preferential expression of the B73 allele in both BM and MB hybrids is likely to be a genuine biological phenomenon. We added a new supplementary figure (Fig. S11) in the revised manuscript.

The method for ASE identification independent of SNPs, as proposed by Anderson et al. (2021), represents a significant attempt in the field. We also tried this approach. Overall, in the VE where we identified the largest number of imprinted genes, the positive rates for MEG and PEG were 80.77% and 79.17%, respectively. In the AL, the positive rates for MEG and PEG were 88.89% and 83.33%, respectively (Fig. R2 a, b). However, Anderson’s method detected tens of times more imprinted genes than SNP-based methods (Fig. R2 c, d), which may lead to a higher false positive rate—especially considering the extensive presence-absence variation (PAV) genes, paralogous genes, and structural variations between B73 and Mo17 genomes. In contrast, the SNPs identified by the SNP-based method are located in more conserved orthologous regions, thus likely yielding a lower false positive rate. In the Discussion section, we have added texts regarding potential biases introduced by using SNP-based methods and a single reference genome, and explored potential solutions (Lines 391-398).

Regarding the scarcity of SE cells in the MB cross, the reviewer raised the possibility that SE-specific expression may be largely driven by the maternal chromosome (M in MB), leading to many M-derived reads being unmappable to the reference genome. As noted earlier, we employed an N-masked genome for alignment, which significantly reduces mapping bias

compared to linear or pseudo-genome approaches. Moreover, the fact that SE cells were successfully identified in both the BM cross and the B73 self-fertilized line indicates that a substantial proportion of SE transcripts originate from the B73 allele. In other words, unless the majority of marker genes in SE are exclusively maternally expressed, our approach should still capture most SE transcripts. In practice, we did observe phenotypic differences in endosperm development between the BM and MB crosses (Fig. R3). Additionally, spatial transcriptome sequencing does not guarantee exact positional correspondence of tissue sections between BM and MB samples, which may also contribute to the observed inconsistency in cell type representation. Therefore, in our imprinting analysis, we focused on the remaining five endosperm cell types, excluding SE from further investigation in this context.

Reviewer 3:

Li et al investigated genomic imprinting in maize endosperm using spatial transcriptomics based on the 10x Visium platform. While the study addresses an interesting biological question, I have substantial concerns regarding the overall experimental design. The authors rely on the Visium platform as the primary basis for cell type annotation. However, Visium has a spatial resolution of approximately 55 μm with a 100 μm center to center distance between spots. and therefore not only fails to achieve true single cell resolution, but is also far from it. Under this limitation, the use of Visium data alone to define cell types in a highly heterogeneous tissue such as maize endosperm is problematic and raises concerns about the validity of the resulting annotations and downstream analyses. In most current studies, single cell or single nucleus RNA sequencing serves as the primary approach for defining cell types, with spatial transcriptomics used as a complementary method to map these identities back to tissue context. In the absence of such single cell reference data, the cell type assignments and downstream interpretations presented here remain questionable.

Response: We really appreciate your insightful comments and kind suggestions. Firstly, we updated the sample preparation details in the Method (Lines 444-460) section. Tissue sections in this study were prepared at a thickness of 10 μm . In addition, the effective capture area of the 10x Genomics Visium slide is a $6.5 \times 6.5 \text{ mm}^2$ square containing 4,992 spots, each with a

diameter of 55 μm . It is worth noting that endosperm cells in maize at 14 days after pollination are generally large, with an average size of 60 μm^2 , which is comparable to the diameter of Visium spots (55 μm). Therefore, it is expected that in many regions, the transcripts captured by individual spots will originate from one or only a few cells, rather than from a mixture of numerous heterogeneous cells.

On the other hand, the main goal of this study remains to identify imprinted genes at the cell type level (such as VE, BETL, AL, etc.) and to compare imprinting differences across cell types, rather than to characterize imprinting at the single-cell resolution. Following your suggestion, we have revised the wording throughout the manuscript—replacing "single cell" to "cell type" where applicable—to ensure more accurate description.

In the future, we plan to employ single-cell or single-nucleus RNA sequencing to identify cell types within the maize endosperm from reciprocal hybrid crosses. This approach will be used to investigate the complexity of genomic imprinting at single cell resolution. However, matching individual cells between reciprocal crosses presents a non-trivial challenge - much more difficult than matching at the cell type level, where correspondence is relatively straightforward. Furthermore, at the single-cell level, both the number of detectable genes and the sequencing read depth are often considerably sparser compared to data aggregated at the cell-type level. Please see our revised sentences in Discussion (Lines 401-406).

Several specific issues illustrate these concerns.

(1) At the spatial resolution of Visium, each spot necessarily captures a mixture of transcripts from multiple neighboring cells. Performing cell type annotation based on such mixed transcriptomes is inherently risky. Even if a best matching reference profile can be identified, this does not guarantee biological correctness, as nearest-neighbor assignment will always produce a match even between fundamentally dissimilar expression profiles. This issue is particularly severe in tissues with sharp cell-type boundaries or gradual differentiation gradients, such as the maize endosperm.

Response: We appreciate your insightful suggestion. As we mentioned above, endosperm cells

in maize at 14 days after pollination are generally large, with an average size of 60 μm , which is comparable to the diameter of Visium spots (55 μm). Therefore, it is expected that in many regions, the transcripts captured by individual spots will originate from one or only a few cells, rather than from a mixture of numerous heterogeneous cells.

Second, to mitigate the risk of mis-classifying functional regions due to mixing effects, we adopted multiple independent criteria when defining these regions. These criteria include: 1. During cell annotation transfer, we used a high-quality marker gene dataset also generated from the Visium platform; 2. Functional regions must exhibit strong spatial continuity and anatomical consistency; 3. Regions should display parental genomic ratios consistent with known biological patterns: a 2:1 maternal-to-paternal ratio in the endosperm cell types, a 1:1 ratio in the embryo cell types, and an essentially maternal-only RNA contribution in maternal tissues such as the seed coat and placento-chalazal region; 4. Expression of multiple well-documented marker genes was further verified by RNA electron in-situ hybridization.

We fully acknowledge that sharp cell-type boundaries and gradual differentiation gradients in maize endosperm may influence cell classification. Therefore, all cell types identified in this study are previously reported well-documented endosperm cell types based on cytological evidence; no novel cell types were defined, thereby avoiding potential biases introduced by technical limitations.

Finally, as noted above, we have discussed the importance of employing single-cell or single-nucleus RNA sequencing in the future to achieve more accurate cell-type classification and to investigate genomic imprinting at single-cell resolution. Please see Lines 401-406 for details.

(2) Trajectory inference based on mixture transcriptomes is even more problematic. Pseudotime or developmental trajectories inferred from spatial spots reflect averaged signals across multiple cell types rather than genuine state transitions of individual cells. As a result, apparent trajectories may be driven by spatial proximity or compositional changes in cell mixtures, rather than true developmental progression.

I provided the relevant information released by 10x Genomics in the following: for the authors' reference:

<https://kb.10xgenomics.com/s/article/360035487572-What-is-the-spatial-resolution-and-configuration-of-the-capture-area-of-the-Visium-v1-Gene-Expression-Slide>

resolution-and-configuration-of-the-capture-area-of-the-Visium-v1-Gene-Expression-Slide

Response: Thank you for your thoughtful suggestion. First, as mentioned in our responses to your questions above, given the relatively large size of maize endosperm cells, it is expected that in many regions the transcripts captured by individual Visium spots will originate from one or only a few cells, rather than from a mixture of numerous heterogeneous cells. Even when a spot contains multiple cells, these cells are generally adjacent and thus likely belong to the same cell type and share similar developmental states. While a spot overlapping the boundary between two or more distinct cell types could introduce cellular heterogeneity into the inferred developmental trajectory, such cases are expected to be rare.

More importantly, the aim of our haplotype-resolved developmental trajectory analysis was to examine differences between parental contributions along developmental paths. Our analysis revealed that parental developmental trajectories showed minimal divergence in less differentiated functional compartments such as BETL and CZ, but exhibited pronounced separation in highly differentiated SE. This suggests that endosperm development is accompanied by increasing divergence between maternal and paternal transcriptional programs. We have further revised the trajectory-related descriptions in the manuscript; please see Lines 339–341 for details.

References

- 1 Krueger, F. & Andrews, S. R. SNPsplite: Allele-specific splitting of alignments between genomes with known SNP genotypes. *F1000Research* **5**, 1479 (2016).
- 2 Fu, Y. *et al.* Spatial transcriptomics uncover sucrose post-phloem transport during maize kernel development. *Nature communications* **14**, 7191 (2023).
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Round 2: Reviewers' Comments and Authors' Responses

Reviewer 1

I thank the authors for their detailed response and for acknowledging the limitations related to the lack of biological replicates, as well as their stated intention to position the study as a proof-of-concept.

Thank you for your support and insightful suggestions. In our revision, we newly generated the snRNA-seq data of hand-dissected endosperm for both BM and MB, with two replicates for each sample. Approximately 79% of PEGs and 70% of MEGs identified by spatial RNA-seq data could be validated at the cell-type level by snRNA-seq data, suggesting an overall high-quality of our imprinting gene calls. In addition, we have toned down the descriptions related to cellular heterogeneity of imprinted genes and have indicated, where appropriate, that our study is a proof-of-concept investigation. Please find our detailed responses below.

However, this intention is not yet consistently reflected in the revised manuscript. The overall framing and interpretation still suggest a level of robustness that is not fully supported by the experimental design. For example, terms such as “high-confidence” imprinted genes (e.g., line 28 in the Abstract and line 96 in the Introduction) remain in use, the manuscript continues to read as a comprehensive characterization of spatial imprinting (e.g., “we constructed a spatial transcriptomic map,” line 93), and conclusions regarding cell-type specificity appear stronger than warranted given the absence of biological replicates. While reciprocal crosses provide valuable support, they do not fully substitute for independent biological replication. Stating in the response that the study is not intended to be definitive is appreciated, but this should be consistently reflected throughout the manuscript, including in the Abstract, Introduction, and interpretation of the results. I therefore encourage the authors to more clearly align the framing, wording, and conclusions with the scope of the study. A more cautious and explicitly proof-of-concept presentation would more accurately reflect the strength of the evidence.

Thank you! Regarding the wording issues in the manuscript, we have appropriately toned down expressions such as “high-confidence” and “comprehensive characterization” where necessary

(Lines 91 - 92). In addition, we have added statements indicating that our study provides proof-of-concept evidence where appropriate (Line 100 and Line 364). We have also revised the titles of the six sections in the manuscript, particularly the third section related to imprinted genes, which has been changed to “Cell-type resolved imprinted genes in maize endosperm” to soften our conclusions regarding the cellular specificity of imprinted genes.

The two spatial transcriptomic datasets from reciprocal crosses cannot serve as strict biological replicates to increase the accuracy of imprinted gene identification. Therefore, we generated snRNA-seq data with two replicates for both BM and MB samples. Using snRNA-seq data, we identified imprinted genes across six endosperm cell types and compared them with those from spatial transcriptomic data. At the cell-type level, 79% of PEGs and 70% of MEGs detected in the spatial transcriptomic data could be validated by snRNA-seq data, confirming that the imprinted genes previously identified from spatial transcriptomic data are generally accurate. Please see lines 225 - 237 for details.

In addition, while the authors have taken steps to address potential biases in the ASE analysis, it would be helpful to more clearly discuss the limitations of the SNP-based approach and the use of a single reference genome, and how these may influence the identification of ASE and imprinted genes.

Thank you! In the Discussion section (Lines 384 - 401), we have thoroughly discussed the impact of the SNP calling strategy and the use of a single reference genome on ASE and imprinting analysis. The content is as follows: “In addition, SNP-based approaches and the use of a single reference genome carry certain limitations, and this choice often involves an important trade-off in the course of a study. First, a prerequisite for a gene to be analyzable is that its 3' end contains high-quality SNPs, which is directly influenced by the size of the SNP set. A larger SNP set tends to result in a higher false-positive rate, whereas a smaller SNP set reduces the number of analyzable genes. Therefore, the size of the SNP set included in the analysis is a critical decision when investigating ASE and imprinting, and should be determined according to the study objectives. Second, the choice between using a single reference genome

and a merged parental genome also entails a trade-off. A single genome cannot simultaneously account for presence/absence variation, copy-number variation, and structural variation between the two parental haplotypes, meaning that certain loci cannot be reliably aligned and are thus excluded from the analysis. The use of merged genomes, in turn, requires that high-quality reference assemblies already exist for both parents, and inconsistencies in assembly or annotation quality between the parental genomes can introduce bias. Overall, the research community has not yet reached a consensus on analytical methods for ASE and imprinting, and the relative applicability of different approaches remains to be systematically evaluated.”

Finally, the authors clarify DNA methylation patterns associated with MEGs in their response. I encourage them to incorporate this analysis into the manuscript (e.g., as supplementary data).

Done. Please see Lines 221 - 224 and Fig. S19 for details.

All other points have been satisfactorily addressed.

We once again want to thank you for your kind advice and support.

Reviewer 3

The authors’ response fails to address the foundational concerns raised regarding the validity of the analytical framework, the assumptions underlying cell type assignment, and the interpretation of trajectory analyses derived from the Visium platform.

We thank the reviewer for their highly constructive suggestions regarding whether Visium spatial transcriptomics can achieve single-cell resolution and the use of spatial data for trajectory analysis. To achieve true single-cell transcriptomic precision and to validate the imprinted genes identified from our spatial transcriptomic data, we newly generated snRNA-seq data for both BM and MB samples, with two replicates per sample. Overall, we achieved

cell-type-level validation rates of 79% for PEGs and 70% for MEGs among the imprinted genes originally identified using the spatial data. Furthermore, we reconstructed the endosperm developmental trajectory using the snRNA-seq data, which yielded results largely consistent with those obtained from the Visium data. Please see our detailed response below.

First, the authors state that maize endosperm cells have an average size of $60\ \mu\text{m}^2$: “It is worth noting that endosperm cells in maize at 14 days after pollination are generally large, with an average size of $60\ \mu\text{m}^2$ ”. This is an area measurement. In contrast, the Visium spot dimension provided ($55\ \mu\text{m}$) refers to a linear diameter. These quantities are not directly comparable and cannot be used to justify spatial equivalence. Using the values provided by the authors, a Visium spot with a $55\ \mu\text{m}$ diameter corresponds to a capture area of approximately $2,375\ \mu\text{m}^2$. A cell size of $60\ \mu\text{m}^2$ would therefore imply that a single spot encompasses transcripts from many cells rather than one or a few. Under the numerical assumptions stated in the response, the conclusion that most spots represent individual or near-individual cells is mathematically unsupported.

We thank the reviewer for pointing out this issue. We apologize for the mistake in our previous manuscript and response letter—the endosperm cells should have a diameter of approximately $60\ \mu\text{m}$ on average², rather than an area of $60\ \mu\text{m}^2$. This error has been corrected in the current revision.

We also fully agree that even though the diameter of endosperm cells ($60\ \mu\text{m}$) is comparable to the spot diameter of Visium ($55\ \mu\text{m}$), it is still difficult to avoid mixing the RNA of multiple cells within one spot. We have provided a detailed statement of this technical limitation in the Methods section (Lines 435 - 451). This is also the reason we supplement our study with new snRNA-seq data to investigate developmental trajectory at single-nucleus level.

Second, even if the Visium spot diameter is numerically comparable to the reported average cell diameter, this does not establish single-cell resolution. A Visium spot represents a two-dimensional circular capture area within a tissue section, whereas cells are three-dimensional,

irregularly shaped structures that may be partially intersected by the sectioning plane. Numerical similarity between spot diameter and average cell diameter does not ensure that captured transcripts originate from a single cell. Partial overlap with adjacent cells, irregular packing, and boundary effects are inherent features of biological tissues. Therefore, diameter equivalence alone cannot be used as evidence that most spots represent individual cells.

Completely agree. We have carefully revised our manuscript to avoid any statement of “Visium spatial transcriptomics having single-cell resolution”.

Third, the response does not address the central methodological concern that mixture transcriptomes fundamentally affect both cell type annotation and trajectory inference. Even if some spots predominantly represent a single cell, no quantitative evidence is provided to demonstrate how frequently this occurs. More importantly, no independent validation is presented to establish that individual spots correspond to biologically discrete cells rather than mixed populations. In the absence of orthogonal evidence, such as single cell reference data or direct experimental confirmation at cellular resolution, the reliability of the reported cell identities and inferred developmental trajectories remains unsupported.

We thank the reviewer for the constructive suggestions. We have analyzed snRNA-seq data and compared with Visium data for cross-validation. Specifically:

1. We newly generated endosperm snRNA-seq data with two replicates each for BM and MB, yielding a total of 38,066 nuclei after quality control (18,554 for BM and 19,512 for MB). Cell type annotation was performed using CellAssign combined with manual curation, and we identified six endosperm cell types similar to those from the Visium data, suggesting that our Visium-based annotation was not substantially affected by the “mixed transcriptome” effect.

2. The cell-type specificity of ASE was independently recapitulated. The cell-type specificity of ASE genes in the snRNA-seq data (59.9%-70.1%) was highly consistent with the Visium results (59.4%-68.1%). Please see Lines 185 - 189.

3. Imprinted gene identification was independently recapitulated. Using the snRNA-seq data, we identified a total of 28 MEGs and 47 PEGs, which is highly consistent with the numbers obtained from Visium (29 MEGs and 56 PEGs). Furthermore, at the cell-type level, we recalled the MEGs and PEGs identified by Visium in the corresponding cell types of the snRNA-seq data. The overall recall rate was 79% for PEGs and 70% for MEGs, with recall rates exceeding 80% in several cell types. When the parental bias ratio of imprinted genes threshold was weakened by 10 percentage points, the recall rates for PEGs and MEGs increased to 92% and 88%, respectively, indicating that the imprinted genes originally identified from our Visium spatial transcriptomic data are generally highly accurate. Please see Lines 225 - 237.

4. All three imprinted lncRNAs identified by Visium were validated by snRNA-seq. Please see Lines 327 - 328 and Lines 333 - 335.

In summary, we cross-validated the Visium data against the snRNA-seq data in four aspects: endosperm cell type identification, cell-type specificity of ASE, imprinted gene identification, and imprinted lncRNA identification. All comparisons showed good consistency, suggesting that our cell-type-level analyses based on Visium spatial transcriptomics were not severely affected by the mixed transcriptome effect.

The broader concern is that the biological conclusions throughout the manuscript are constructed on the implicit assumption that a Visium spot represents one or at most a very small number of cells. This assumption underlies the cell type annotations, the spatial pattern interpretations, and the inferred developmental trajectories. However, as discussed above, this premise is not empirically established. If individual spots contain mixed transcriptomes, then the derived cell identities and developmental inferences may reflect compositional averaging rather than true cellular states. Consequently, the interpretative framework of the study depends on an assumption that has not been demonstrated, which substantially weakens the reliability of the reported biological conclusions.

Thank you for your suggestion. As mentioned in our responses above, in the revised manuscript we have clarified all statements that associate Visium spatial transcriptomics with single-cell

resolution. Furthermore, the snRNA-seq data serve as a powerful cross-validation, demonstrating the accuracy of the ASE and imprinted genes identified from the Visium transcriptomic data, as well as the high degree of cell-type specificity conclusions inferred from these genes.

For the trajectory analysis, we have now fully based our analysis on the snRNA-seq data in the revised manuscript, and the results largely recapitulate the conclusions obtained from the spatial transcriptomic data, suggesting that the difference in parental transcriptional programs gradually increases during maize endosperm development.

References

Fu, Y., et al. (2023). "Spatial transcriptomics uncover sucrose post-phloem transport during maize kernel development." Nature communications **14**(1): 7191.