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Peer Review File

Plastoglobules Compartmentalize Nitrogen Assimilation in Maize

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Plastoglobules Compartmentalize Nitrogen Assimilation for Nitrogen-Use Efficiency in Maize

Plastoglobules (PGs) are known primarily for their role in antioxidant metabolism. This manuscript identifies a new and fascinating role in nitrogen-use efficiency (NUE). It convincingly demonstrates that two key enzymes in nitrogen fixation — nitrate reductase 2 (NIR2) and glutamine synthase 1 (GLN1) — are specifically recruited to plastoglobules, where they are hypothesised to channel enzymatic reactions to increase the efficiency of the process. Using cryo-EM, the manuscript demonstrates that GLN1 forms a decameric complex when purified from overexpressing E. coli cells.

Comments
This an interesting manuscript reporting on a what may be considered a scientific breakthrough, namely how nitrogen-use efficiency is optimized in plants and how NUE could be further improved by genetic engineering. The paper is well written and contains high quality data. But several important points need to be addressed.
A clear rationale for the association of NIR2 and GLN1 with PG is not given and it is important to explain in the discussion how this association improves metabolic channelling and NUE.
Line 131: nitrogen-dependent alterations to the morphology and number of PGs. The size of the PGs increased with increasing nitrogen concentrations, but the reason for this is unclear. The manuscript could be improved further by assessing the contents of the PGs, which are likely to be of a lipid or antioxidant nature. The relationship between PG content and NUE appears to be a fundamental open question.
Lines 253 and 260: Do the intrinsically disordered regions (IDRs) at the N-termini (1–57 in NIR2 and 1–55 in GLN1) correspond to the mature regions of the proteins or the chloroplast targeting signals at the N-termini of the full-length proteins (see also comment on Extended Data Figure 9)? This important issue needs to be clarified and explained more clearly in the Results and Methods sections. Fig. 4 a and c: Along these lines: Is cytoplasmic localisation observed in the absence of IDR? In the absence of IDR, it is expected to be found inside the chloroplast but not associated with the PG.
Line 275: The semi-in vivo CO-IP suggests that the first 55 amino acids (IDR) of GLN1 are required for interaction with NIR2, but this is somewhat unclear as they are also required for targeting to PG, without which the two proteins could not interact in the first place. Therefore, this is not entirely conclusive, and the text should be modified accordingly.
Lines 38 and1013: “Cryo-EM structures show PG-localized GLN1 forms a decameric complex”. The statement in the abstract is incorrect. The GLN1 forming a decameric complex was not “PG-localized” but rather it was purified from E. coli cells that overexpress it. Furthermore, if GLN1 were PG-localized, it would be expected to be in a complex with NIR2, since the two proteins have been shown to interact elsewhere in the paper. In conclusion, it is unclear whether PG-localised GLN1 would also form a decameric complex.

Fig. 2c: are these single chloroplasts or single cells?
Fig. 4 a and c: Is localisation in the cytoplasm observed in the absence of IDR?
Extended Data Fig.9: Schematic representations of GLN1 to GLN6 are shown. GLN1 is the only one with an intrinsically disordered region (IDR) at its N-term. This (again) raises the question of whether the IDR corresponds to the chloroplast targeting signal or a portion of the mature protein.

Referee #2

(Remarks to the Author)
Efficient nitrogen assimilation is essential for crop productivity and global food security, yet its subcellular organization remains poorly understood. In this study, the authors identified plastoglobules (PGs), small lipid droplet–like structures within chloroplasts, as evolutionarily conserved metabolic hubs that coordinate nitrogen utilization. PGs expand dynamically in response to nitrogen availability in maize, and this response is conserved across monocot and dicot species. Using an optimized purification approach, the authors profiled the maize PG proteome and, for the first time, identified NIR2 and GLN1 localized within PGs. Both enzymes are targeted to PGs via IDRs. Cryo-electron microscopy revealed that the PG-localized GLN1 assembles into a decameric complex, which interacts with NIR2 to form an efficient metabolic channel, thereby enhancing assimilation efficiency. Furthermore, natural variation was found to generate two isoforms of NIR2. NIR2T1 contains the IDR and localizes to PGs, and NIR2T2 which lacks the IDR and localizes in the cytoplasm. Importantly, natural variation in NIR2 correlates with NUE, and its overexpression markedly increases both biomass and NUE.

This work introduces a powerful and widely applicable strategy for developing high-NUE crops. More importantly, its paradigm-shifting insights open a new frontier in metabolic engineering, showing how subcellular compartmentalization can be leveraged to improve enzymatic efficiency and NUE. Overall, this manuscript is of considerable significance and value. However, it would benefit from further strengthening in the following aspects:

Major points：
1. The discovery of NIR2 and GLN1 within the PG proteome is an exciting and important finding. To further substantiate their localization, it would be valuable to include additional controls assessing plastid fraction purity, such as immunoblotting with compartment-specific markers and SDS-PAGE fractionation, to more rigorously exclude the possibility of contamination from thylakoid or stromal proteins. The authors are encouraged to cite the recent PG proteome studies by Devarasu et al. (2025a, 2025b), which defined a core stress-responsive PG proteome in maize. Highlighting the distinctive strengths of the present methodology relative to these works, along with a comparative discussion of NIR2 and GLN1 abundance and enrichment patterns, would help contextualize the current findings. In addition, the submission of raw proteomics data and relevant processing parameters will be important to ensure reproducibility and further support the conclusions drawn.

2. The observation of natural variation of NIR2 is intriguing. To strengthen the study, I recommend that the authors include near-isogenic lines (NILs) or NIR2T2-overexpression lines, if feasible, to provide complementary evidence that clarifies the specific contributions of NIR2T1 and NIR2T2. In addition, a more in-depth discussion of why certain germplasm preferentially expresses the NIR2T1 isoform while others favor NIR2T2, considering possible genetic, evolutionary, or breeding-related factors would enhance the biological interpretation.

3. The manuscript presents compelling functional evidence for PG-localized NIR2T1, showing that NIR2T1 overexpression enhances plant height and biomass under low, normal, and high nitrogen conditions compared to the wild type. I am curious whether NIR2T1 overexpression consistently improves NUE and yield performance under field conditions, particularly regarding yield-related traits under different nitrogen levels.

4. Structural analysis reveals that the decameric GLN1 complex within PGs closely resembles cytosolic GS1a in catalytic mechanism, suggesting comparable enzymatic activity between the PG-localized and cytosolic forms. It would be interesting to know whether other NIR and GLN family members obtain the N-terminal IDR, and whether IDR primarily mediates subcellular localization or additionally affects catalytic efficiency. To strengthen the proposed model, in vitro enzyme activity assays comparing full-length and IDR-deleted variants of NIR2 and GLN1 are recommended to determine whether the IDR play a role in catalysis or serves solely as a localization determinant. Furthermore, investigating the subcellular localization of NIR and GLN family members under different nitrogen conditions would help clarify whether their association with PGs is dynamically regulated by nitrogen availability.

5. The manuscript presents a compelling analysis of nitrogen-related processes. To further enhance the physiological relevance of this work, it would be valuable to consider placing these findings in the broader context of metabolic integration. Given that PGs appear to function as a lipid metabolic hub, a more extensive discussion of carbon–nitrogen co-regulation at the systemic level could help situate the study within the larger framework of plant metabolic networks.

6. The study convincingly shows the dynamic response of PGs to nitrogen status. Building on these solid findings, it would be interesting to explore in future work the upstream signaling mechanisms through which nitrogen status regulates PG expansion. An open question remains whether carbon metabolism also participates in this regulatory process. A discussion of potential signaling pathways involved would further strengthen the biological insight of this compelling study.

Minor points:

Overall, the manuscript is well organized and clearly written. However, several minor issues still need to be addressed.
1. Throughout the manuscript, the clarity of statistical reporting could be improved, particularly in defining “biologically independent samples”. For example, in Fig. 1c (n = 20 biologically independent samples) and Fig. 3d (n = 3 biologically independent samples), it is unclear whether these numbers represent individual plants measured within a single experiment, or the total number of samples combined across multiple independent experiments.
2. For biochemical experiments (e.g., Fig. 4d and 4g), it would be important to specify in the figure legends that the

experiments were independently repeated three times with similar results. Likewise, for the microscopy data, please indicate how many tobacco leaves were used and how often the observations were repeated.
3. In Fig. 1d, the nitrogen concentration is presented without units.
4. In Fig. 5b, could the authors clarify the average T1/T2 ratio observed in the overall population? This information would help determine whether the ratios observed in the selected inbred lines are relatively high or low.
5. In Fig. 3c, the authors show that gln2–gln6 genes encoding proteins not localized to PGs showed no obvious morphological defects, whereas gln2 resulted in reduced plant height. Similarly, in Fig. 5j and k, the quantitative data clearly show that NIR2T1 overexpression lines produce higher fresh weight than the wild type under all nitrogen conditions; however, the seedling phenotype shown in panel k does not appear markedly different. The authors may wish to provide additional representative images or clarify this discrepancy.
6. In Extended Fig. 7 and Fig. 8, the x-axis label on the bar charts should be corrected from HN to NN. The gene names gn2 and gn4 should be corrected to gln2 and gln4.
7. In Fig. 4 and Extended Fig. 12, the western blot panels need improvement. The bands are not well aligned horizontally, and the lanes display noticeable variations in molecular weight.
8. Lines 122-123 “transmission electron microscopy (TEM) of chloroplasts in leaves of maize seedling...” can be rephrased to “transmission electron microscopy (TEM) on chloroplasts of maize seedling leaves...”
9. Lines 186-189，The logical flow of the sentence could be improved for better clarity. Rephrasing is suggested.
10. Line 318: “was performed using partial inbred lines” should be “selected inbred lines”.
11. Line 406: “underscorethe the functional importance” should be corrected to “underscore the functional importance”.
12. Discussion section: The repeated mention of the role of PG in nitrogen assimilation should be omitted.
13. Some typos should be corrected. For example, Line 257: “For NIR2，variants...” should be “For NIR2, variants...”; Line 316: “NIR2T2 lacking 139 amino acids…”should be “NIR2T2 lacks 139 amino acids...”; Line 644: “CO-IP” should be “Co-IP”.

Referee #3

(Remarks to the Author)
Improving nitrogen use efficiency is becoming a priority for crop improvement. This study breaks new ground by shedding light on nitrogen assimilation in chloroplasts of maize, a major crop. Chloroplastic plastoglobules (PGs) were previously viewed as hubs of lipid metabolism. In this study by Chen et al., the authors describe a new role of PGs as a critical site in nitrogen assimilation. The authors use a set of cutting-edge and accepted standard methodology to identify and characterize two main proteins of maize underlying and orchestrating the nitrogen assimilation function of maize PGs through nitrate reduction and glutamate synthesis. The methods used in this tour-de-force study range from advanced proteomics, modern structural biology, genetics, genomics, greenhouse experiments and cell biology. The results presented are of immediate interest to many people in crop research and plant science. Due to the importance of food security in face of climate change, the negative influence of nitrogen overfertilization on marine food webs and ultimately climate stability, and due to finite fossil fuels used for the production of nitrogen fertilizers, the study is of great societal importance. In my opinion, these implications extend well beyond plant research, offering value to a broad scientific community.

In short, the authors investigated how nitrogen availability influences PG number and size in maize and identified two key PG-associated proteins, NIR2 and GLN1. Through colocalization assays in tobacco, they demonstrated that NIR2 and GLN1 proteins target PGs, likely via newly discovered N-terminal intrinsically disordered regions. The study further reports the cryo-EM structure of maize GLN1, revealing an oligomeric architecture typical for plant GLN proteins. By assessing GLN1 oligomerization under ammonium treatment, the authors suggest a potential link between PG-associated proteins and nitrogen use efficiency. Finally, they then switch back to NIR2 and analyse its splice variants to highlight the functional importance of the NIR2 disorder region only translated from the T1 isoform under variable nitrogen conditions and that relevant preferential splicing variation in NIR2 exists in the maize germplasm underlying the nitrogen use efficiency trait.

The conclusions made by the authors are generally original and often strongly supported by their own data. Although the clarity of how this study links PGs and nitrogen assimilation is unprecedented, the authors are sometimes overemphasizing the novelty of their own findings. Afterall, a very recent study already studied in detail that chloroplastic nitrogen assimilation in maize is mediated by ferredoxin (ZmFd4) and NIR proteins (NIR1, NIR2 also shown in extended data) (Jia et al. Nature Plants 11, 643-659, 2025). Just last year, a work in Arabidopsis showed responses of PGs to nitrogen starvation (Coulon et al. Journal of Experimental Botany, Vol 75, No 20 pp, 6542-6562, 2024), although Coulon and colleagues focused on lipid metabolism as a readout for PG function.
I recommend publication. However, there are a few major and minor points that should be amended before publication.

Major points
1. I find the authors overemphasize the novelty and impact of their study. I suggest to choose more precise phrasing that relies less on buzz words like ‘paradigm’, (counted four times in lines 43, 107, 365, 448), ‘for the first time’ (counted four times lines 103, line 170, 174, 394), ‘universal’ (line 44). Each of these occurrences could be counterfeited, e.g., is it really a ‘universal’ crop improvement strategy when only maize was tested? Can is really be ‘unveiling a new paradigm for subcellular metabolic engineering’ when the authors provide a first example of this strategy (how can something already be a paradigm when it is done for the first time)? I think the authors have reason to emphasize the novelty and impact of their study, but should use such terms only where appropriate. Reconsider the wide use of the word ‘unequivocally’.
2. In line 288-290, the authors wrote ‘we performed high-resolution cryo-electron microscopy (cryo-EM) analysis of purified PG-localized GLN1 from maize chloroplasts‘. In the method paragraph ‘cryo-EM sample preparation’ (line 1014-1016), the authors wrote ‘the GLN1 gene was cloned into the pET28a–SUMOstar vector …’.
Please clarify exactly from which organism the protein for cryo-EM was obtained; recombinant versus endogenous protein

source? How can E-coli protein be PG-localized? Please also briefly describe in the main text relevant detail how the protein was purified. E.g. affinity purification (tag?), size-exclusion chromatography (column?) for improved readability. I am also not satisfied with one SDS page shown in Extended Data Fig.10,a. Please show full recombinant protein purification pipeline with intermediate results: SDS page of affinity purification and SDS page of SEC fractions. Please enlarge Extended Data Fig.10, c (negative staining) so we can see the individual particles and monodispersity of the sample. Scale bar in negative staining without label.
3. Please include the proteomic raw data (Figure 2b), and an explain it more. I only found three long sheets 'group1', 'group2', and 'WT' in the source data, seems to be the proteomic data of IP-MS for figure 4, but not for figure 2. With this limited information, I could not trace how the 13 proteins in figure 2 were selected.
4. Please clearly explain how PG number and area was quantified. Can you rule out that under high nitrogen regime, there are not just simply more and larger chloroplasts? Does the PG number per chloroplast volume really increase too or, in other words, do larger chloroplasts not automatically contain more PGs? Phrase accordingly.
5. Jia et al. (nature plants 2025) reported ZmNIR1 and ZmNIR2 chloroplast localization in transient tobacco assays. This is contradicting with the current study, stating that only NIR2 is localized to chloroplasts. Please explain this contradiction
6. I am desperately missing a loading control in Fig 4, g and Fig 4, h. Without it, the data cannot be interpreted and I have to disagree with the conclusions. Increased decamer signal could also stem from increased protein concentration in loading. In the corresponding text (lines 299-303), I disagree with several statements. 'cross-linking showed that GLN1 exists in different assembly states': No! It is rather, with increased cross-linker concentration, more higher order complexes could be stabilized. We learn nothing about in vivo assembly of the complex from cross linking. Also, 'increased levels of exogenous NH4+ substrate led to enhanced GLN1 oligomerization' No! It seems like NH4+ stabilizes the hexamer and smaller complexes, but not octamer or decamer. Loading control! Also, what is the band at 72kDa and why does it increase with NH4+ concentration?
Discussion reads mostly like a summary of the data and for me falls quite flat. It could be much more exciting. Particularly, I am lacking a higher-level contextualization of the data. For example, how is the dual function of PGs in lipid metabolism and nitrogen assimilation balanced? We know since a long time that low nitrogen leads to leaf yellowing, is this related to PG function in nitrogen assimilation? Why do certain maize cultivars have decreased nitrogen use efficiency; was this accidentally selected by historic breeding? How about nitrogen use efficiency and GLN1/NIR2 in legumes, that rely less on nitrogen fertilization due to nodule bacteria? Under what environmental/agricultural scenarios is low NUE useful, why did it evolve in some wild populations?
7. I am missing the data described in lines 250-255. Please include AF3 models.
8. Figure 4, b and line 261: Does deletion of IDR recruitment or even disturb PG formation?
9. Figure 5, g: Show data of individual lines here. Due to the number of genetic differences, it seems inappropriate to bulk different lines into one plot. Statistics become problematic.
10. Line 304-309: Why no NIR2 is co-purifying in the complex? Please be aware that complex formation is formally never shown in the manuscript. Simply pulldown experiments were made.

Minor points
What's the difference between WT and CK? What does CK stand for? In Fig 3, d, what is the reason that the gln1 and nir1 are compared with CK, but others with WT?

Throughout the text, clarify the concept of metabolic channelling better and avoid confusion with plasma membrane channels/transporter.

Use 'PG' and 'PGs' (singular versus plural) appropriately. I spotted many such mistakes throughout the text. For example, it should be 'PG localization', not 'PGs localization'.

Fig. 1, a: Why does the chloroplast in the TEM image at 2mM N look so different from other images (white empty cavities)? Is this a preparation artifact?
Fig. 2, a: Typo in 'Plant Materital' should be 'Material'.
Fig. 4, a: Legend is far too small! Better use same font size for all labels in all figures.
Fig. 4, b, c: delta55 should be 'GLN1delta55' to avoid confusion with NIR2 in the same figure.
Fig. 3, e; Fig. 4, b; c; Fig. 5, c: Confocal microscopy pictures need to be more zoomed in. We cannot recognize PG composition in this view.
Fig 3, a: The log2 Exp scheme is too small for print.
Extended Data Fig.5: Some mutants show which amino acids were changed into stop codon, but some not.
Extended Data Fig.8, a: Typos in labels 'gn2' and 'gn4' should be 'gln2' and 'gln4'. In Extended Data Fig.8, b, label 'CK' is appearing (see above).
Line 41: Add 'maize' in front of 'NIR2' for context.
Line 42: First use of abbreviation NUE. Avoid introducing abbreviations in abstract.
Line 49: Avoid using 'efficient' and 'efficiency' in the same sentence.
Line 57-58: 'Maize, recognized as the world's most widely cultivated crop globally'. 1) 'most cultivated' means grown on largest agricultural area or most tons produced? 2) please include a source for this information, e.g. FAO.org has excellent data supporting such claims 3) 'world's' and 'globally' are redundant. 4) 'recognized'=subjective. Better rely on data.
Line 59: For general readers, it could be useful to briefly explain the term 'C4 plants'?, Perhaps discussing the link between C4 metabolism and nitrogen utilization could be something for the discussion too?
Line 75: Unclear what 'it' is referring to.
Line 114: Which N source was used in the experiments? Ammonia, Nitrate, urea? Specify at least once in main text and in figure legends.
Line 130: avoid using reference 26 in the middle of construct name.

Line 130: ‘L.’ stands for Linnaeus? Since Nicotiana tabacum and the other species used here are standard model plants, I find the mentioning of the first describing author of the species somewhat obsolete (we do not have to worry about confusion here). If the authors want to really indicate the first- describing author, please do so consistently. For example, in line 130 they authors write ‘Nicotiana tabacum L.’ and in line 141 they write ‘Glycine max (L.) Merr.’ and ‘Nicotiana tabacum L.)’. Please correct the typos.
Line 133: Avoid using ‘under’ as it is confusing here. Better say ‘at’.
Line 143: Abbreviations LN and NN were already introduced in line 116.
Line 192 and elsewhere: Avoid lab jargon ‘Agroinfiltrated’.
Line 203 and Figure 3: Figures 3, a-e are not appearing in sequence of text.
Line 242f: Overinterpretation! The authors have not tested a causal relationship between localization and function, for example through genetically complementing with both NIR2 and GLN1 variants with mislocalization. Merely correlative data is shown.
Line 271: I would argue that IP-MS is still ‘biased’ by extraction method, data processing etc.
Line 272 and 277: unclear if ‘transgenic plants’ means maize or tobacco?
Line 313 and elsewhere: the term ‘natural variation’ seems inappropriate since no wild populations were studies. What the authors did is to look at ‘genetic variation in cultivated germplasm of maize’. Please correct the use throughout the manuscript (e.g., line 329, 340) In line 438, better say ‘inbred lines’ than ‘natural populations’.
Line 339: Wrong figure reference. Should be Extended Data Fig. 12, a.
Line 356: LN, NN and HN were already introduced. Strike out.
Line 406: typo ‘underscorethe’
Line 615, missing unit after ‘scale bar, 1’

Comments to the PDB report. Minor inconsistencies. Does not change the interpretation of the structural data or the manuscript altogether:
1. In the PDB report page 5, G203 in chain A also P204 in chain B are marked as a residue with a poor fit to the EM map. Please check is there any flexibility of the adjacent prolines P202 and P204 in Chain A and B. Please consider the atom-atom clash between chain B P204 and M245 in page 12. These might be the outcomes from same reason.
2. In the PDB report page 5, E410 in chain A with a poor fitting might be due to its sidechain. E410 side chain OE1 and OE2 are out of the map. Side chains of K418 and L419 are also located at the outside of the map.
3. Chain B G47, as the first residue, is entirely uncovered by the map. Perhaps the authors can start to build the model from residue 48? Please check all the G47 residues among different chains, they overall with poor fits.
4. Region of chain F from residue 410-413 is not in the map. Also, please see the pdb report page 20 chain F E412 with a non-rotameric sidechain. Same in chain F, side chains of K418 and L419 are out of the map.
5. Please check the issue about atom clash. The structure model has a long list of close contacts. Among the 284 contacts, the clash overlap between chain F, residue F218 and chain G, residue K188 is too large (1.14 Å!).
6. There are 27 side chain outliers in all chains (page 19).

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)
This is an interesting study in which a substantial amount of work has been conducted to demonstrate that plastoglobules (PGs) are key regulators of nitrite reduction and subsequent assimilation in maize leaves. The topic falls within the broader goal of improving crop nitrogen (N) use efficiency for sustainable agricultural productivity, particularly in maize - a crop of major global economic importance. Maize is known for its high demand for both N fertilizers and water. However, the Introduction remains too general and does not fully reflect the current state of knowledge on maize nitrogen use efficiency (NUE). In particular, it does not adequately address the importance and validation of candidate genes involved in the ammonia assimilatory pathway, as well as amino acid biosynthesis and interconversion. Furthermore, although the significance of cellular compartmentation in inorganic N assimilation and organic N recycling is mentioned, I disagree with the claim that our understanding of these processes remains very limited (lines 86–90). The literature review is insufficient, both from physiological and molecular perspectives, especially regarding the two cell types found in maize (bundle sheath and mesophyll cells). Additionally, the phrase “atomic resolution level” is unclear and requires clarification. We have the general impression that the authors have structured their introduction in a way that suggests their work on plastoglobules in relation to primary nitrogen assimilation is entirely novel—when, in fact, this is not completely the case. Moreover, the limited but existing literature in the field is not even cited. The same remarks apply to the Discussion, although both the novelty of the findings and their significance are presented convincingly. In summary, the Introduction and Discussion sections would benefit from a thorough revision to better reflect the existing literature in relation to the originality of the findings.
Major comments:

1) The results presented in Figure 1 are largely descriptive and do not provide significant novel insights, particularly regarding the phenotypic evaluation of plants in response to varying nitrogen (N) nutrition levels. The same observation applies to the data shown in Extended Data Figures 1 and 2. Furthermore, the rationale behind the experiments presented in these two figures is not clearly introduced and remains ambiguous in the context of the broader study. The growth conditions of the plants in these figures appear inconsistent with those described elsewhere in the study, which raises questions about

their comparability. A major limitation of this study is the complete omission of the occurrence of morphologically distinct mesophyll and bundle sheath chloroplasts. This oversight is particularly problematic given the emphasis placed on the importance of cellular and subcellular compartments in the Introduction. Leaf sections where two types of cells are visible should be presented to both visualize and reliably quantify plastoglobules in the corresponding chloroplasts. The same issue applies to the fluorescence images presented in Figure 1 and the other figures of the manuscript
2) While the objectives of the study presented in Figure 2 are well-founded, my previous comment also applies here. There are several established methods available for isolating the two types of maize cells and their respective chloroplasts. This major issue becomes even more critical when considering the nitrogen-assimilating enzymes analyzed in the subsequent proteomic study.
3) Although the selection and characterization of the Nir and Gln mutants are only briefly described—and their homozygosity remains unclear—they appear to be acceptable. To improve the clarity of the results, the accession numbers for the different genes should be provided, especially for those encoding plastidic or cytosolic glutamine synthetase (GS). This is important because the nomenclature for these genes varies across published studies. Along these lines, it should be explicitly stated in both the results section and the figure legends that Gln1 encodes a plastidic GS (GS2). It is somewhat surprising that most genes encoding cytosolic GS are expressed at higher levels in roots compared to leaves.
4) The experiments regarding the PG-dependent metabolic channel for primary nitrogen assimilation are convincing. However, the cell-type compartmentalization of this pathway is not addressed, despite the fact that nitrate and nitrite reduction primarily occur in the leaf mesophyll. It is therefore likely that the observed biological process also takes place in this cell type. Nevertheless, the possibility that this process occurs in bundle sheath cells cannot be excluded. In these cells, GS2 is present in the chloroplasts, while the NIR enzyme is considered to be absent. This raises questions about the potential cooperation between these two enzymatic activities within plastoglobules (PGs), further emphasizing the importance of cell-specific distribution in nitrogen assimilation within a C4 plant.
5) Line 302 and Figure 4h: The claim that GLN1 assembly occurs in a substrate-concentration-dependent manner should be interpreted with caution. The ammonium concentrations used in this study are significantly higher than the enzyme's Km for this substrate (which is in the micromolar range) and are therefore likely physiologically irrelevant. Additionally, the production of photorespiratory ammonium in bundle sheath cells—at least during the early stages of plant development—and the resulting cellular compartmentalization of ammonium need to be carefully considered. Unfortunately, these aspects were not adequately addressed in the present study.
6) The results concerning the natural variation of NIR2 are quite convincing. However, the cytosolic localization of NIR2T2 appears to be questionable in light of the known electron donor for the corresponding enzyme activity, which is a plastidial ferredoxin in both photosynthetic and non-photosynthetic tissues.
7) In the section dedicated to the functional validation of the PG-localized NIRT1, the appropriate control should be the NIRT2 overexpressors. It would be advisable to indicate the nitrogen (N) concentrations (LN, NN, and HN) directly in the text, rather than only in the legend of Figure 5.
8) The discussion emphasizes the importance of cellular compartmentalization in the control of primary nitrogen (N) assimilation. However, the study is limited to events occurring in the chloroplast, without considering that maize leaves contain two types of cells and thus two types of chloroplasts, each with distinct metabolic and physiological functions. While it has been clearly demonstrated that plastoglobules play a key role in controlling nitrite reduction and ammonia assimilation within one type of chloroplast (probably in mesophyll cells), the broader physiological implications at the leaf level and potentially at the whole-plant level remain poorly understood. The text also mentions compartmentalization mechanisms, but these will need to be further assessed using a combination of metabolic analyses and metabolic modeling approaches.

In summary, the technical and scientific aspects of the work are both novel and excellent. However, the plant biochemistry and physiology, along with their interpretation, are insufficiently supported by the available data. This limitation leads to an overestimation of the potential breeding and agronomic applications.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I now fully endorse this study. The revised manuscript is a major improvement on the original version. The authors have addressed all of my comments in their 75 page rebuttal. They have done so in a specific and very satisfactory manner, having carried out a substantial amount of additional experimentation in the process. Overall, the manuscript is novel and comprehensive, covering everything from biochemistry to field testing. It provides a blueprint for engineering plastoglobules (PGs) for metabolic channelling in crop species.
Improvements regarding my comments:
1) Questions surrounding the localisation of NIR2 and GLN1 have been answered, and the separate sequences required for chloroplast targeting and plastoglobule association have been experimentally identified.
2) A clear rationale has been provided for the association of NIR2 and GLN1 with PG.
3) The relationship between PG content and NUE has also been experimentally addressed.
4) Questions regarding the semi-in vivo CO-IP have been answered.
5) The discussion has been significantly enhanced.

Referee #2

(Remarks to the Author)
The authors have thoroughly addressed all my concerns and the manuscript has been significantly improved. I am satisfied with the current version and strongly support its publication in Nature.

This study represents an important contribution to the field of plant and crop science. It provides new insights into the spatial regulation of nitrogen metabolism at the intracellular level and introduces an innovative framework that goes beyond conventional gene discovery approaches.

The newly added data are of high quality, and the statistical analyses are appropriate and well presented.

Only a few minor issues remain before publication:
1. In Fig. 1a, the capitalization of the first letter appears inconsistent. Please check and ensure consistent formatting across all figures.
2. The titles of Extended Data Fig. 6 (“Gene structure of nir and gln mutants”) and Extended Data Fig. 9 and 1 (“Different nitrogen concentration treatments on nir/gln mutants”) could be revised for clarity.
3. The manuscript currently uses both WT and CK to denote controls. For consistency, it may be preferable to use WT throughout, with figure legends indicating different genetic backgrounds if necessary.

Referee #3

(Remarks to the Author)
The authors have thoroughly addressed all scientific concerns, either through improved reasoning or, in many cases, by presenting new data. The PDB report has also been significantly improved through improvements to the 3D density and the model fitting. I particularly appreciate the re-examination of the contradictory data from Jia et al. (Nature Plants, 2025), which has improved the quality and significance of the study

All minor issues regarding typos and labelling have been corrected. However, the authors are submitting a heavily revised manuscript (substantial textual changes). I advise the authors to carefully check their discussion for grammar and typos, should they be considered for print.

Overall, I have no remaining major points and recommend publication.

Referee #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5

(Remarks to the Author)
I am fully satisfied with the revisions made to the manuscript and the additional experiments conducted, particularly regarding the dual compartmentation of nitrogen (N) assimilation in maize leaves. Overall, this is excellent work that significantly advances our understanding of nitrogen use efficiency in maize and its regulatory mechanisms, a research field in which very few major breakthroughs have been made recently. However, I still disagree with two statements made in response to one of my previous comments. These two minor points listed below can easily be addressed in the final revised version of the manuscript.

1) “Cytosolic GS1 serves as the predominant active form in roots, responsible for assimilating root-derived NH4+to prevent ammonium toxicity under high concentrations,”

This statement correctly reflects the predominance of GS1 in roots. However, it is highly unlikely that a toxic build up of ammonium occurs in the soil, and consequently in roots, under standard physiological or agronomic conditions, unless maize is grown in soils or habitats where ammonium naturally accumulates.

2) “chloroplast-localized GS2 is expressed in green tissues and represents the most abundant isoform in leaves, where it functions to reassimilate ammonium released during nitrate reduction in plastids and during photorespiration (Moreira et al., 2022).”

I strongly disagree with this statement. A thorough review of the literature clearly shows that the proportion of GS1 and GS2 activity in maize leaves remains very similar in both mesophyll cells (MC) and bundle sheath cells (BSC), although some variations may occur depending on plant developmental stage, leaf age, and nitrogen nutrition mode. This primarily concerns two cytosolic GS isoenzymes, which are referred to in several older studies, sometimes under different nomenclatures: for example GS1.3 (or GS112/1B) original gene accession number (d14577x65928) and GS1.4 (or GS107/1A), (d14576x65929). Furthermore, it is well established that maize belongs to the group of plants that predominantly assimilate nitrogen in the leaves.

To facilitate your review and to outline the structure of the revised manuscript, we have used text in different colors and included a table of contents with reference to the corresponding sections. The explanations are provided below：

Black text	Indicate the original comments from reviewers
Bule text	Indicate our direct response to the reviewers's comments

Table of content

Referee #1 (Remarks to the Author):

Plastoglobules Compartmentalize Nitrogen Assimilation for Nitrogen-Use Efficiency in Maize

Plastoglobules (PGs) are known primarily for their role in antioxidant metabolism. This manuscript identifies a new and fascinating role in nitrogen-use efficiency (NUE). It convincingly demonstrates that two key enzymes in nitrogen fixation — nitrate reductase 2 (NIR2) and glutamine synthase 1 (GLN1) — are specifically recruited to plastoglobules, where they are hypothesised to channel enzymatic reactions to increase the efficiency of the process. Using cryo-EM, the manuscript demonstrates that GLN1 forms a decameric complex when purified from overexpressing E. coli cells.

Comments

This an interesting manuscript reporting on a what may be considered a scientific breakthrough, namely how nitrogen-use efficiency is optimized in plants and how NUE could be further improved by genetic engineering. The paper is well written and contains high quality data. But several important points need to be addressed.

Response to the Reviewer

We are deeply grateful for your encouraging feedback and the constructive suggestions provided. These insights have been instrumental in helping us further refine our study and strengthen the empirical foundation of our work.

During this five-month revision period, in accordance with your suggestions and comments, we have made the following major revisions and improvements: (1) In-depth elucidation of the chloroplast and plastoglobules (PGs) localization mechanisms of ZmNIR2 and ZmGLN1. (2) Provision of data demonstrating the influence of nitrogen on the lipid composition of PGs. (3) Supplementation with multiple in vivo and in vitro interaction assays to further verify the interaction between ZmNIR2 and ZmGLN1. Additionally, we have further expanded and

enhanced the “Introduction” and “Discussion” sections.

We believe these substantial additions have significantly enhanced the mechanistic clarity and overall impact of our study. We sincerely thank you for your rigorous guidance, which has been pivotal in helping us reach this more definitive stage of our research.

1.A clear rationale for the association of NIR2 and GLN1 with PG is not given and it is important to explain in the discussion how this association improves metabolic channelling and NUE.

Response to the Reviewer’s Comment:

We thank the reviewer for raising this important point. Here is the rationale for NIR2/GLN1 association with PGs and its role in metabolon and NUE. We will discuss this from the following aspects:

Structural and biochemical basis for PG association: NIR2 and GLN1 are targeted to PGs via distinct hydrophobic regions (N-HR1~6 for NIR2; G-HR1 and G-HR2 for GLN1), as demonstrated by systematic truncation and fluorescence localization experimental results (Fig. 4 and Extended Data Figs. 12, 13). These hydrophobic domains function cooperatively to anchor the enzymes to the lipid monolayer surface of PGs. The cryo-EM structure of GLN1 reveals a decameric assembly that positions its active sites outward, making it architecturally compatible with membrane-docked metabolic complex formation. NIR2 and has direct although interaction with GLN1 within the PG microdomain. Thus, PGs provide a dedicated suborganellar scaffold that concentrates these two sequential enzymes in space.

PG localization enables metabolon: By co-localizing NIR2 and GLN1 on the same PG surface, the physical distance between the two active sites is minimized. This arrangement creates a privileged microchannel that allows direct transfer of NH_4^+ from NIR2 to GLN1, effectively “substrate channeling”. Channeling increases the local substrate concentration at the GLN1 active site, accelerates the overall flux from nitrite to glutamine, and protects cells from toxic NH_4^+ accumulation.

In fact, the view of PGs as centers of lipid metabolism has been extensively

studied, affirming the critical role of PG compartmentalization in metabolic synthesis(van Wijk *et al.*, 2017). Taking lipid metabolism as an example, in the biosynthesis of Plastochromanol (PC8), plastoquinone (PQ) can be reduced to plastoquinol (PQH2) via type II NAD(P)H dehydrogenase C1 (NDC1). PQH2 then acts as a direct substrate and is catalyzed by tocopherol cyclase (VTE1) to generate PC8 (Shanmugabalaji *et al.*, 2022). Notably, NDC1 and VTE1, the two enzymes that function sequentially in the lipid metabolic pathway, are colocalized in PGs, which may thereby enable more efficient biosynthesis of PC8. Similarly, NIR2 and GLN1 discussed in this study, which are also localized to PGs, operate in sequential steps of the nitrogen metabolism pathway. We provide new evidence demonstrating a interaction between NIR2 and GLN1, which may facilitate the formation of an enzyme-enzyme association. Such a working model has been previously elucidated for several multi-enzyme complexes within chloroplasts. The compartmentalized structure of PGs enables the tight spatial coupling of the two enzymatic reactions.

Physiological and agronomic consequences for NUE: The metabolon conferred by PG co-localization translates directly into improved nitrogen use efficiency (NUE). *nir2* and *gln1* loss-of-function mutants exhibit severe growth defects and nitrogen-unresponsive phenotypes even under high nitrate supply, whereas mutants of non-PG-localized isoforms (*nir1*, *gln2-6*) are phenotypically normal (Fig. 3, Extended Data Figs. 9, 10). This genetic evidence establishes that PG-specific localization is necessary for efficient nitrogen assimilation. Moreover, overexpression of the PG-targeted isoform $NIR2^{T1}$ enhances both PG biogenesis and biomass accumulation across nitrogen regimes, and field trials confirm biomass gains under standard fertilization (Fig. 5j-m). These data demonstrate that the PG channeling mechanism is not only physiologically relevant but also agronomically important.

In summary, PGs themselves are dynamic, their number increases with nitrogen availability in mesophyll cell chloroplasts (Fig. 1c-f), and this response is conserved across C_3 and C_4 species (Extended Data Fig. 2). GLN1 oligomerization into active decamers also increases with nitrogen supply. Thus, the rationale of this system

operates through a feed-forward loop: nitrogen sufficiency → PG proliferation + GLN1 assembly → enhanced NIR2/GLN1 channeling capacity → faster NH_4^+ assimilation → further growth. This regulatory logic places compartmentation itself as a control point for NUE, operating above the level of individual enzyme kinetics.

Incorporating the suggestions from all reviewers, we have rewritten the "Discussion" section. Due to space limitations, we have condensed the key points mentioned above and integrated them into the Discussion.

References Involved：

van Wijk, K. J. & Kessler, F. Plastoglobuli: Plastid Microcompartments with Integrated Functions in Metabolism, Plastid Developmental Transitions, and Environmental Adaptation. *Annu. Rev. Plant Biol.* **68**, 253–289 (2017).

Shanmugabalaji, V. , Zita, W. , Collombat, J. , & Kessler, F. . Plastoglobules: a hub of lipid metabolism in the chloroplast. *Advances in Botanical Research* **101**, 91-119 (2022).

2. Line 131: nitrogen-dependent alterations to the morphology and number of PGs. The size of the PGs increased with increasing nitrogen concentrations, but the reason for this is unclear. The manuscript could be improved further by assessing the contents of the PGs, which are likely to be of a lipid or antioxidant nature. The relationship between PG content and NUE appears to be a fundamental open question.

Response to the Reviewer's Comment:

We fully agree with the reviewer's perspective. As an internal chloroplast compartment containing diverse proteins and metabolites, changes in the number and size of PGs under varying nitrogen treatments are inevitably accompanied by alterations in their internal composition. As the reviewer noted, this likely involves changes in lipids or antioxidants, among other substances. This insight provides a valuable direction for us to further explore the relationship between PG composition and nitrogen use efficiency (NUE).

Previous studies have shown that changes in the number and morphology of PGs primarily occur during two processes: lipid remodeling during plastid differentiation and in response to stress. Regarding the former, reports indicate that

the number of PGs decreases during the transition from etioplast to chloroplast. This process releases structural thylakoid lipids (e.g., MGDG) and their precursors (e.g., TG, DG) for thylakoid membrane synthesis (Lichtenthaler, 1968). Concurrently, antioxidant lipids such as tocopherols increase to protect the nascent membranes from damage (Eugeni Piller *et al.*, 2014). During the transition from chloroplast to chromoplast, PGs enlarge and recruit various carotenoid biosynthetic enzymes, participating in the synthesis, esterification, storage, and degradation of carotenoids (Egea *et al.*, 2010). During the transition from chloroplast to gerontoplast, the number of PGs increases. Senescence leads to the disassembly of thylakoid membranes, chlorophyll degradation releasing free phytol, and hydrolysis of galactolipids producing free fatty acids (Ghosh *et al.*, 2001). In the context of stress responses, it has been reported that PG size increases under conditions such as drought, high light stress, and nitrogen starvation, and subsequently decreases upon stress relief (Rottet *et al.*, 2015). Subsequently, researchers discovered that under nitrogen starvation in Arabidopsis, MGDG content significantly decreases, while the levels of TG and phytyl/sterol esters increase. Furthermore, within these accumulating lipids, there is an increased proportion of C16:3 fatty acids in phytyl esters and C18:3 fatty acids in TG (Coulon *et al.*, 2024).

However, the changes in PGs in MC chloroplasts under varying nitrogen levels during normal vegetative growth (Fig. 1 a-c), as explored in this study, have not been reported previously, and thus no information on compositional alterations under these conditions could be obtained from existing literature.

[Redacted text]

[Redacted text]

In summary, this is a highly promising research direction, and we have already conducted extensive data collection to facilitate further investigation. However, we also aim to interpret the results and derived conclusions with caution. The current manuscript is intended to focus primarily on the relationship between PGs and NUE, while detailed analyses of changes in PG internal metabolites will be reserved for future publications. If you have any specific suggestions regarding this topic, we would be very grateful to receive them, and we will incorporate your feedback to refine our subsequent research efforts.

[Redacted text and figure]

References Involved：

Lichtenthaler H.K. , Plastoglobuli and fine structure of plastids, *Endeavour* **27**, 144–149 (1968).

Eugeni Piller, L., Glauser, G., Kessler, F. & Besagni, C. Role of plastoglobules in metabolite repair in the tocopherol redox cycle. *Front Plant Sci* **5**, 298 (2014).

Egea, I.; Barsan, C.; Bian, W.; Purgatto, E.; Latché, A.; Chervin, C.; Bouzayen, M.; Pech, J.-C. Chromoplast differentiation: current status and perspectives. *Plant Cell Physiol* **51**, 1601–1611 (2010).

Ghosh, S., Mahoney, S. R., Penterman, J. N., Peirson, D. & Dumbroff, E. B. Ultrastructural and biochemical changes in chloroplasts during Brassica napus senescence. *Plant Physiology and Biochemistry* **39**, 777–784 (2001).

Coulon, D.; Nacir, H.; Bahammou, D.; Jouhet, J.; Bessoule, J.-J.; Fouillen, L.; Bréhélin, C. Roles of plastoglobules and lipid droplets in leaf neutral lipid accumulation during senescence and nitrogen deprivation. *J Exp Bot* **75**, 6542–6562 (2024).

Rottet, S., Besagni, C. & Kessler, F. The role of plastoglobules in thylakoid lipid remodeling during plant development. *Biochim Biophys Acta* **1847**, 889–899 (2015).

Li, J. *et al.* OsFBN7-OsKAS I module promotes formation of plastoglobules clusters in rice chloroplasts. *New Phytol* **239**, 1771–1789 (2023).

Pralon, T. *et al.* Mutation of the Atypical Kinase ABC1K3 Partially Rescues the PROTON GRADIENT REGULATION 6 Phenotype in Arabidopsis thaliana. *Front Plant Sci* **11**, 337 (2020).

3. Lines 253 and 260: Do the intrinsically disordered regions (IDRs) at the N-termini (1–57 in NIR2 and 1–55 in GLN1) correspond to the mature regions of the proteins or the chloroplast targeting signals at the N-termini of the full-length proteins (see also comment on Extended Data Figure 9)? This important issue needs to be clarified and explained more clearly in the Results and Methods sections. Fig. 4 a and c: Along these lines: Is cytoplasmic localisation observed in the absence of IDR? In the absence of IDR, it is expected to be found inside the chloroplast but not associated with the PG.

Response to the Reviewer’ s Comment:

We appreciate your insightful question regarding this critical issue. In our initial investigation into the localization domains, our experimental results demonstrated that the N-terminal intrinsically disordered regions (IDRs) of NIR2 and GLN1 respectively determine their chloroplast and PG localization. However, we had not delved deeper into this aspect at the time. Regarding the analysis of the chloroplast transit peptide（cTP）contained in NIR2 and GLN1 and the mechanism of their PG localization after entering the chloroplast, we have carefully revised the relevant section of the manuscript based on a combination of multiple cTP prediction analyses methods and fluorescence localization experimental results.

Definition and functional demarcation of the cTP versus the mature proteins (Fig. 4 a,c) : (1) Chloroplast transit peptide (cTP). For NIR2, the predicted cTP spans amino acids 1-47; cleavage is predicted between residues 37 and 38 (TargetP-2.0). For GLN1, the cTP comprises approximately the first 51 amino acids; our structural and truncation data indicate the cleavage site lies between residues 30 and 31 (Fig. 4a). These cTPs are proteolytically removed after chloroplast import and are not present in the mature, functional proteins. (2) Mature N-terminal: In fact, for most proteins, the chloroplast signal peptide is not completely cleaved off; a portion is retained to participate in the conformational formation of the mature protein (Teixeira *et al.*, 2013). The region immediately downstream of the cTP cleavage site (NIR2 residues ~38-47; GLN1 residues ~31-51) is retained in the mature protein. In GLN1, part of this region (G-HR1, aa 40- 53) is hydrophobic and plays a dual role in decamer assembly and PG anchoring.

Localization phenotype upon removal of the IDR/cTP region (Fig. 4c,d and Extended Data Figs. 12, 13) : In the revised manuscript, we demonstrated that deletion of the entire N-terminal region causes cytoplasmic retention. This is because the cTP is absent, so the protein cannot enter the chloroplast at all. This is not a PG-targeting defect per se, but rather a failure of chloroplast import. For chloroplast-localized proteins, a subset forms their final conformation and remains in the stroma to function after cleavage of their cTP, while another subset is further targeted to internal compartments such as thylakoids (Teixeira *et al.*, 2013). By contrast, proteins targeted to PGs often anchor into PGs via hydrophobic regions, as previously reported for *Zm*PSY2 and *At*PGL34 (Yu *et al.*, 2023; Vidi *et al.*, 2007). Critically, our truncation series separates these two functions: Deletion of the cTP alone resulted a failure of chloroplast import (Fig. 4a,c,d and Extended Data Fig. 12c,d, 13c,d). Deletion of internal hydrophobic regions while retaining the cTP (e.g., For NIR2, ΔN-HR1-6; For GLN1, ΔG-HR1-2) resulted in protein being imported into the chloroplast but failing to localize to PGs, remaining diffusely distributed in the stroma (Fig. 4b,c,d and Extended Data Fig. 12b, 13b).

References Involved：

Teixeira, P. F. & Glaser, E. Processing peptidases in mitochondria and chloroplasts. *Biochim. Biophys. Acta.* **1833**, 360–370 (2013).

Yu, J. S., You, M. K., Lee, Y. J. & Ha, S.-H. Stepwise protein targeting into plastoglobules are facilitated by three hydrophobic regions of rice phytoene synthase 2. *Front Plant Sci.* **14**, 1181311 (2023).

Vidi, P.-A., Kessler, F. & Bréhélin, C. Plastoglobules: a new address for targeting recombinant proteins in the chloroplast. *BMC Biotechnol* **7**, 4 (2007).

4.Line 275: The semi-in vivo CO-IP suggests that the first 55 amino acids (IDR) of GLN1 are required for interaction with NIR2, but this is somewhat unclear as they are also required for targeting to PG, without which the two proteins could not interact in the first place. Therefore, this is not entirely conclusive, and the text should be modified accordingly.

Response to the Reviewer’ s Comment:

Thank you for raising this question, we would like to clarify our semi-in vivo Co-IP experimental procedure for you. First, we extracted active proteins from maize leaves to prepare a total plant protein lysate. Subsequently, we added in vitro expressed ZmGLN1 protein to this lysate for immunoprecipitation. At this stage, no intact organellar structures remain in the protein mixture; ZmNIR2 and ZmGLN1 proteins are mixed together within the lysate in a state that allows contact. Therefore, the results reflect their interaction in planta without the confounding factor of their targeting capabilities.

We have conducted more comprehensive experimental validation regarding whether NIR2 and GLN1 interact. We performed assays using IP-MS, pull-down, semi-in vivo CO-IP and Flow-induced dispersion analysis (FIDA) to validate the interaction between these key nitrogen assimilation enzymes, ZmNIR2 and ZmGLN1. we performed unbiased immunoprecipitation-mass spectrometry (IP-MS) using transgenic plants overexpressing Flag-tagged NIR2. As expected, GLN1 was identified among the interacting proteins (Data see Source data Group1, Group2 and WT). Considering that chloroplast proteins are processed by stromal processing

peptidases upon entering the chloroplast, leading to the cleavage of certain peptide segments, we also verified the interaction using the mature proteins after removal of the chloroplast transit peptides. Δ30 (ZmGLN1)-His, purified from E. coli, was co-precipitated with ZmNIR2 from lysates of FLAG-NIR2T1OE plants. The in vitro pull-down assays confirmed that recombinant Δ37 (ZmNIR2)-Flag can pull down recombinant Δ30 (ZmGLN1)-HA. Furthermore, Flow-induced Dispersion Analysis (FIDA) quantified the binding affinity between recombinant ZmNIR2-Flag and ZmGLN1-HA, yielding a dissociation constant (Kd) of approximately 0.054 uM in the buffer system (in 50 mM HEPES, pH 8.0, 100 mM NaCl). These results collectively indicate an interaction between NIR2 and GLN1 (Fig 4l-n). .

5.Lines 38 and1013: "Cryo-EM structures show PG-localized GLN1 forms a decameric complex". The statement in the abstract is incorrect. The GLN1 forming a decameric complex was not "PG-localized" but rather it was purified from E. coli cells that overexpress it. Furthermore, if GLN1 were PG-localized, it would be expected to be in a complex with NIR2, since the two proteins have been shown to interact elsewhere in the paper. In conclusion, it is unclear whether PG-localised GLN1 would also form a decameric complex.

Response to the Reviewer's Comment:

We thank the reviewer for pointing out the inexact phrasing. The GLN1 protein used for cryo-EM structure determination was indeed recombinantly expressed and purified from an E. coli system, not directly isolated from plant plastoglobules (PGs). Therefore, we have modified the text in the abstract as below: "Cryo-EM structures of recombinant GLN1 show a decameric architecture."

However, we believe the cryo-EM structures of the recombinant maize GLN1 recapitulate the oligomerization state of the endogenous form in the plastoglobules. It is generally accepted that recombinant proteins retain their endogenous oligomeric states in most cases. We have shown that GLN1 exhibits a single decameric state both in size-exclusive chromatography and dynamic light scattering results, consistent with

the cryo-EM structure. Moreover, the cryo-EM structure exhibits large interfaces among protomers in the decameric complex, suggesting the oligomerization is not an artifact. The decameric architecture is also supported by the crystal structure of the decameric GS1a in maize, which has ~ 75.56 % sequence identity to GLN1(Unno *et al.*, 2006). To further validate the conclusion, we, we examined the native oligomeric state of GLN1 in planta in both CK (control) and gln1 maize. In CK, a single band near 440 kDa is detected using a GLN1-specific antibody, suggesting that GLN1 exhibits a high oligomerization state (the molecular weight of a monomer is 46 kDa), most likely a decamer as observed in the cryo-EM structure (Fig. 4j).

References Involved：

Unno, H. *et al.* Atomic Structure of Plant Glutamine Synthetase. *Journal of Biological Chemistry* **281**, 29287–29296 (2006).

6.Fig. 2c: are these single chloroplasts or single cells?

Response to the Reviewer’ s Comment:

These are all single chloroplasts. To present the localization results more clearly, we have displayed the subcellular localization data from a single chloroplast. A corresponding image with a wider field of view is provided in the Extended Data Fig. 4, 5 and the specific chloroplast from which the crop was taken is indicated.

7.Fig. 4 a and c: Is localisation in the cytoplasm observed in the absence of IDR?

Response to the Reviewer’ s Comment:

Subcellular localization results indicate that upon removal of the IDR, NIR2 and GLN1 predominantly localize to the cytoplasm. Since the IDR encompasses the chloroplast transit peptide (cTP) required for chloroplast import, the localization of the proteins became somewhat disordered following cTP removal.

8.Extended Data Fig.9: Schematic representations of GLN1 to GLN6 are shown. GLN1 is the only one with an intrinsically disordered region (IDR) at its N-term. This

(again) raises the question of whether the IDR corresponds to the chloroplast targeting signal or a portion of the mature protein.

Response to the Reviewer’ s Comment:

Thank you for the reviewer's reminder; your assessment is entirely correct. We performed cTP predictions for all GLNs. The results indicate that only GLN1 contains an N-terminal cTP, whereas the other GLNs do not, which is consistent with the subcellular fluorescence localization results. In the submitted version, we have also further refined the figures illustrating the chloroplast and PG localization domains of NIR2 and GLN1 (Fig. 4).

Referee #2 (Remarks to the Author):

Efficient nitrogen assimilation is essential for crop productivity and global food security, yet its subcellular organization remains poorly understood. In this study, the authors identified plastoglobules (PGs), small lipid droplet–like structures within chloroplasts, as evolutionarily conserved metabolic hubs that coordinate nitrogen utilization. PGs expand dynamically in response to nitrogen availability in maize, and this response is conserved across monocot and dicot species. Using an optimized purification approach, the authors profiled the maize PG proteome and, for the first time, identified NIR2 and GLN1 localized within PGs. Both enzymes are targeted to PGs via IDRs. Cryo-electron microscopy revealed that the PG-localized GLN1 assembles into a decameric complex, which interacts with NIR2 to form an efficient metabolic channel, thereby enhancing assimilation efficiency. Furthermore, natural variation was found to generate two isoforms of NIR2. NIR2T1 contains the IDR and localizes to PGs, and NIR2T2 which lacks the IDR and localizes in the cytoplasm. Importantly, natural variation in NIR2 correlates with NUE, and its overexpression markedly increases both biomass and NUE.

This work introduces a powerful and widely applicable strategy for developing high-NUE crops. More importantly, its paradigm-shifting insights open a new frontier in metabolic engineering, showing how subcellular compartmentalization can be leveraged to improve enzymatic efficiency and NUE. Overall, this manuscript is of considerable significance and value. However, it would benefit from further strengthening in the following aspects:

Response to the Reviewer

We are profoundly encouraged by your recognition. We sincerely thank you for the comprehensive and thoughtful summary of our study's significance regarding global food security and subcellular organization.

We have fully embraced your suggestions for "further strengthening" the manuscript. During this extensive revision process, following your valuable

suggestions, we have made the following key revisions: (1) Optimized and clarified the PG purification and proteomic analysis. (2) Added robust genetic evidence to support the nitrogen-use efficiency potential. (3) Expanded the "Discussion" sections to include broader implications and open questions

We believe these enhancements have solidified the conceptual framework you highlighted, and we are deeply grateful for your guidance in helping us refine this work.

Major points：

1.The discovery of NIR2 and GLN1 within the PG proteome is an exciting and important finding. To further substantiate their localization, it would be valuable to include additional controls assessing plastid fraction purity, such as immunoblotting with compartment-specific markers and SDS-PAGE fractionation, to more rigorously exclude the possibility of contamination from thylakoid or stromal proteins. The authors are encouraged to cite the recent PG proteome studies by Devarasu *et al.* (2025a, 2025b), which defined a core stress-responsive PG proteome in maize. Highlighting the distinctive strengths of the present methodology relative to these works, along with a comparative discussion of NIR2 and GLN1 abundance and enrichment patterns, would help contextualize the current findings. In addition, the submission of raw proteomics data and relevant processing parameters will be important to ensure reproducibility and further support the conclusions drawn.

Response to the Reviewer' s Comment:

We appreciate your thoughtful suggestion. Prior to first submission, we did not find any relevant literature on PG extraction and components identification in maize. Furthermore, nitrogen-related components were not mentioned in the PG proteomic identification tables of other species.

(1) Purity assessment of isolated fractions using specific markers

Considering that maize, as a C4 plant, differentiates into two cell types: mesophyll cells (MCs) and bundle sheath cells (BSCs), and that primary nitrogen assimilation primarily occurs in MCs, we employed a mechanical disruption method to isolate relatively pure chloroplasts from MCs. These chloroplasts were then used for further PG extraction. In SDS-PAGE experiments, we used antibodies against PEPC and Rubisco as specific markers for MCs and BSCs, respectively, to verify the purity of our MC chloroplasts isolation. To assess the purity of the extracted PGs, we used specific markers: tocopherol cyclase (VTE1) for PGs, Translocon at the Outer envelope membrane of Chloroplasts 75 (TOC75) for the chloroplast outer membrane, and chlorophyll a/b-binding (LHCB1) for the thylakoid membrane (Pierre-Alexandre Vidi *et al.*, 2006; Sarah Rottet *et al.*, 2016). The results demonstrate that we successfully isolated highly enriched PGs from MCs (Extended Data Fig.3 b,c).

(2) Advantages of PG proteomic identification

While we are interested in the composition of maize PGs, our primary objective was to identify nitrogen-associated components based on the PG nitrogen dynamic response phenotype. Therefore, compared to established PG extraction strategies and proteomic identification methods used in other species, we aimed to capture as many proteins as possible. We employed a timsTOF HT mass spectrometer integrated with a fourth-generation ultra-high ion capacity TIMS-XR analyzer and utilized Data-Independent Acquisition (DIA) technology. DIA is one of the highly regarded mass spectrometry acquisition techniques in recent years, capable of efficiently detecting relatively low-abundance protein molecules in complex samples. However, due to the instrument's high sensitivity, our samples also yielded a substantial number of low-abundance contaminant proteins. Consequently, we cross-referenced our results with established PGs' proteins identified in multiple *Arabidopsis* studies (A. Jimmy Ytterberg *et al.*, 2006; Pierre-Alexandre Vidi *et al.*, 2006; Peter K. Lundquist *et al.*, 2012; Roberto Espinoza-Corral *et al.*, 2021) and their corresponding homologous proteins in maize. We found that the majority of these homologous proteins ranked within the top 800 in terms of protein expression abundance, and their cumulative protein quantity accounted for approximately 88.3% of the total protein

captured. This demonstrates that our PG component identification results are relatively accurate.

(3) Principles of PG Proteomic Analysis

Furthermore, we performed GO functional enrichment analysis to categorize these 800 proteins and identified multiple functional terms, including cytoskeletal proteins, lipid metabolism, and antioxidant synthesis, among others. Additionally, a small number of nitrogen metabolism-related components were detected. Given that PGs are monolayer lipid vesicles budding from thylakoids and are structurally intimately associated with them, it is challenging to completely avoid co-isolation of thylakoid components during purification. Therefore, unlike PG component identification studies in other species and those by Devarasu *et al.* (2025a, 2025b), we further conducted subcellular localization experiments to verify the actual protein localization. We validated proteins with relatively high abundance within each functional term. This process allowed us to exclude proteins from the mass spectrometry data that were not genuinely localized to PGs and ultimately confirmed PG-localized proteins supported by fluorescence markers, which included the nitrogen metabolism components NIR2 and GLN1 (Fig. 2b,c and Extended Data Fig. 5). Both NIR2 and GLN1 proteins were also present in the proteomic data of Devarasu *et al.* (2025a, 2025b); however, their abundance varied significantly under different experimental conditions. Therefore, due to differences in plant materials, developmental stages, and growth environments, a direct cross-comparison of protein abundance levels is not feasible.

References Involved：

Romanowska, E., Parys, E. (2011). Mechanical Isolation of Bundle Sheath Cell Strands and Thylakoids from Leaves of C4 Grasses. In: Carpentier, R. (eds) Photosynthesis Research Protocols. Methods in Molecular Biology, vol 684. Humana Press, Totowa, NJ.

Vidi, P.-A. *et al.* Tocopherol Cyclase (VTE1) Localization and Vitamin E Accumulation in Chloroplast Plastoglobule Lipoprotein Particles. Journal of Biological Chemistry 281, 11225 – 11234 (2006).

Rottet, S. *et al.* Identification of Plastoglobules as a Site of Carotenoid Cleavage.

Frontiers in Plant Science 7, (2016).

Jimmy, A., Peltier, J.-B. & J., K. Protein Profiling of Plastoglobules in Chloroplasts and Chromoplasts. A Surprising Site for Differential Accumulation of Metabolic Enzymes. Plant Physiology 140, 984 – 997 (2006).

K., P. *et al.* The Functional Network of the Arabidopsis Plastoglobule Proteome Based on Quantitative Proteomics and Genome-Wide Coexpression Analysis . Plant Physiology 158, 1172 – 1192 (2012).

Espinoza‐Corral, R., Schwenkert, S. & K., P. Molecular changes of Arabidopsis thaliana plastoglobules facilitate thylakoid membrane remodeling under high light stress. The Plant Journal 106, 1571 – 1587 (2021).

Devadasu, E. *et al.* Dynamic changes to the plastoglobule lipidome and proteome in maize over a dehydration – rehydration cycle. Journal of Experimental Botany (2025)

Devadasu E, Susanto FA, Schilmiller AL, Mahey M, Johnny C, Lundquist PK. Dynamic changes to the plastoglobule lipidome and proteome in maize during heat stress and recovery. J Exp Bot. 2025 Oct 14:eraf452.

2.The observation of natural variation of NIR2 is intriguing. To strengthen the study, I recommend that the authors include near-isogenic lines (NILs) or NIR2T2-overexpression lines, if feasible, to provide complementary evidence that clarifies the specific contributions of NIR2T1 and NIR2T2. In addition, a more in-depth discussion of why certain germplasm preferentially expresses the NIR2T1 isoform while others favor NIR2T2, considering possible genetic, evolutionary, or breeding-related factors would enhance the biological interpretation.

Response to the Reviewer’s Comment:

We thank the reviewer for their insightful suggestions. Below we address each point in turn.

(1) Complementary genetic evidence: Overexpression of the *NIR2^{T2}* isoform

We fully agree that additional genetic lines would further solidify the functional assignment of the two *NIR2* splice isoforms. In fact, we have already generated and phenotyped *NIR2^{T2}* overexpression lines in the same genetic background (B73) as the *NIR2^{T1}*-overexpression lines. These data are presented in Extended Data Fig. 18d–g. Consistent with our hypothesis, NIR2^{T2}OE plants showed no significant differences in plant height, biomass, or nitrogen responsiveness compared to

wild-type controls, whereas *NIR2*^{T1}-overexpression lines exhibited marked enhancement of all these traits. These results provide direct evidence that the presence of the cTP (and consequently entering chloroplasts for PG localization) is essential for the positive effect of *NIR2* on NUE, and that simply increasing the amount of the *NIR2*^{T2}-encoded cytosolic protein is not sufficient to improve NUE.

We acknowledge that near-isogenic lines (NILs) carrying defined *NIR2* splicing haplotypes would offer an elegant, non-transgenic validation. However, such NILs are not yet available in our current study. Given the strong correlation between the *NIR2*^{T1}/*NIR2*^{T2} transcript ratio and nitrogen-dependent biomass across 111 inbred lines and field-validated yield differences (Fig. 5f,g), we are confident that the existing genetic and transgenic data already provide compelling evidence for the causal role of the *NIR2*^{T1} isoform.

(2) Deeper discussion of germplasm-specific isoform preferences

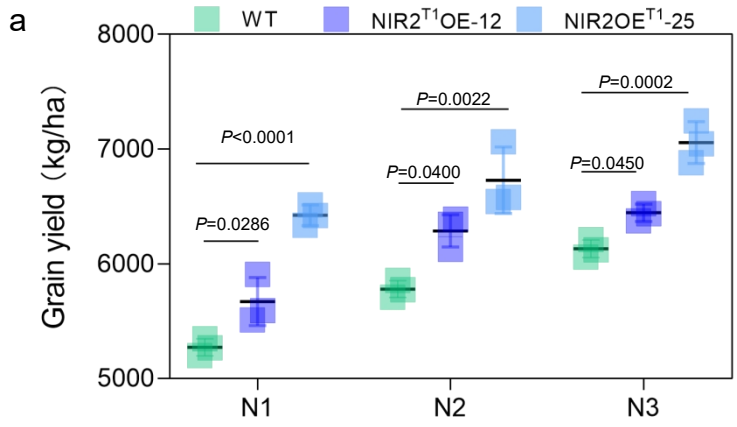
All 44 teosinte accessions examined show 72–99% *NIR2*^{T1} (Extended Data Fig. 18c), indicating that teosinte uniformly favors *NIR2*^{T1} and high *NIR2*^{T1} expression is the ancestral state and that the *NIR2*^{T2}-enriched haplotypes arose after domestication, likely through selection or drift. Several non-mutually exclusive possibilities may explain this phenomenon: 1) The *NIR2* locus may be linked to other domestication or improvement genes that were selected for traits such as early vigor, erect architecture, or disease resistance. The *NIR2*^{T2}-favoring haplotype could have been carried along passively; 2) Modern high-input agricultural systems provide abundant nitrogen, which may have reduced the fitness penalty associated with lower NUE, allowing *NIR2*^{T2}-enriched haplotypes to persist; 3) We have to see that the isoform ratio is a continuous trait, not a binary switch. This suggests that cis-regulatory variation (e.g., promoter strength, splicing efficiency) rather than presence/absence of the alternative splice site itself underlies the observed differences; 4) The fact that elite inbreds such as B73 and CML247 maintain high *NIR2*^{T1} proportions demonstrates that it is possible to combine high NUE with other desirable agronomic traits. Hence, introgression or promoter editing to increase *NIR2*^{T1} proportion represents a feasible and currently underexploited breeding strategy.

We have incorporated the suggestions from all reviewers, and have rewritten the "Discussion" section. Due to space limitations, we have condensed the key points mentioned above and integrated them into the "Discussion".

3.The manuscript presents compelling functional evidence for PG-localized NIR2T1, showing that NIR2T1 overexpression enhances plant height and biomass under low, normal, and high nitrogen conditions compared to the wild type. I am curious whether NIR2T1 overexpression consistently improves NUE and yield performance under field conditions, particularly regarding yield-related traits under different nitrogen levels.

Response to the Reviewer' s Comment:

In this study, we have consistently used plant biomass as an indicator of nitrogen use efficiency, while yield is also a trait of considerable importance. To further investigate the yield potential of $NIR2^{T1}$ overexpressing lines under field conditions, we conducted a multi- nitrogen gradient experiment in Sanya. The trial employed direct seeding without basal fertilizer, with nitrogen applied at three rates—50%, 75%, and 100%—during the V3, V8, and VT stages. The results showed that under the same nitrogen supply levels, the $NIR2^{T1}$ overexpression lines exhibited higher yield potential compared to WT controls (Fig. a in this chapter). Moving forward, we will carry out more systematic field evaluations and variety combination trials centered on $NIR2^{T1}$, with the goal of improving nitrogen use efficiency in existing elite cultivars.



a, Quantification of Grain yield of WT, NIR2OE^{T1}-12 and NIR2OE^{T1}-25 under three nitrogen concentration treatments in Sanya (N1, 50% fertilization; N2, 75% fertilization; N3, 100% fertilization). Bars sharing the same lower-case letters indicate no significant difference ($P > 0.05$) based on one-way ANOVA with Tukey's test.

3.Structural analysis reveals that the decameric GLN1 complex within PGs closely resembles cytosolic GS1a in catalytic mechanism, suggesting comparable enzymatic activity between the PG-localized and cytosolic forms. It would be interesting to know whether other NIR and GLN family members obtain the N-terminal IDR, and whether IDR primarily mediates subcellular localization or additionally affects catalytic efficiency. To strengthen the proposed model, in vitro enzyme activity assays comparing full-length and IDR-deleted variants of NIR2 and GLN1 are recommended to determine whether the IDR play a role in catalysis or serves solely as a localization determinant. Furthermore, investigating the subcellular localization of NIR and GLN family members under different nitrogen conditions would help clarify whether their association with PGs is dynamically regulated by nitrogen availability.

Response to the Reviewer’ s Comment:

(1) Functional investigation of the N-terminal domains of NIR2 and GLN1

In light of suggestions from other reviewers, we have undertaken more rigorous revisions and conducted deeper investigations into the regions of NIR2 and GLN1 responsible for chloroplast localization and PG targeting.

We have precisely delineated the chloroplast localization domains and PG-targeting signals of NIR2 and GLN1. The IDRs of NIR2 and GLN1 indeed determine their chloroplast localization. Through chloroplast transit peptide prediction (cTP) analyses, we identified the cTP of NIR2 as the N-terminal 1-47 amino acids, and that of GLN1 as the N-terminal 1-51 amino acids. The predicted cleavage site for the NIR2 transit peptide lies between the 37th and 38th amino acids at the N-terminus. The predicted cleavage site for the GLN1 transit peptide is between the 43rd and 44th amino acids at the N-terminus. However, analysis of the ZmGLN1 cryo-electron

microscopy structure revealed amino acids 31-43 are involved in decamer formation. Therefore, Therefore, we hypothesize that the cleavage site is located between amino acids 30 and 31 (Fig. 4a,c). Among chloroplast-localized proteins, some form their final conformation and remain in the stroma to function after cleavage of their transit peptide, while others are further targeted to internal compartments such as thylakoids (Teixeira *et al.*, 2013). Chloroplast proteins are processed by stromal processing peptidases upon entry into the chloroplast, resulting in the removal of specific peptide segments. Consequently, the mature NIR2 and GLN1 proteins do not include the cleaved N-terminal chloroplast transit peptides. Thus, the function of the transit peptide is solely for chloroplast targeting and does not affect subsequent enzymatic reactions.

(2) Investigation of subcellular localization changes of NIRs and GLNs under nitrogen treatment

Investigating the relationship between nitrogen and the subcellular localization of NIRs and GLNs is highly warranted. Therefore, we employed a tobacco transient expression system to test all eight proteins (NIR1-2, GLN1-6) under three nitrogen concentrations. Our results indicate that nitrogen treatment does not alter the inherent subcellular localization of these proteins (Extended Data Fig 11). However, for GLN1 and NIR2 specifically, the number of PGs increases when nitrogen concentration is elevated, which aligns with the findings presented in Fig. 1e.

4. The manuscript presents a compelling analysis of nitrogen-related processes. To further enhance the physiological relevance of this work, it would be valuable to consider placing these findings in the broader context of metabolic integration. Given that PGs appear to function as a lipid metabolic hub, a more extensive discussion of carbon–nitrogen co-regulation at the systemic level could help situate the study within the larger framework of plant metabolic network.

Response to the Reviewer’ s Comment:

We sincerely thank you for raising this valuable point, which allows us to

elaborate on the broader metabolic context of our findings. The ultimate goal of plant photosynthetic carbon fixation is to convert inorganic carbon into organic carbon for storage and utilization in various forms. Carbon fixed through photosynthesis is primarily channeled into three major metabolic streams: sugar synthesis, amino acid synthesis and lipid synthesis.Within this framework, plastoglobules (PGs) have been established as crucial hubs for chloroplast lipid metabolism, serving as sites for the enrichment, turnover, and storage of lipids such as triacylglycerols, phospholipids, and isoprenoids. Beyond their role in lipid homeostasis, PGs participate actively in the biogenesis, maintenance, and lipid remodeling of thylakoid membranes(van Wijk *et al.*, 2017), thereby forming a direct functional link between lipid metabolism and photosynthetic carbon metabolism

The co-localization of carbonic anhydrases (CAHs) with NIR2 and GLN1 within mesophyll PGs suggests that these structures serve as platforms for carbon–nitrogen metabolic crosstalk (Fig. 2b,c). By concentrating enzymes from both pathways in a single subcompartment, PGs may enable rapid metabolic channeling of inorganic carbon and nitrogen intermediates, thereby synchronizing amino acid biosynthesis with photosynthetic carbon availability. Thus, PGs appear to mediate a form of cross-pathway coupling that coordinates carbon and nitrogen utilization at the subcellular level. While our study provides initial evidence for this coupling, the detailed mechanisms underlying PG-mediated carbon–nitrogen co-regulation remain an important and open question for future research.

We have incorporated the suggestions from all reviewers, and have rewritten the “Discussion” section. Due to space limitations, we have condensed the key points mentioned above and integrated them into the “Discussion”.

References Involved：

van Wijk, K. J. & Kessler, F. Plastoglobuli: Plastid Microcompartments with Integrated Functions in Metabolism, Plastid Developmental Transitions, and Environmental Adaptation. *Annu Rev Plant Biol* **68**, 253–289 (2017).

5.The study convincingly shows the dynamic response of PGs to nitrogen status. Building on these solid findings, it would be interesting to explore in future work the upstream signaling mechanisms through which nitrogen status regulates PG expansion. An open question remains whether carbon metabolism also participates in this regulatory process. A discussion of potential signaling pathways involved would further strengthen the biological insight of this compelling study.

Response to the Reviewer’ s Comment:

We thank the reviewer for this thoughtful and forward-looking comment. We fully agree that defining the signaling pathways that couple nitrogen status to PG dynamics is a critical next step, and that the potential involvement of carbon metabolism adds an important layer of biological complexity.

The question of how nitrogen signaling pathways regulate PGs is indeed a valuable scientific inquiry. Existing research has shown a direct correlation between the state of PGs and the abundance of endogenous proteins. The nitrogen assimilation enzymes NIR2 and GLN1, identified in this study as specifically localized to PGs, exhibit a trend of nitrate-induced expression across multiple species, and their enzymatic activity is also linked to nitrate availability. For example, the accumulation level of ZmGS2 is induced by nitrate, and its enzyme activity is detectable only in the presence of nitrate (Prinsi and Espen, 2015). NLP, a core component of the nitrate signaling pathway, has been reported in multiple species. For instance, phosphorylated AtNLP7 translocates into the nucleus and activates AtNIR1 expression (Marchive *et al.*, 2013), while ZmNLP5/6/8 can directly bind to the nitrate-responsive element (NRE) in the promoter of ZmNIR2, enhancing its transcription (Cao *et al.*, 2017; Ge *et al.*, 2020). Additionally, early nitrate-responsive signaling components such as squamosa promoter binding protein-like 9 (SPL9), TGACG sequence-specific binding protein 1 (TGA1), TGA4, auxin signaling F-box 3 (AFB3), and NAC domain containing protein 3 (NAC4) (Krouk *et al.*, 2010; Vidal *et al.*, 2010, 2013; Alvarez *et al.*, 2014) may mediate the precise regulation of PG states by nitrogen through modulating the expression of nitrogen assimilation enzymes

within PGs, thereby forming a multi-layered nitrogen signaling network.

The SnRK1 protein kinase plays a crucial role in energy sensing and carbon metabolism regulation in plants. Recent studies indicate that SnRK1 not only participates in carbon metabolism but also influences nitrogen metabolism to some extent. In vivo and in vitro experimental results confirm that SnRK1 can phosphorylate and inactivate nitrate reductase, thereby regulating nitrate metabolism (Sugden *et al.*, 1999). Notably, the catalytic subunit KIN10 of SnRK1 can phosphorylate NLP7, promoting its cytoplasmic localization and degradation, which in turn inhibits its activity (Wang *et al.*, 2022). In summary, carbon metabolism signaling pathways can also regulate the expression of carbon and nitrogen metabolic enzymes within PGs to modulate PG states.

We have incorporated the suggestions from all reviewers, and have rewritten the Discussion section. Due to space limitations, we have condensed the key points mentioned above and integrated them into the Discussion. We have also proposed several questions emerging from this work in the rephrased Discussion. The first one is: what are the upstream signaling components that couple nitrogen status to PG proliferation?

References Involved：

Prinsi, B. & Espen, L. Mineral nitrogen sources differently affect root glutamine synthetase isoforms and amino acid balance among organs in maize. *BMC Plant Biol* **15**, 96 (2015).

Marchive, C.; Roudier, F.; Castaings, L.; Bréhaut, V.; Blondet, E.; Colot, V.; Meyer, C.; Krapp, A. Nuclear retention of the transcription factor NLP7 orchestrates the early response to nitrate in plants. *Nat Commun* **4**, 1713 (2013).

Cao, H.; Qi, S.; Sun, M.; Li, Z.; Yang, Y.; Crawford, N. M.; Wang, Y. Overexpression of the Maize ZmNLP6 and ZmNLP8 Can Complement the Arabidopsis Nitrate Regulatory Mutant nlp7 by Restoring Nitrate Signaling and Assimilation. *Front Plant Sci* **8**, 1703 (2017).

Ge, M.; Wang, Y.; Liu, Y.; Jiang, L.; He, B.; Ning, L.; Du, H.; Lv, Y.; Zhou, L.; Lin, F.; Zhang, T.; Liang, S.; Lu, H.; Zhao, H. The NIN-like protein 5 (ZmNLP5) transcription factor is involved in modulating the nitrogen response in maize. *Plant J* **102**, 353–368 (2020).

Krouk, G., Mirowski, P., LeCun, Y., Shasha, D. E. & Coruzzi, G. M. Predictive network modeling of the high-resolution dynamic plant transcriptome in response to nitrate. *Genome Biol* **11**, R123 (2010).

Vidal, E. A.; Araus, V.; Lu, C.; Parry, G.; Green, P. J.; Coruzzi, G. M.; Gutiérrez, R. A. Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **107**, 4477–4482 (2010).

Vidal, E. A., Moyano, T. C., Riveras, E., Contreras-López, O. & Gutiérrez, R. A. Systems approaches map regulatory networks downstream of the auxin receptor AFB3 in the nitrate response of Arabidopsis thaliana roots. *Proc Natl Acad Sci U S A* **110**, 12840–12845 (2013).

Alvarez, J. M.; Riveras, E.; Vidal, E. A.; Gras, D. E.; Contreras-López, O.; Tamayo, K. P.; Aceituno, F.; Gómez, I.; Ruffel, S.; Lejay, L.; Jordana, X.; Gutiérrez, R. A. Systems approach identifies TGA1 and TGA4 transcription factors as important regulatory components of the nitrate response of Arabidopsis thaliana roots. *Plant J* **80**, 1–13 (2014).

Sugden, C., Donaghy, P. G., Halford, N. G. & Hardie, D. G. Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. *Plant Physiol* **120**, 257–274 (1999).

Wang, H.; Han, C.; Wang, J.-G.; Chu, X.; Shi, W.; Yao, L.; Chen, J.; Hao, W.; Deng, Z.; Fan, M.; Bai, M.-Y. Regulatory functions of cellular energy sensor SnRK1 for nitrate signalling through NLP7 repression. *Nat Plants* **8**, 1094–1107 (2022).

Minor points:

Overall, the manuscript is well organized and clearly written. However, several minor issues still need to be addressed.

1. Throughout the manuscript, the clarity of statistical reporting could be improved, particularly in defining “biologically independent samples”. For example, in Fig. 1c (n = 20 biologically independent samples) and Fig. 3d (n = 3 biologically independent samples), it is unclear whether these numbers represent individual plants measured within a single experiment, or the total number of samples combined across multiple independent experiments. data are means ± SD from 3 biological replicates

Response to the Reviewer’ s Comment:

Thank you for raising this critical point. We fully agree that clearly defining "biologically independent samples" is essential to ensuring the rigor of the study. We have accordingly revised the relevant figure legends and have also thoroughly reviewed the entire manuscript to ensure that all reporting of sample sizes (*n*) remains consistent with this definition. These revisions aim to fully eliminate ambiguity and enhance the transparency and reproducibility of the experimental design and statistical analysis. Once again, we appreciate your valuable input, which has helped us improve the quality of the manuscript.

For example, For Fig. 1c, The legend now reads: "Data are mean ± s.d. (n = 18 independent biological chloroplasts from a single, randomized experiment.)".

2. For biochemical experiments (e.g., Fig. 4d and 4g), it would be important to specify in the figure legends that the experiments were independently repeated three times with similar results. Likewise, for the microscopy data, please indicate how many tobacco leaves were used and how often the observations were repeated.

Response to the Reviewer' s Comment:

We thank the reviewer for this important suggestion to enhance the reproducibility of our work. We have now provided the requested details in the respective figure legends and the Methods section:

For all relevant biochemical assays (e.g., Fig. 4j,k), we have added the following statement to the figure legends: "The experiment was independently repeated three times with similar results. A representative result is shown."

For the microscopy data, we have updated the corresponding figure legends (e.g., Fig. 1e) to state: "Representative fluorescence images (e)". "Observations were made on at least 10 cells from 3 independently transformed tobacco leaves."

3. In Fig. 1d, the nitrogen concentration is presented without units.

According to your suggestion, we have added the units to the Fig. 1d.

4. In Fig. 5b, could the authors clarify the average T1/T2 ratio observed in the overall

population? This information would help determine whether the ratios observed in the selected inbred lines are relatively high or low.

We performed calculations on the 111 selected inbred lines, revealing that the average proportion of T1 is approximately 68.18%. This information indeed provides a more intuitive reference for assessing whether the selected inbred lines exhibit relatively high or low proportions, and it has been added to the main text.

5. In Fig. 3c, the authors show that gln2–gln6 genes encoding proteins not localized to PGs showed no obvious morphological defects, whereas gln2 resulted in reduced plant height. Similarly, in Fig. 5j and k, the quantitative data clearly show that NIR2T1 overexpression lines produce higher fresh weight than the wild type under all nitrogen conditions; however, the seedling phenotype shown in panel k does not appear markedly different. The authors may wish to provide additional representative images or clarify this discrepancy.

Thank you for noting this. In the data presented in Fig. 3d, the *gln2* mutant does indeed exhibit a certain degree of reduction in plant height compared to the WT. Here, I would like to clarify and correct my description regarding this mutant. My intended point was that, relative to mutants like *nir2* and *gln1* which show exceptionally obvious growth defects, mutations in *gln2* through *gln6* do not cause particularly pronounced morphological defects. My original phrasing was indeed not sufficiently precise, and we have revised the relevant descriptions accordingly. "Crucially, mutants of other genes encoding proteins not localized to PGs (e.g., *nir1*, *gln2-6*) maintained a normal phenotype without observable defects (Fig. 3c,d)." is revised as "In contrast, mutants of other genes (e.g.,*nir1*, *gln3-6*) exhibited a normal phenotype without observable defects. Although *gln2* exhibited a reduction in plant height, there was no significant difference in biomass (Fig. 3c,d). "

In Figs. 5j and 5k, we have replaced the images with more representative ones along with the corresponding data.

6.In Extended Fig. 7 and Fig. 8, the x-axis label on the bar charts should be corrected

from HN to NN. The gene names gn2 and gn4 should be corrected to gln2 and gln4.

Thank you for pointing out this issue. We deeply apologize for this error and have corrected it. The correct nitrogen concentrations have now been indicated in Extended Data Fig. 9 and Fig. 10

7. In Fig. 4 and Extended Fig. 12, the western blot panels need improvement. The bands are not well aligned horizontally, and the lanes display noticeable variations in molecular weight.

We thank you for pointing out the this issue. We have re-prepared the samples, completed the corresponding experiments, and updated the Western blot images with higher-quality versions.

It should be noted that, in response to feedback from other reviewers, the data in Fig. 4 have been replaced with results demonstrating the oligomeric state of GLN1 in planta, as determined using endogenous antibodies (Fig. 4j,k).

8.Lines 122-123 “transmission electron microscopy (TEM) of chloroplasts in leaves of maize seedling...” can be rephrased to “transmission electron microscopy (TEM) on chloroplasts of maize seedling leaves...”

We have revised textual inaccuracies noted in the review.

9.Lines 186-189，The logical flow of the sentence could be improved for better clarity. Rephrasing is suggested.

The textual inaccuracies pointed out in the review have been addressed.

10.Line 318: “was performed using partial inbred lines” should be “selected inbred lines”.

We have corrected the textual inaccuracies identified in the review.

11.Line 406: “underscorethe the functional importance” should be corrected to “underscore the functional importance”.

We have revised textual inaccuracies noted in the review.

12.Discussion section: The repeated mention of the role of PG in nitrogen assimilation should be omitted.

Thank you for your reminder. The relevant section has been deleted.

13. Some typos should be corrected. For example, Line 257: “For NIR2， variants...” should be “For NIR2, variants...”; Line 316: “NIR2T2 lacking 139 amino acids…”should be “NIR2T2 lacks 139 amino acids...”; Line 644: “CO-IP” should be “Co-IP”.

We have revised textual inaccuracies noted in the review.

Referee #3 (Remarks to the Author):

Improving nitrogen use efficiency is becoming a priority for crop improvement. This study breaks new ground by shedding light on nitrogen assimilation in chloroplasts of maize, a major crop. Chloroplastic plastoglobules (PGs) were previously viewed as hubs of lipid metabolism. In this study by Chen *et al.*, the authors describe a new role of PGs as a critical site in nitrogen assimilation. The authors use a set of cutting-edge and accepted standard methodology to identify and characterize two main proteins of maize underlying and orchestrating the nitrogen assimilation function of maize PGs through nitrate reduction and glutamate synthesis. The methods used in this tour-de-force study range from advanced proteomics, modern structural biology, genetics, genomics, greenhouse experiments and cell biology. The results presented are of immediate interest to many people in crop research and plant science. Due to the importance of food security in face of climate change, the negative influence of nitrogen overfertilization on marine food webs and ultimately climate stability, and due to finite fossil fuels used for the production of nitrogen fertilizers, the study is of great societal importance. In my opinion, these implications extend well beyond plant research, offering value to a broad scientific community.

In short, the authors investigated how nitrogen availability influences PG number and size in maize and identified two key PG-associated proteins, NIR2 and GLN1. Through colocalization assays in tobacco, they demonstrated that NIR2 and GLN1 proteins target PGs, likely via newly discovered N-terminal intrinsically disordered regions. The study further reports the cryo-EM structure of maize GLN1, revealing an oligomeric architecture typical for plant GLN proteins. By assessing GLN1 oligomerization under ammonium treatment, the authors suggest a potential link between PG-associated proteins and nitrogen use efficiency. Finally, they then switch back to NIR2 and analyse its splice variants to highlight the functional importance of the NIR2 disorder region only translated from the T1 isoform under variable nitrogen conditions and that relevant preferential splicing variation in NIR2 exists in the maize

germplasm underlying the nitrogen use efficiency trait.

The conclusions made by the authors are generally original and often strongly supported by their own data. Although the clarity of how this study links PGs and nitrogen assimilation is unprecedented, the authors are sometimes overemphasizing the novelty of their own findings. Afterall, a very recent study already studied in detail that chloroplastic nitrogen assimilation in maize is mediated by ferredoxin (ZmFd4) and NIR proteins (NIR1, NIR2 also shown in extended data) (Jia *et al.* Nature Plants 11, 643-659, 2025). Just last year, a work in Arabidopsis showed responses of PGs to nitrogen starvation (Coulon *et al.* Journal of Experimental Botany, Vol 75, No 20 pp, 6542-6562, 2024), although Coulon and colleagues focused on lipid metabolism as a readout for PG function.

I recommend publication. However, there are a few major and minor points that should be amended before publication.

Response to the Reviewer

We are deeply honored and encouraged by your recognition. We are especially grateful for the insightful and constructive suggestions provided, which have been instrumental in helping us refine our narrative and elevate the overall scientific rigor of the manuscript.

We sincerely appreciate your reminder regarding the contextualization of our work. We have taken this advice to heart and have carefully refined the manuscript to ensure a more balanced and objective presentation. Specifically, we have integrated and discussed the foundational studies by Jia *et al*. (2025) and Coulon *et al*. (2024), ensuring that our discovery is properly situated within the evolving landscape of nitrogen metabolism and plastoglobules (PGs) research.

Furthermore, we have tempered the language throughout the text, replacing overstated claims with precise, evidence-based descriptions to avoid any overemphasis on novelty. To address the major points you raised regarding the physical evidence for enzyme assembly, we have provided definitive new data using Blue Native-PAGE (BN-PAGE) and in vivo immunodetection. We believe these

revisions have significantly enhanced the mechanistic clarity and academic depth of our study, and we thank you for your guidance in helping us achieve this higher standard.

Major points

1.I find the authors overemphasize the novelty and impact of their study. I suggest to choose more precise phrasing that relies less on buzz words like ‘paradigm’, (counted four times in lines 43, 107, 365, 448), ‘for the first time’ (counted four times lines 103, line 170, 174, 394), ‘universal’ (line 44). Each of these occurrences could be counterfeited, e.g., is it really a ‘universal’ crop improvement strategy when only maize was tested? Can is really be ‘unveiling a new paradigm for subcellular metabolic engineering’ when the authors provide a first example of this strategy (how can something already be a paradigm when it is done for the first time)? I think the authors have reason to emphasize the novelty and impact of their study, but should use such terms only where appropriate. Reconsider the wide use of the word ‘unequivocally’.

Response to the Reviewer’ s Comment:

We sincerely appreciate your insightful and constructive comments on the wording of our manuscript. We fully agree with your point that some phrases used to describe the novelty and impact of our study were overemphasized and not sufficiently precise, and we apologize for the inappropriate use of such buzz words as paradigm, “for the first time” and “universal” in the text.

Following your valuable suggestion, we have carefully revised the entire manuscript to replace and refine all the overstated expressions mentioned. For each revised phrase, we have adopted more accurate, cautious and evidence-based wording that is strictly consistent with our experimental results. We have also removed the redundant repetition of such terms to ensure the objectivity and rigor of the

manuscript’s expression.

All the revised contents are marked with track changes in the revised manuscript for the reviewer’s easy checking. We thank the reviewer again for this critical comment, which has significantly helped us improve the language expression and academic prudence of our work.

2. In line 288-290, the authors wrote ‘we performed high-resolution cryo-electron microscopy (cryo-EM) analysis of purified PG-localized GLN1 from maize chloroplasts‘. In the method paragraph ‘cryo-EM sample preparation’ (line 1014-1016), the authors wrote ‘the GLN1 gene was cloned into the pET28a–SUMOstar vector …’.

Please clarify exactly from which organism the protein for cryo-EM was obtained; recombinant versus endogenous protein source? How can E-coli protein be PG-localized? Please also briefly describe in the main text relevant detail how the protein was purified. E.g. affinity purification (tag?), size-exclusion chromatography (column?) for improved readability. I am also not satisfied with one SDS page shown in Extended Data Fig.10,a. Please show full recombinant protein purification pipeline with intermediate results: SDS page of affinity purification and SDS page of SEC fractions. Please enlarge Extended Data Fig.10, c (negative staining) so we can see the individual particles and monodispersity of the sample. Scale bar in negative staining without label.

Response to the Reviewer’s Comment:

We thank the reviewer for the questions and suggestions. The protein used for cryo-EM structure determination was over-expressed and purified from E. coli, not directly isolated from plant plastoglobules (PGs). We have revised the misleading description "PG-localized GLN1" throughout the text. Upon request by the reviewer, we have added a detailed description of the recombinant GLN1 expression and purification process in the main text to improve the article's readability: “To gain structural insights into the functional mechanism of ZmGLNl and its role in nitrogen assimilation, we performed high-resolution cryo-electron microscopy (cryo-EM)

analysis of recombinant ZmGLN1 protein purified from E. coli through a multi-step chromatography process, including affinity, ion exchange, and size-exclusion chromatography." Moreover, we provided the step-by-step SDS-PAGE results for the purification process in the revised Extended Data Fig. 15.

In addition, Extended Data Fig. 10c (now Extended Data Fig. 15a) does not show negative-stain results, but rather micrographs from cryo-EM samples. In the revised version, we have enlarged the micrograph, and the scale bar is shown in the upper left corner of the image.

3.Please include the proteomic raw data (Figure 2b), and an explain it more. I only found three long sheets 'group1', 'group2', and 'WT' in the source data, seems to be the proteomic data of IP-MS for figure 4, but not for figure 2. With this limited information, I could not trace how the 13 proteins in figure 2 were selected.

Response to the Reviewer' s Comment:

We thank the Reviewer for highlighting the absence of the proteomic raw data (Fig. 2b) in our original work. We have now included this data in the source data.

Prior to submission, we did not find any relevant literature on plastoglobules (PGs) extraction and component identification in maize. Furthermore, nitrogen-related components were not mentioned in the PG proteomic identification tables of other species.While we are interested in the composition of maize PGs, our primary objective was to identify nitrogen-associated components based on the PG nitrogen dynamic response phenotype. Therefore, compared to established PG extraction strategies and proteomic identification methods used in other species, we aimed to capture as many proteins as possible. We employed a timsTOF HT mass spectrometer integrated with a fourth-generation ultra-high ion capacity TIMS-XR analyzer and utilized Data-Independent Acquisition (DIA) technology. DIA is one of the highly regarded mass spectrometry acquisition techniques in recent years, capable of efficiently detecting relatively low-abundance protein molecules in complex samples. However, due to the instrument's high sensitivity, our samples also yielded a substantial number of low-abundance contaminant proteins. Consequently, we

cross-referenced our results with established PG proteins identified in multiple Arabidopsis studies (Ytterberg *et al.*, 2006; Vidi *et al.*, 2006; Peter K. Lundquist *et al.*, 2012; Espinoza-Corral *et al.*, 2021) and their corresponding homologous proteins in maize. We found that the majority of these homologous proteins ranked within the top 800 in terms of protein expression abundance, and their cumulative protein quantity accounted for approximately 88.3% of the total protein captured. This demonstrates that our PG component identification results are relatively accurate.

Furthermore, we performed GO functional enrichment analysis to categorize these 800 proteins and identified multiple functional terms, including cytoskeletal proteins, lipid metabolism, and antioxidant synthesis, among others. Additionally, a small number of nitrogen metabolism-related components were detected. Given that PGs are monolayer lipid vesicles budding from thylakoids and are structurally intimately associated with them, it is challenging to completely avoid co-isolation of thylakoid components during purification. Therefore, unlike PG component identification studies in maize by Devarasu *et al.* (2025a, 2025b), we further conducted subcellular localization experiments to verify the actual protein localization. We validated proteins with relatively high abundance within each functional term. This process allowed us to exclude proteins from the mass spectrometry data that were not genuinely localized to PGs and ultimately confirmed PG-localized proteins supported by fluorescence markers. This constitutes the source and selection process for the 13 proteins presented in Fig. 2.

References Involved：

Vidi, P.-A. et al. Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. *J. Biol. Chem*. 281, 11225 – 11234 (2006).

Ytterberg, A. J., Peltier, J.-B. & van Wijk, K. J. Protein profiling of plastoglobules in chloroplasts and chromoplasts. A surprising site for differential accumulation of metabolic enzymes. *Plant Physiol.* **140**, 984–997 (2006).

Lundquist, P. K. et al. The functional network of the *Arabidopsis* plastoglobule proteome based on quantitative proteomics and genome-wide coexpression analysis. *Plant Physiol*. **158**, 1172 – 1192 (2012).

Espinoza-Corral, R., Schwenkert, S. & Lundquist, P. K. Molecular changes of *Arabidopsis thaliana* plastoglobules facilitate thylakoid membrane remodeling under high light stress. *Plant J.* **106**, 1571–1587 (2021).

Devadasu, E. et al. Dynamic changes to the plastoglobule lipidome and proteome in maize over a dehydration-rehydration cycle. *J. Exp. Bot.* eraf453 (2025a)

Devadasu, E. et al. Dynamic changes to the plastoglobule lipidome and proteome in maize over a dehydration-rehydration cycle. *J. Exp. Bot.* eraf453 (2025b)

3.Please clearly explain how PG number and area was quantified. Can you rule out that under high nitrogen regime, there are not just simply more and larger chloroplasts? Does the PG number per chloroplast volume really increase too or, in other words, do larger chloroplasts not automatically contain more PGs? Phrase accordingly.

Response to the Reviewer’ s Comment:

We thank you for pointing out the this issue. For electron microscopy sample preparation, we randomly selected three maize seedlings from each nitrogen treatment and collected the middle segment of the third leaf for processing. For each sample, 2-3 micrographs were taken at 3000× magnification. A total of 18 chloroplasts with relatively uniform size and median cross-sections were chosen to quantify plastoglobule numbers and chloroplast area.For chlorophyll measurement, the middle portion of the same third leaf used for electron microscopy was sampled. After removing the leaf vein, 0.08 g of leaf tissue was weighed for chlorophyll content analysis.

The results indicated that both chloroplast area and chlorophyll content increased significantly as nitrogen concentration rose from 0.04 mM to 4 mM. However, no further notable enhancement was observed within the 4 mM to 16 mM nitrogen range (Extended Data Fig.1 a,b). These findings suggest that once nitrogen availability becomes sufficient for maize, the chloroplast size in mesophyll cells stabilizes within a certain range, whereas the number of plastoglobules continues to increase.

Our findings align with previous studies: in green tissues, transcription factors, light signals, and hormonal signals form a complex network that regulates the transcription of chloroplast- and photosynthesis-related genes, thereby finely controlling the development and number of chloroplasts in each cell (Cackett *et al.*,

2021). Furthermore, the total chloroplast surface area per cell is positively correlated with mesophyll cell size, and the proportion of the cell surface area occupied by chloroplasts remains essentially constant, independent of both cell size and cell age (Honda *et al.*, 1971).

References Involved：

Cackett, L., Luginbuehl, L. H., Schreier, T. B., Lopez-Juez, E. & Hibberd, J. M. Chloroplast development in green plant tissues: the interplay between light, hormone, and transcriptional regulation. *New Phytol* **233**, 2000–2016 (2022).

Honda, S. I., Hongladarom-Honda, T., Kwanyuen, P. & Wildman, S. G. Interpretations on chloroplast reproduction derived from correlations between cells and chloroplasts. *Planta* **97**, 1–15 (1971).

4.Jia *et al.* (nature plants 2025) reported ZmNIR1 and ZmNIR2 chloroplast localization in transient tobacco assays. This is contradicting with the current study, stating that only NIR2 is localized to chloroplasts. Please explain this contradiction

Response to the Reviewer’ s Comment:

Upon your identification of this contradiction, we have accorded it particular importance. Thank you for raising this important question. In our initial observations of ZmNIR1, we noted that the fluorescence signal was predominantly localized to the cytoplasm. We then employed TargetP-2.0, which predicted a very low probability (0.0806) for this protein containing a chloroplast transit peptide. This indicated that the protein is scarcely predicted to localize to chloroplasts. Consequently, this prediction led us to draw an insufficiently cautious conclusion initially.

First, we performed tobacco cell transient expression assays with sequentially increasing bacterial culture concentrations and observed a corresponding increase in chloroplast-localized protein. Second, we conducted Western blot analysis to detect the expressed protein. In addition to a band at the expected size of the target protein, a distinct band was also detected at approximately 25 kDa, which is attributed to the cleavage of the GFP tag. This finding explains why the initially expressed ZmNIR1 exhibited fluorescent signals in both the cytoplasm and nucleus. Finally, we further utilized Deeploc-2.1 for prediction. The results indicate that the protein is predicted to

contain a chloroplast transit peptide for plastid localization, with a probability of 0.9758. However, the predicted transit peptide signal is primarily located within amino acids 70-100 at the N-terminus. Given that the chloroplast transit peptides of most chloroplast proteins are situated at the very N-terminus, this explains the discrepancy in the prediction by TargetP-2.0.

Following the recognition of our oversight, we reached out to the corresponding author of the Jia et al. study for a detailed academic discussion. They kindly provided the original plasmids used in their work, which allowed us to rigorously re-verify the subcellular localization of ZmNIR1 under our experimental conditions. **We confirmed that ZmNIR1 indeed localizes to chloroplasts (Fig. 3e). Surprisingly, we also observed a minor fraction of the protein localized to PGs. This indicates that ZmNIR1 also possesses the ability to localize to PGs, albeit to a lesser extent (Extended Data Fig. 8a).**

This finding prompted us to reconsider why ZmNIR1 was not identified in our previous PG proteomic analysis and why the *nir1* mutant did not exhibit significant phenotypic defects. First, we examined the expression profiles of ZmNIR1 and ZmNIR2. Our results show that ZmNIR2 is highly expressed in leaves, whereas ZmNIR1 is primarily expressed in roots (Extended Data Fig. 8b). Second, we retrieved and queried our proteomic data, confirming that the PG proteomic dataset does contain ZmNIR1. However, its detected protein abundance was only about 2,000, compared to about 200,000 for ZmNIR2 (Extended Data Fig. 8c). This large difference led us to initially categorize the ZmNIR1 signal as low-abundance non-specific noise and exclude it during the preliminary screening of the proteomic data. Taken together, the phenotypic data from genetic materials and the PG protein content indicate that while both ZmNIR1 and ZmNIR2 are capable of PG localization, ZmNIR2 plays the decisive role.

In conclusion, we sincerely thank you for raising this point. It has not only helped us correct the localization of ZmNIR1 but has also significantly contributed to refining our understanding of its function. The results further demonstrate that the functionality of such proteins depends not only on PG localization but also critically

on their abundance within PGs.

6. I am desperately missing a loading control in Fig 4, g and Fig 4, h. Without it, the data cannot be interpreted and I have to disagree with the conclusions. Increased decamer signal could also stem from increased protein concentration in loading. In the corresponding text (lines 299-303), I disagree with several statements. 'cross-linking showed that GLN1 exists in different assembly states': No! It is rather, with increased cross-linker concentration, more higher order complexes could be stabilized. We learn nothing about in vivo assembly of the complex from cross linking. Also, 'increased levels of exogenous NH4+ substrate led to enhanced GLN1 oligomerization' No! It seems like NH4+ stabilizes the hexamer and smaller complexes, but not octamer or decamer. Loading control! Also, what is the band at 72kDa and why does it increase with NH4+ concentration?

Response to the Reviewer's Comment:

We are extremely grateful to the reviewer for this critical and correct assessment. The original cross-linking experiments in Figs. 4g and 4h were indeed fundamentally flawed for the interpretation we attempted, primarily due to the lack of a loading control and the inherent limitations of cross-linking for inferring in vivo assembly states, as the reviewer rightly points out.

To address these issues in the most rigorous way possible, we have not simply added a loading control to the flawed experiment. Instead, we have performed completely new experiments using a fundamentally better approach and have replaced the original Figs. 4g and 4h in their entirety. The new data are now presented in revised Fig. 4j,k. To directly address the reviewer's concern regarding protein concentration and loading consistency, we included immunoblotting against UGPase as a loading control for the total protein extracts (Fig. 4j, k). This ensures that the observed changes in decamer abundance are a truereflection of the protein's oligomeric state rather than an artifact of unequal loading.

We employed Blue Native-PAGE (BN-PAGE) followed by immunoblotting

using a ZmGLN1-specific endogenous antibody. This method directly visualizes the native oligomeric states of the protein from cell extracts without the use of chemical cross-linkers, thereby eliminating the artifacts introduced by variable cross-linker efficiency and concentration. The identification of a stable 440 kDa band in native plant extracts—matching the predicted mass of the decameric complex resolved by our cryo-EM analysis—provides definitive evidence that ZmGLN1 exists predominantly as a decamer in its native environment. This cross-validation confirms that our structural insights are physiologically relevant and accurately describe the functional state of the enzyme within PGs. Furthermore, the total absence of the ~440 kDa signal in the *gln1* mutant, combined with the consistency of the non-specific background bands, further validates the high specificity of our antibody and the reliability of our protein quantification. To investigate how this oligomeric state responds to nitrogen availability, we treated maize seedlings with three nitrogen concentrations (2 mM, 4 mM, and 8 mM). The results demonstrate a clear, nitrogen-dependent increase in ZmGLN1 decamer abundance (Fig. 4k), providing robust empirical support for our model that nitrogen availability scales the capacity of the PG-localized metabolic hub through enhanced enzyme assembly.

7.Discussion reads mostly like a summary of the data and for me falls quite flat. It could be much more exciting. Particularly, I am lacking a higher-level contextualization of the data. For example, how is the dual function of PGs in lipid metabolism and nitrogen assimilation balanced? We know since a long time that low nitrogen leads to leaf yellowing, is this related to PG function in nitrogen assimilation? Why do certain maize cultivars have decreased nitrogen use efficiency; was this accidentally selected by historic breeding? How about nitrogen use efficiency and GLN1/NIR2 in legumes, that rely less on nitrogen fertilization due to nodule bacteria? Under what environmental/agricultural scenarios is low NUE useful, why did it evolve in some wild populations?

Response to the Reviewer’s Comment:

We agree entirely that the original Discussion was too descriptive and failed to

convey the broader significance and open questions that make this story exciting. In the revised manuscript, we have completely restructured and rewritten the Discussion to move beyond a simple recapitulation of results. We now explicitly address the high-level conceptual points raised by the reviewer, as summarized below.

(1) how is the dual function of PGs in lipid metabolism and nitrogen assimilation balanced?

PGs function as multitasking metabolic microdomains rather than specialists. We argue that the lipid metabolism function (storage of prenylquinones, tocopherols, triacylglycerols) and the nitrogen assimilation function are not in conflict but are mechanistically coupled. PG expansion requires lipid biosynthesis, which consumes carbon skeletons and reductants; nitrogen assimilation also demands carbon skeletons (2-oxoglutarate) and ATP/NADPH. The nitrogen-dependent proliferation of PGs links nitrogen status to the expansion of lipid storage capacity, illustrating a broader principle: plants coordinate carbon and nitrogen metabolism not only through transcriptional networks but also through dynamic remodeling of subcellular architecture. Future work should explore whether the 'PG-targeting code' defined here can be exploited to engineer synthetic C–N metabolic channels for improved crop performance

(2) Since a long time that low nitrogen leads to leaf yellowing, is this related to PG function in nitrogen assimilation?

In this study, we directly connect the classic phenotype of nitrogen-deficiency chlorosis to our discovery. The *nir2* mutant, even under high nitrate, phenocopies nitrogen starvation (yellow leaves, stunted growth). This demonstrates that impaired PG-localized nitrite reductase is sufficient to trigger the entire suite of nitrogen-deficiency symptoms. We therefore propose that the leaf yellowing observed under low nitrogen is not merely a passive consequence of reduced chlorophyll synthesis but an active starvation signal initiated by the collapse of PG-mediated nitrogen assimilation. This reframes a century-old physiological observation in molecular terms.

(3) Why do certain maize cultivars have decreased nitrogen use efficiency; was

this accidentally selected by historic breeding?

All 44 teosinte accessions examined show 72–99% *NIR2*^{T1} (Extended Data Fig. 18c), indicating that teosinte uniformly favors *NIR2*^{T1} and high *NIR2*^{T1} expression is the ancestral state and that the *NIR2*^{T2}-enriched haplotypes arose after domestication, likely through selection or drift. Several non-mutually exclusive possibilities may explain this phenomenon: 1) The *NIR2* locus may be linked to other domestication or improvement genes that were selected for traits such as early vigor, erect architecture, or disease resistance. The *NIR2*^{T2}-favouring haplotype could have been carried along passively; 2) Modern high-input agricultural systems provide abundant nitrogen, which may have reduced the fitness penalty associated with lower NUE, allowing *NIR2*^{T2}-enriched haplotypes to persist; 3) We have to see that the isoform ratio is a continuous trait, not a binary switch. This suggests that *cis-regulatory variation* (e.g., promoter strength, splicing efficiency) rather than presence/absence of the alternative splice site itself underlies the observed differences; 4) The fact that elite inbreds such as B73 and CML247 maintain high *NIR2*^{T1} proportions demonstrates that it is possible to combine high NUE with other desirable agronomic traits. Hence, introgression or promoter editing to increase *NIR2*^{T1} proportion represents a feasible and currently underexploited breeding strategy.

(4) What about legumes and other nitrogen-autonomous systems?

This is an excellent comparative point. Legumes possess a bacterially derived nitrogen assimilation pathway in nodules. We speculate that the plastidic GS/GOGAT cycle in legumes may be partially redundant with the cytosolic GS1 isoforms that process ammonium exported from bacteroids. Whether PGs in legume mesophyll cells also harbour NIR and GS2, and whether these contribute to whole-plant nitrogen balance, is completely unknown. We frame this as an exciting frontier for evolutionary systems biology: how do plants with symbiotic nitrogen fixation rewire the subcellular compartmentalization of primary nitrogen assimilation?

(5) Is low NUE ever useful? Why did T2-enriched haplotypes arise in teosinte?

We now directly confront the apparent paradox that wild teosinte, which never experienced synthetic fertilizer, uniformly maintains the high-NUE T1 isoform. If

high NUE is universally beneficial, why do T2-enriched haplotypes exist at all in modern germplasm? We propose two non-exclusive explanations: 1) Genetic hitchhiking: The T2 haplotype may be linked to alleles that confer tolerance to other stresses (drought, cold, herbivory) that were more prevalent in certain maize cultivation environments. Under stress, prioritizing nitrogen conservation over maximizing growth may represent an adaptive strategy. Thus, low NUE in the vegetative stage could be a trade-off for stress tolerance. 2) Relaxed selection: Modern high-input agricultural systems provide abundant nitrogen, which may have reduced the fitness penalty associated with lower NUE, allowing T2-enriched haplotypes to persist.

We have incorporated the suggestions from all reviewers, and have rewritten the Discussion section. Due to space limitations, we have condensed the key points mentioned above and integrated them into the Discussion.

8.I am missing the data described in lines 250-255. Please include AF3 models.

We agree with the reviewer's comment. After careful consideration, we have removed this figure from the revised manuscript because it is not essential for supporting the main conclusions of this work. This change helps to streamline the presentation and focus on the key results. The relevant content has been deleted in the revised version.

9.Figure 4, b and line 261: Does deletion of IDR recruitment or even disturb PG formation?

Response to the Reviewer's Comment:

You have noted a subtle yet important question, and we indeed need to provide some clarification to explain this phenomenon. In the tobacco transient expression system, the absence of the IDR does not affect the formation of PGs. The perceived differences in the number of PGs across various expression combinations, as observed in the images, arise from our imaging procedure. When acquiring multichannel fluorescent images, the focal plane for capturing cytoplasmic signals and the focal

plane for capturing chloroplast signals are physically separated. To present both signals in a single composite image, we employ a 3D acquisition method involving long-range, multi-layer scanning. The images are then merged using maximum intensity projection. This scanning approach captures more chloroplasts, making the number of PGs appear higher.

10.Figure 5, g: Show data of individual lines here. Due to the number of genetic differences, it seems inappropriate to bulk different lines into one plot. Statistics become problematic.

Response to the Reviewer’ s Comment:

We thank you for this valuable suggestion. We agree that combining data from independent transgenic lines with distinct genetic backgrounds in a single plot was not statistically appropriate, as it could mask line-to-line variation.

In direct response to this comment, we have revised Figure 5g to now show the data for each individual transgenic line separately, rather than as a combined average. This change provides a more transparent view of the variation between independent lines and strengthens the statistical validity of our conclusions.We believe this presentation is now methodologically sound and clearly addresses your concern.

11. Line 304-309: Why no NIR2 is co-purifying in the complex? Please be aware that complex formation is formally never shown in the manuscript. Simply pulldown experiments were made.

Response to the Reviewer’s Comment:

Regarding the question about why NIR2 was not co-purified in the complex in lines 304-309: The in vitro formaldehyde (FA) cross-linking used to assess the assembly state of GLN1 was performed with recombinantly expressed GLN1 protein alone, which explains why NIR2 was not co-purified in that experiment.

We are grateful for your question regarding the potential interaction between GLN1 and NIR2 within PGs. This critical point prompted us to conduct a systematic re-examination using additional experimental approaches, including semi-in vivo

Co-IP, in vitro pull-down assays, and Flow-induced Dispersion Analysis (FIDA) (Fig. 4l-n). Specifically, Δ30 (ZmGLN1)-His, purified from E. coli, was co-precipitated with ZmNIR2 from lysates of FLAG-NIR2T1OE plants. The in vitro pull-down assays confirmed that recombinant Δ37 (ZmNIR2)-Flag can pull down recombinant Δ 30 (ZmGLN1)-HA. Furthermore, Flow-induced Dispersion Analysis (FIDA) quantified the binding affinity between recombinant ZmNIR2-Flag and ZmGLN1-HA, yielding a dissociation constant (Kd) of approximately 0.054 uM in the buffer system (in 50 mM HEPES, pH 8.0, 100 mM NaCl). These results collectively indicate an interaction between NIR2 and GLN1.

Minor points

1.What's the difference between WT and CK? What does CK stand for? In Fig 3, d, what is the reason that the gln1 and nir1 are compared with CK, but others with WT?

The reason is that the genetic backgrounds of the materials presented differ. The *nir2* mutants and the *gln2-6* mutants share the same background, and their corresponding control is designated as WT. Conversely, the *gln1* mutant and the *nir1* mutant share an identical background, and their corresponding control is designated as CK. The relevant information has been indicated in the Methods section:The mutants *nir2-1, nir2-2*, *gln2*, *gln3*, *gln4*, *gln5* and *gln6* seeds were obtained from the mutant library (http://maizeems.qlnu.edu.cn/) and generated in the WT (B73 background). The ZmNIR2^{T1}OE and ZmNIR2^{T2}OE overexpression lines were generated in the WT (B73 background). The mutants *nir1* and *gln1* were created by Center for Crop Functional Genomics and Molecular Breeding of China Agricultural University and generated in the CK (ND101 background).

2.Throughout the text, clarify the concept of metabolic channelling better and avoid confusion with plasma membrane channels/transporter.

Thank you for your valuable suggestion. To further refine the description of this working model, we conducted an extensive literature review and ultimately decided to

adopt the term “metabolon” (Moehlenbrock *et al.*, 2011; Pareek *et al.*, 2021). A metabolon refers to a sequential complex of enzymes in a native metabolic pathway that assembles through protein-protein interactions into a tightly coupled complex. This structural organization facilitates substrate channeling, conferring three major advantages: (a) increasing the local substrate concentration near enzyme active sites; (b) protecting unstable reaction intermediates; and (c) significantly reducing the diffusion distance of substrates between enzymes. Collectively, these features enhance the overall efficiency and reaction rate of the metabolic pathway. The evidence presented in this study precisely aligns with the concept of a metabolon.

References Involved：

Moehlenbrock, M. J. et al. Metabolon catalysts: an efficient model for multi-enzyme cascades at electrode surfaces. *ChemCatChem.* **3**, 561–570 (2011).

Pareek, V. et al. metabolon: predictions, deductions, and evidence. *Molecular Cell* **81**, 3775–3785 (2021).

Use ‘PG’ and ‘PGs’ (singular versus plural) appropriately. I spotted many such mistakes throughout the text. For example, it should be ‘PG localization’, not ‘PGs localization’.

We have revised textual inaccuracies noted in the review.

Fig. 1, a: Why does the chloroplast in the TEM image at 2mM N look so different from other images (white empty cavities)? Is this a preparation artifact?

Thank you for the insightful observation. We have re-examined the previous data. Lower-magnification field-of-view images revealed that a small proportion of chloroplasts exhibited increased stromal lamellar gaps. After carefully reviewing the raw data and consulting with electron microscopy technicians, we hypothesize that the cavities observed in the chloroplasts in that image were likely artifacts introduced during sample processing or slide preparation and do not represent the true biological structure under that treatment condition. Consequently, the electron micrograph for the 2 mM treatment in the original Fig. 1a lacked representativeness. We have now replaced it with an image from a representative region of another sample prepared in

the same batch under the same treatment group.

Fig. 2, a: Typo in ‘Plant Materital’ should be ‘Material’.

We have revised textual inaccuracies noted in the review.

Fig. 4, a: Legend is far too small! Better use same font size for all labels in all figures.

We have adjusted the font size for all labels in all figures.

Fig. 4, b, c: delta55 should be ‘GLN1delta55’ to avoid confusion with NIR2 in the same figure.

Given the adjustments to the data, we have removed this content.

Fig. 3, e; Fig. 4, b; c; Fig. 5, c: Confocal microscopy pictures need to be more zoomed in. We cannot recognize PG composition in this view.

Regarding this issue, we have added a zoomed-in panel to more clearly display the PG composition

Fig 3, a: The log2 Exp scheme is too small for print.

We have revised textual inaccuracies noted in the review.

Extended Data Fig.5: Some mutants show which amino acids were changed into stop codon, but some not.

We have thoroughly reviewed, revised, and supplemented this section.

Extended Data Fig.8, a: Typos in labels ‘gn2’ and ‘gn4’ should be ‘gln2’ and ‘gln4’. In Extended Data Fig.8, b, label ‘CK’ is appearing (see above).

We have revised textual inaccuracies noted in the review.

Line 41: Add ‘maize’ in front of ‘NIR2’ for context.

We have revised textual inaccuracies noted in the review.

Line 42: First use of abbreviation NUE. Avoid introducing abbreviations in abstract.

We have revised textual inaccuracies noted in the review.

Line 49: Avoid using ‘efficient’ and ‘efficiency’ in the same sentence.

We have revised textual inaccuracies noted in the review.

Line 57-58: ‘Maize, recognized as the world’s most widely cultivated crop globally’. 1) ‘most cultivated’ means grown on largest agricultural area or most tons produced? 2) please include a source for this information, e.g. FAO.org has excellent data supporting such claims 3) ‘world’s’ and ‘globally’ are redundant. 4) ‘recognized’=subjective. Better rely on data.

We have revised the phrasing and cited relevant reports in support.

Line 59: For general readers, it could be useful to briefly explain the term ‘C4 plants’?, Perhaps discussing the link between C4 metabolism and nitrogen utilization could be something for the discussion too?

In response to the valuable suggestions from the other reviewers, we have revised the manuscript to address the specificity of C_4 plants and supplemented it with relevant experimental data for support. Furthermore, we have expanded the discussion on this point to further explore the potential biological significance of this mechanism in C_4 plants and its similarities to and differences from the C_3 system.

Line 75: Unclear what ‘it’ is referring to.

We have revised textual inaccuracies noted in the review.

Line 114: Which N source was used in the experiments? Ammonia, Nitrate, urea? Specify at least once in main text and in figure legends.

The nitrogen source used was KNO_3, as indicated in both the main text and the figure legends

Line 130: avoid using reference 26 in the middle of construct name.

We have revised textual inaccuracies noted in the review.

Line 130: 'L.' stands for Linnaeus? Since Nicotiana tabacum and the other species used here are standard model plants, I find the mentioning of the first describing author of the species somewhat obsolete (we do not have to worry about confusion here). If the authors want to really indicate the first- describing author, please do so consistently. For example, in line 130 they authors write 'Nicotiana tabacum L.' and in line 141 they write 'Glycine max (L.) Merr.' and 'Nicotiana tabacum L.)'. Please correct the typos.

Thank you for the suggestion. The phrasing has been revised accordingly, and the Latin name is provided upon first mention.

Line 133: Avoid using 'under' as it is confusing here. Better say 'at'.

We have revised textual inaccuracies noted in the review.

Line 143: Abbreviations LN and NN were already introduced in line 116.

We have revised textual inaccuracies noted in the review.

Line 192 and elsewhere: Avoid lab jargon 'Agroinfiltrated'.

We have revised textual inaccuracies noted in the review.

Line 203 and Figure 3: Figures 3, a-e are not appearing in sequence of text.

The text sequence at this point has been adjusted

Line 242f: Overinterpretation! The authors have not tested a causal relationship between localization and function, for example through genetically complementing with both NIR2 and GLN1 variants with mislocalization. Merely correlative data is shown.

The text at this location has been deleted.

Line 271: I would argue that IP-MS is still 'biased' by extraction method, data processing etc.

We have conducted more comprehensive experimental validation regarding whether NIR2 and GLN1 interact. We performed assays using pull-down, semi-in vivo CO-IP and Flow-induced dispersion analysis (FIDA) to validate the interaction between these key nitrogen assimilation enzymes, ZmNIR2 and ZmGLN1. Considering that chloroplast proteins are processed by stromal processing peptidases upon entering the chloroplast, leading to the cleavage of certain peptide segments, we also verified the interaction using the mature proteins after removal of the chloroplast transit peptides. The results indicate interaction between NIR2 and GLN1.

Line 272 and 277: unclear if 'transgenic plants' means maize or tobacco?

It's maize transgenic plants

Line 313 and elsewhere: the term 'natural variation' seems inappropriate since no wild populations were studies. What the authors did is to look at 'genetic variation in cultivated germplasm of maize'. Please correct the use throughout the manuscript (e.g., line 329, 340) In line 438, better say 'inbred lines' than 'natural populations'.

We have revised textual inaccuracies noted in the review.

Line 339: Wrong figure reference. Should be Extended Data Fig. 12, a.

We have revised textual inaccuracies noted in the review.

Line 356: LN, NN and HN were already introduced. Strike out.

We have revised textual inaccuracies noted in the review.

Line 406: typo 'underscorethe'

We have revised textual inaccuracies noted in the review.

Line 615, missing unit after 'scale bar, 1'

We have revised textual inaccuracies noted in the review.

Comments to the PDB report. Minor inconsistencies. Does not change the interpretation of the structural data or the manuscript altogether:

1. In the PDB report page 5, G203 in chain A also P204 in chain B are marked as a residue with a poor fit to the EM map. Please check is there any flexibility of the adjacent prolines P202 and P204 in Chain A and B. Please consider the atom-atom clash between chain B P204 and M245 in page 12. These might be the outcomes from same reason.

2. In the PDB report page 5, E410 in chain A with a poor fitting might be due to its sidechain. E410 side chain OE1 and OE2 are out of the map. Side chains of K418 and L419 are also located at the outside of the map.

3. Chain B G47, as the first residue, is entirely uncovered by the map. Perhaps the authors can start to build the model from residue 48? Please check all the G47 residues among different chains, they overall with poor fits.

4. Region of chain F from residue 410-413 is not in the map. Also, please see the pdb report page 20 chain F E412 with a non-rotameric sidechain. Same in chain F, side chains of K418 and L419 are out of the map.

5. Please check the issue about atom clash. The structure model has a long list of close contacts. Among the 284 contacts, the clash overlap between chain F, residue F218 and chain G, residue K188 is too large (1.14 Å!).

6. There are 27 side chain outliers in all chains (page 19).

Response to the Reviewer's Comment:

Thank you for your thorough review of our previously submitted model and for your valuable feedback. We have comprehensively examined and revised the model in response to each of your suggestions. Firstly, we have improved the overall quality of

the electron density map (EM map) through additional data processing steps, providing a clearer and more reliable foundation for model building. Secondly, we have addressed all the issues highlighted in the report involving, including re-adjusting the conformations of main chains and side chains to resolve poor fitting with the map resolving unreasonable atomic clashes (particularly the severe clash between F218 in Chain F and K188 in Chain G), and deleting residues that lack map support. The revised structure exhibits substantially improved stereochemical quality. In the updated wwPDB EM validation report:

- Clashscore is 4
- Ramachandran outliers are 0%
- Side-chain outliers are 0%
- Q-score (model–map agreement) is 0.61

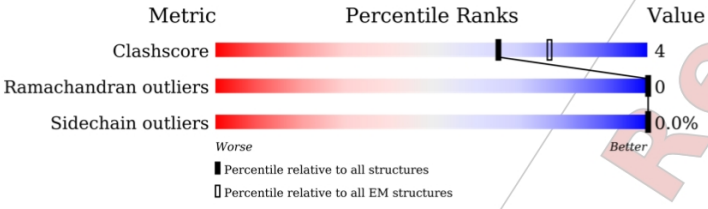
These validation metrics place the model among high-quality cryo-EM structures at comparable resolution and indicate excellent backbone geometry, side-chain conformation, and model–map agreement. The revised cryo-EM map, refined coordinates, and updated wwPDB EM validation report are resubmitted for review.

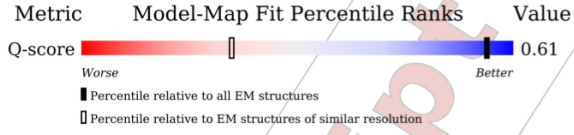
1 Overall quality at a glance

The following experimental techniques were used to determine the structure:
ELECTRON MICROSCOPY

The reported resolution of this entry is 1.98 Å.

Percentile scores (ranging between 0-100) for global validation metrics of the entry are shown in the following graphic. The table shows the number of entries on which the scores are based.





Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5 (Remarks to the Author):

This is an interesting study in which a substantial amount of work has been conducted to demonstrate that plastoglobules (PGs) are key regulators of nitrite reduction and subsequent assimilation in maize leaves. The topic falls within the broader goal of improving crop nitrogen (N) use efficiency for sustainable agricultural productivity, particularly in maize - a crop of major global economic importance. Maize is known for its high demand for both N fertilizers and water. However, the Introduction remains too general and does not fully reflect the current state of knowledge on maize nitrogen use efficiency (NUE). In particular, it does not adequately address the importance and validation of candidate genes involved in the ammonia assimilatory pathway, as well as amino acid biosynthesis and interconversion. Furthermore, although the significance of cellular compartmentation in inorganic N assimilation and organic N recycling is mentioned, I disagree with the claim that our understanding of these processes remains very limited (lines 86–90). The literature review is insufficient, both from physiological and molecular perspectives, especially regarding the two cell types found in maize (bundle sheath and mesophyll cells). Additionally, the phrase “atomic resolution level” is unclear and requires clarification. We have the general impression that the authors have structured their introduction in a way that suggests their work on plastoglobules in relation to primary nitrogen assimilation is entirely novel—when, in fact, this is not completely the case. Moreover, the limited but existing literature in the field is not even cited. The same remarks apply to the Discussion, although both the novelty of the findings and their significance are presented convincingly. In summary, the Introduction and Discussion sections would benefit from a thorough revision to better reflect the existing literature in relation to the originality of the findings.

Response to the Reviewer:

We sincerely thank your recognition of the "substantial amount of work" conducted and the importance of our study for global agricultural productivity. We are especially grateful for your meticulous and critical critique of our Introduction and

Discussion sections. Your feedback provided a vital roadmap for us to move beyond a general narrative and ground our findings in the deep context of maize NUE and C4 physiology.

We have taken your suggestions for a "thorough revision" very seriously, and we believe these changes have significantly elevated the manuscript's scholarly quality. Specifically:

Overhaul of Introduction and Discussion: We have completely restructured these sections to provide a comprehensive review of the ammonia assimilatory pathway and the role of key candidate genes in maize NUE. We have removed any overstated claims of absolute novelty and instead focused on how our discovery of PG-mediated compartmentalization provides a missing mechanistic link within the established framework of plant metabolism.

Refining C4 Cell-Type Specificity: We have integrated a much more nuanced discussion of the two cell types in maize (MCs and BSCs). To address your concern, we provided new experimental evidence demonstrating that nitrogen-responsive PG dynamics are primarily manifested in MCs, thereby providing the physiological resolution that was previously lacking.

Technical Clarification: We have addressed the ambiguity regarding the phrase "atomic resolution level" by removing this term entirely from the manuscript. Instead, we now provide the precise physical parameters of our structural data (1.98 Å resolution) to ensure the highest level of scientific accuracy.

Literature Integration: We have conducted an exhaustive literature search and cited the relevant but previously missing studies, ensuring that our work properly acknowledges the foundational contributions of the scientific community.

We are deeply indebted to your suggestions. This rigorous revision process has not only addressed the initial limitations but has also instrumentally strengthened the logical cohesion and academic depth of our study.

Major comments:

1) The results presented in Figure 1 are largely descriptive and do not provide significant novel insights, particularly regarding the phenotypic evaluation of plants in response to varying nitrogen (N) nutrition levels. The same observation applies to the data shown in Extended Data Figures 1 and 2. Furthermore, the rationale behind the experiments presented in these two figures is not clearly introduced and remains ambiguous in the context of the broader study. The growth conditions of the plants in these figures appear inconsistent with those described elsewhere in the study, which raises questions about their comparability. A major limitation of this study is the complete omission of the occurrence of morphologically distinct mesophyll and bundle sheath chloroplasts. This oversight is particularly problematic given the emphasis placed on the importance of cellular and subcellular compartments in the Introduction. Leaf sections where two types of cells are visible should be presented to both visualize and reliably quantify plastoglobules in the corresponding chloroplasts. The same issue applies to the fluorescence images presented in Figure 1 and the other figures of the manuscript.

Response to the Reviewer’ s Comment:

We sincerely thank you for these critical and comprehensive comments, which have helped us significantly improve the rigor and clarity of our manuscript. Below we provide point-by-point responses to the reviewers' comments, along with the corresponding revisions.:

(1) Identification of nitrogen utilization indicators in maize and current limitations

our current research efforts are focused on two main areas. One area investigates the molecular mechanisms underlying nitrogen use efficiency in maize at the cellular level. The other area involves the mapping and cloning of superior nitrogen efficiency genes from population materials. In this latter line of research, we monitor multiple physiological traits potentially directly related to nitrogen utilization, including biomass, plant height, protein content, amino acid content, root-to-shoot ratio, stem

diameter, leaf color, root architecture, and yield. Data for these traits are collected across multiple locations and seasons to facilitate the mining of key genes. We have already identified several superior nitrogen efficiency genes, with some findings published — for example, the high-protein gene *THP9* cloned from a wild maize population was published in Nature in 2022 (Huang *et al.*, 2022). Nevertheless, we still cannot definitively identify the key physiological indicator(s) for nitrogen efficiency in maize.

While tillering has been largely established as a key indicator for nitrogen efficiency in rice , which links to plant biomass and yield, and several key genes have been identified accordingly, the situation in maize is more complex. Nitrogen demand in maize persists throughout its lifecycle, and the requirement varies across different growth stages, leading to shifts in the corresponding key nitrogen-responsive traits. We will continue to deepen our research along this path, using more robust data to elucidate the key indicators for nitrogen utilization in maize and the corresponding regulatory networks.In the present study, the two nitrogen-responsive traits most critical to our research content are leaf color and biomass. It was precisely the pronounced change in leaf color under different nitrogen concentrations that initially prompted us to examine chloroplast morphology in detail, conducting extensive observations of internal structures. This ultimately led, through multiple independent replicates, to the consistent identification of dynamic changes in PGs in response to nitrogen, which we then pursued with further in-depth investigation.

(2) Investigating the conservation of PG nitrogen-responsive dynamics in MC chloroplasts in C3 and C4 plants

Existing reports have revealed that the structure, core components, and functions of PGs are highly conserved across multiple species. Similar dynamic responses of PGs to environmental changes have also been observed in different species (Leggett *et al.*, 2006, Steinmüller *et al.*, 1985, Eymery *et al.*, 1999, Zhang *et al.*, 2010, Locy *et al.*, 1996, Lichtenthaler *et al.*, 1981). This inspired us to investigate whether the dynamic response of PGs to nitrogen observed in maize could also be detected in other species. Based on this hypothesis, we selected two monocot crops (rice and

wheat) and two dicot crops (soybean and tobacco) for preliminary validation in Extended Data Figures 1 and 2. Notably, we observed dynamic PG responses to nitrogen in all four crop types, which further underscores the research significance and general relevance of the underlying molecular mechanism.

Given our limited cultivation experience with rice, wheat, soybean, and tobacco, and considering their distinct growth patterns and nitrogen requirements, we took particular care in designing the experiments. More importantly, the dynamic response of PGs is sensitive to environmental conditions. To minimize unrelated variations caused by factors such as light intensity, humidity, and temperature, we ensured that each crop was grown under optimal conditions while subjected to different nitrogen treatments. After consulting with specialized research teams familiar with each crop, we developed corresponding nitrogen treatment protocols and provided suitable growth environments tailored to each species. This explains why the experimental setups differed slightly among the four crops.

（3）The necessity of cellular compartmentalization

Crucially, we fully acknowledge your suggestion regarding the classification of chloroplast types in the two cell types. Maize, as a typical C4 plant, exhibits Kranz anatomy in its leaves, consisting of outer mesophyll cells and inner bundle sheath cells tightly surrounding the vascular bundles. The chloroplasts in these two cell types show significant differentiation in thylakoid and grana structures, with bundle sheath chloroplasts containing abundant starch grains. During our initial observations of PGs, we noted these two chloroplast types. However, since the bundle sheath chloroplasts are densely packed with starch grains and their PGs did not exhibit clear changes in number or morphology with varying nitrogen concentrations, we did not investigate this aspect further. Given that mesophyll cells are the primary site for initial nitrogen assimilation, we focused our observations and in-depth studies on them.

Nevertheless, we sincerely thank you for pointing out this oversight. We have now conducted a more comprehensive study and expanded this section. As shown in the relevant figures, bundle sheath chloroplasts contain numerous starch grains. Under lower nitrogen concentrations (0.04 mM, 2 mM), thylakoids are sparse, and the

chloroplasts are largely filled with starch grains, which may be related to reduced carbon demand for amino acid assimilation under low nitrogen conditions (Hirel et al., 2005). The number of PGs per chloroplast ranges from 0 to 3. With increasing nitrogen concentrations (4 mM, 8 mM, and 16 mM), thylakoid structures become more abundant, and the number of PGs per chloroplast remains between 1 and 6 (Extended Data Fig. 1c,d,e). Across the five nitrogen levels tested (0.04 mM to 16 mM), we did not observe a consistent, nitrogen-dependent increase in PG numbers like that seen in mesophyll cells. The relatively low abundance of PGs in BSC chloroplasts at 0.04 mM and 4 mM nitrogen treatments is more likely attributed to reduced thylakoid formation under low nitrogen conditions, which limits the membrane area available for PG budding from thylakoids, rather than reflecting a direct decrease in PG biogenesis. It is also noteworthy that the cross-sectional area of bundle sheath chloroplasts showed no significant variation across the five nitrogen concentrations, which contrasts with the marked reduction in mesophyll chloroplast cross-sectional area under low nitrogen conditions.

References Involved：

Huang, Y. et al. *THP9* enhances seed protein content and nitrogen-use efficiency in maize. *Nature* **612**, 292–300 (2022).

Austin, J. R., Frost, E., Vidi, P.-A., Kessler, F. & Staehelin, L. A. Plastoglobules are lipoprotein subcompartments of the chloroplast that are permanently coupled to thylakoid membranes and contain biosynthetic enzymes. *Plant Cell* **18**, 1693–1703 (2006).

Steinmüller, D. & Tevini, M. Composition and function of plastoglobuli : I. Isolation and purification from chloroplasts and chromoplasts. *Planta* **163**, 201–207 (1985).

Eymery, F. & Rey, P. Immunocytolocalization of CDSP 32 and CDSP 34, two chloroplastic drought-induced stress proteins in *Solanum tuberosum* plants. *Plant Physiol. Biochem.* **37**, 305–312 (1999).

Zhang, R., Wise, R. R., Struck, K. R. & Sharkey, T. D. Moderate heat stress of Arabidopsis thaliana leaves causes chloroplast swelling and plastoglobule formation. *Photosynth. Res.* **105**, 123–134 (2010).

Locy, R. D., Chang, C. C., Nielsen, B. L. & Singh, N. K. Photosynthesis in

salt-adapted heterotrophic tobacco cells and regenerated plants. *Plant Physiol.* **110**, 321–328 (1996).

Lichtenthaler, H. K. et al. Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. *Photosynth. Res.* **2**, 115–141 (1981).

Hirel, B.; Martin, A.; Terce-Laforgue, T.; Gonzalez-Moro, M.B.; Estavillo, J.M. Physiology of maize I: A comprehensive and integrated view of nitrogen metabolism in a C4 plant. *Physiol. Plant*. **124**,167–177 (2005)

2) While the objectives of the study presented in Figure 2 are well-founded, my previous comment also applies here. There are several established methods available for isolating the two types of maize cells and their respective chloroplasts. This major issue becomes even more critical when considering the nitrogen-assimilating enzymes analyzed in the subsequent proteomic study.

Response to the Reviewer’ s Comment:

Based on the results presented regarding the aforementioned issues, although the PGs in bundle sheath cell chloroplasts did not exhibit nitrogen level-dependent dynamic changes, we agree with your suggestion that PGs from the two chloroplast types should be extracted separately. Due to structural differences, mesophyll cells (MCs) and bundle sheath cells (BSCs) can be separated either by cellulase enzymatic digestion (Becker *et al.*, 1993) or by mechanical stripping using a blender at different power settings (Romanowska *et al*, 2011). While cellulase treatment may affect cellular physiological states, to avoid potential impacts on subsequent proteomic measurement and analysis, we opted for physical separation using a blender at varying power levels. MCs can be stripped under low-power blending, whereas BSCs require multiple rounds of high-power blending for effective detachment. We collected a large quantity of fresh maize leaves, removed the leaf tips, bases, and midribs, and used 200 g of material for subsequent cell separation and PG extraction. We successfully isolated four batches of highly concentrated MC chloroplasts and one batch of BSC chloroplasts. Detection using cell type-specific protein markers showed that PEPC was only detected in MCs, while Rubisco was exclusively detected in BSCs, confirming the high purity of our chloroplast separation (Extended Data

Fig.3).

Although we successfully extracted chloroplasts from both MC and BSC, we encountered challenges during subsequent PG purification. PGs from MC chloroplasts were abundant and a single batch of crude extract yielded a substantial amount of PGs. Even after pooling four batches of crude extract for refined purification, we still obtained a considerable quantity of high-purity PGs. In contrast, no visually observable oily floating layer was obtained from BSC extracts after the first or second rounds of ultracentrifugation. Using the PG-specific antibody VTE1 for detection, a clear protein band was observed in the PG fraction from MCs, while only a very faint band was detected in the supernatant from BSC extracts. This is consistent with the electron microscopy observations and the final extraction yield. To further analyze the protein contents of PGs from both cell types, we submitted both samples for mass spectrometry analysis. However, due to the extremely low protein concentration in the BSC PG sample, it was insufficient for reliable instrument detection (Fig. A in this chapter). Nevertheless, the issue you raised remains worthy of attention and further investigation. In future work, we will continue attempts to isolate BSC PGs and compare their internal components with those from MC PGs. Such research will contribute to a deeper understanding of the physiological processes of nitrogen assimilation in C_4 plants.

In summary, this study focuses exclusively on the analysis of MC PGs and the mechanistic investigation of key nitrogen assimilation enzymes. Nonetheless, given that MCs serve as the primary site for initial nitrogen assimilation in both C4 and C3 plants, we believe the discovery of this phenomenon and the elucidation of its underlying mechanisms remain highly significant.

References Involved：

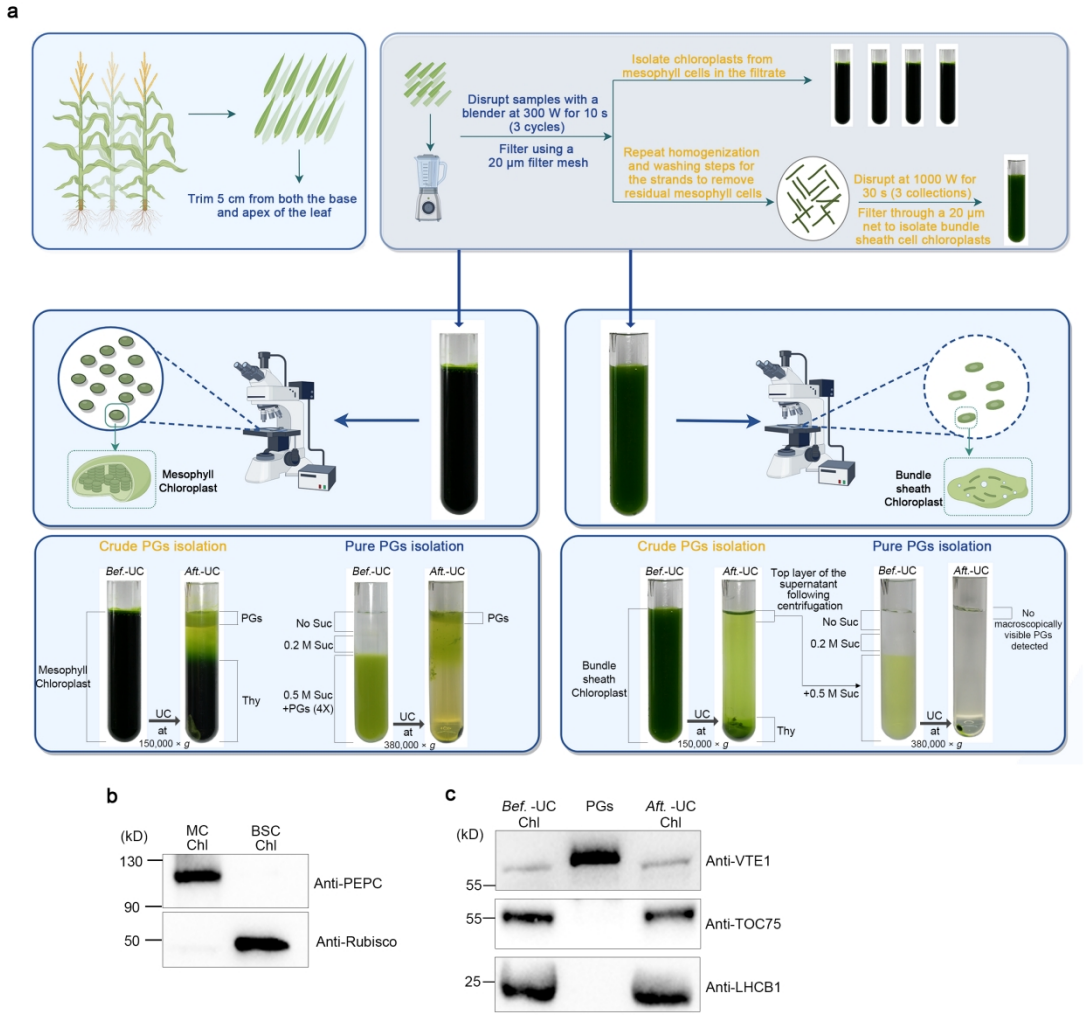
Becker, ThomasW., Perrot-Rechenmann, C., Suzuki, A. & Hirel, B. Subcellular and immunocytochemical localization of the enzymes involved in ammonia assimilation in mesophyll and bundle-sheath cells of maize leaves. *Planta* **191**, (1993).

Romanowska, E. & Parys, E. Mechanical isolation of bundle sheath cell strands and thylakoids from leaves of C4 grasses. *Methods Mol Biol* **684**, 327–337 (2011).

The revisions to the Figure are as follows：

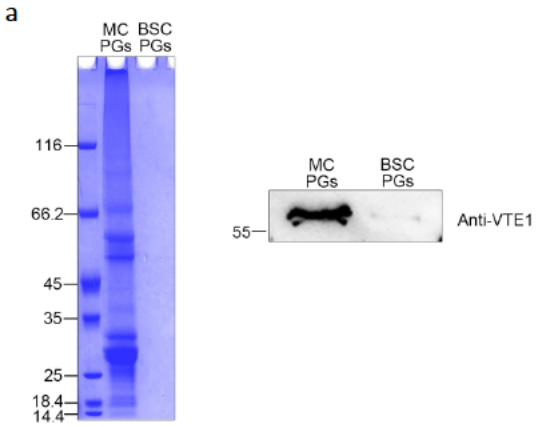
(see the Extended Data Fig.3)

Extended Data Fig.3



a, Schematic diagram for the extraction and purification of PGs from maize MCs and BSCs. Abbreviations: *Bef.*-UC, before ultracentrifugation; *Aft.*-UC, after ultracentrifugation; Thy, thylakoids; Suc, sucrose. **b**, Purity assessment of isolated thylakoids from MCs and BSCs. PEPC and Rubisco were used as specific marker proteins for MCs and BSCs, respectively. **c**, Western blot analysis of chloroplasts

before ultracentrifugation (*Bef.*-UC Chl), purified PGs fractions (PGs) and thylakoids after PGs depletion (*Aft.*-UC Chl). Tocopherol cyclase VTE1, TOC75, and LHCB1 were used as the marker proteins for PGs, chloroplast outer membrane, and thylakoid membrane, respectively.



a, Protein analysis of PGs from MC and BSC. Equal volumes of MC PGs and BSC PGs samples were separated by SDS-PAGE. Total proteins were visualized by Coomassie Brilliant Blue staining, and the amount of PGs was indicated using a PGs-specific VTE1 antibody.

Although the selection and characterization of the Nir and Gln mutants are only briefly described—and their homozygosity remains unclear—they appear to be acceptable. To improve the clarity of the results, the accession numbers for the different genes should be provided, especially for those encoding plastidic or cytosolic glutamine synthetase (GS). This is important because the nomenclature for these genes varies across published studies. Along these lines, it should be explicitly stated in both the results section and the figure legends that Gln1 encodes a plastidic GS (GS2). It is somewhat surprising that most genes encoding cytosolic GS are expressed at higher levels in roots compared to leaves.

Response to the Reviewer’ s Comment:

We thank the reviewer for these constructive suggestions to improve clarity.

First, we have now explicitly stated in the "Methods" section that all mutants used in this study were homozygous. and we have described the genotyping method by PCR amplification and Sanger sequencing. Second, the official accession numbers for all key genes discussed have been provided in parentheses in the "Methods". As suggested, we have explicitly stated in both the "Results" text and the relevant figure legends that Gln1 encodes the plastidic glutamine synthetase (GS2). Notably, the maize gene identifiers and their corresponding updated designations used in this study are those of the V5 version.

Given that the majority of cytosolic GS isoforms exhibit higher expression levels in roots, we have also reviewed recent literature on this topic. Nitrate and ammonium are the two primary inorganic nitrogen sources in plants. In many species, including maize, a significant portion of the nitrate absorbed by roots is transported to the shoots for reduction in mesophyll cells, while another fraction is reduced to ammonium in the roots. This ammonium is subsequently assimilated into organic nitrogen via GS (Li *et al.*, 2025; Liu *et al.*, 2025). Cytosolic GS1 serves as the predominant active form in roots, responsible for assimilating root-derived $NH_4^+$to prevent ammonium toxicity under high concentrations. In contrast, chloroplast-localized GS2 is expressed in green tissues and represents the most abundant isoform in leaves, where it functions to reassimilate ammonium released during nitrate reduction in plastids and during photorespiration (Moreira *et al.*, 2022).

References Involved：

Li, J. et al. Genetic and molecular mechanisms underlying nitrogen use efficiency in maize. *J. Genet. Genom.* **52**, 276 – 286 (2025).

Liu, H., Gao, X., Fan, W. & Fu, X. Optimizing carbon and nitrogen metabolism in plants: From fundamental principles to practical applications. *J. Integr. Plant Biol* **67**, 1447 – 1466 (2025).

Moreira, E., Coimbra, S. & Melo, P. Glutamine synthetase: an unlikely case of functional redundancy in *Arabidopsis thaliana*. *Plant Biol.* **24**, 713 – 720 (2022).

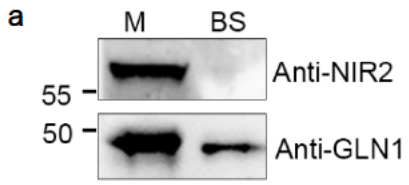
3) The experiments regarding the PG-dependent metabolic channel for primary nitrogen assimilation are convincing. However, the cell-type compartmentalization of this pathway is not addressed, despite the fact that nitrate and nitrite reduction primarily occur in the leaf mesophyll. It is therefore likely that the observed biological process also takes place in this cell type. Nevertheless, the possibility that this process occurs in bundle sheath cells cannot be excluded. In these cells, GS2 is present in the chloroplasts, while the NIR enzyme is considered to be absent. This raises questions about the potential cooperation between these two enzymatic activities within plastoglobules (PGs), further emphasizing the importance of cell-specific distribution in nitrogen assimilation within a C4 plant.

Response to the Reviewer’ s Comment:

Your suggestion serves as an important reminder of the necessity to consider both the functions of PGs and their cellular compartmentalization. As a C4 plant, maize has two distinct leaf cell types: mesophyll cells (MCs) and bundle sheath cells (BSCs). Using endogenous antibodies against NIR2 and GLN1, we performed immunodetection on isolated chloroplasts from these two cell types (Fig. a in this chapter). The results are consistent with your observation: NIR2 was detected exclusively in MCs, while GLN1 was present in both cell types. Previous studies have established that key enzymes involved in primary nitrogen assimilation, NR and NIR, are localized only in mesophyll cells (Harel *et al.*, 1977), whereas GLN is found in both cell types (Becker *et al.*, 1993). The cellular compartmentalization of these crucial nitrogen assimilation enzymes indicates that the “metabolic channel” mode involving NIR2 and GLN1 operates solely within the PGs of MCs. This precisely provides a plausible explanation for the absence of a dynamic nitrogen response observed in BSC PGs.

This study has validated the dynamic nitrogen response of PGs in the MCs of C_4 plants. Preliminary results in C_3 plants suggest that this model may also be applicable. An established cellular compartmentalization model for nitrogen assimilation posits that glutamine is likely synthesized from nitrate within MCs during primary nitrogen assimilation, as these cells contain NR, NIR, and GLN1. In contrast, the high levels of

Fd-GOGAT present in BSCs may contribute significantly to glutamate synthesis in this process. The clear spatial separation of primary glutamine and glutamate synthesis in maize leaves necessitates the transport of nitrogenous compounds (e.g., glutamine and/or glutamate) between MCs and BSCs (Becker *et al.*, 2000). The nitrogen-responsive dynamic changes specifically identified in MC PGs in this work, regulated by the primary assimilation enzymes NIR2 and GLN1, represent an extension of the existing compartmentalization model. This discovery deepens our understanding to the subcellular level of chloroplasts, specifically, within PGs. Maize, a C_4 plant, exhibits enhanced capabilities for the uptake, metabolism, and re-translocation of carbon and nitrogen metabolites compared to most C_3 plants. This advantage may well stem from the synergistic combination of two features: the nitrogen-efficient regulatory function afforded by PG subcellular compartmentalization, and the cellular compartmentalization inherent to nitrogen assimilation.



a, Purity assessment of isolated thylakoids from mesophyll cells (MC) and BSC (BSC). NIR2 and GLN1 were detect by Western blot with anti-GLN1 antibody and anti-NIR2 antibody.

References Involved:

Harel, E., Lea, P. J. & Miflin, B. J. The localisation of enzymes of nitrogen assimilation in maize leaves and their activities during greening. *Planta* **134**, 195–200 (1977).

Becker, ThomasW., Perrot-Rechenmann, C., Suzuki, A. & Hirel, B. Subcellular and immunocytochemical localization of the enzymes involved in ammonia assimilation in mesophyll and bundle-sheath cells of maize leaves. *Planta* **191**, (1993).

Becker, T. W., Carrayol, E. & Hirel, B. Glutamine synthetase and glutamate dehydrogenase isoforms in maize leaves: localization, relative proportion and their role in ammonium assimilation or nitrogen transport. *Planta* **211**, 800–806 (2000).

4) Line 302 and Figure 4h: The claim that GLN1 assembly occurs in a substrate-concentration-dependent manner should be interpreted with caution. The ammonium concentrations used in this study are significantly higher than the enzyme's Km for this substrate (which is in the micromolar range) and are therefore likely physiologically irrelevant. Additionally, the production of photorespiratory ammonium in bundle sheath cells—at least during the early stages of plant development—and the resulting cellular compartmentalization of ammonium need to be carefully considered. Unfortunately, these aspects were not adequately addressed in the present study.

Response to the Reviewer's Comment:

We sincerely thank the reviewer for this insightful and forward-looking comment. Indeed, using endogenous antibodies to directly assess the oligomeric state of GLN1 under normal physiological conditions in maize, as well as its response to nitrogen variation, represents the most direct and effective approach.

Using a GLN1-specific endogenous antibody, we examined the native oligomeric state of GLN1 in planta in both CK (control) and gln1 mutant maize materials. In CK, a single band was observed near 440 kDa, which was absent in the gln1 mutant. Based on the estimated molecular weight of the GLN1 monomer (46 kDa), this band corresponds to a decamer (Fig. 4j). This result is consistent with the protein structure observed by cryo-electron microscopy, indicating that GLN1 exists stably as a decamer in plants. To investigate how this oligomeric state changes under varying nitrogen conditions, we treated maize seedlings with three nitrogen concentrations (2 mM, 4 mM, and 8 mM) following the cultivation method described in Fig. 1. Equal amounts of leaf tissue were then used to assess GLN1 oligomerization. The results demonstrate that an increase in GLN1 decamers in response to higher nitrogen levels(Fig. 4k).

Regarding the potential difference in GLN1 oligomerization state due to variations in ammonia concentration across cellular compartments, as you mentioned, we agree this is a plausible consideration. However, we must acknowledge an oversight: the original cross-linking experiments were indeed fundamentally flawed for the interpretation we attempted, primarily due to the lack of a loading control and the inherent limitations of cross-linking for inferring in vivo assembly states. This issue has been sufficiently resolved by using the endogenous antibody detection method. While differences in ammonia concentration across compartments may influence GLN1 protein abundance, they do not affect its oligomeric state, as GLN1 exists stably as a decamer in maize plants.

5) The results concerning the natural variation of NIR2 are quite convincing. However, the cytosolic localization of NIR2T2 appears to be questionable in light of the known electron donor for the corresponding enzyme activity, which is a plastidial ferredoxin in both photosynthetic and non-photosynthetic tissues.

Response to the Reviewer’s Comment:

Thank you for your positive recognition of the findings related to natural variation in NIR2 in this study, which provides significant confidence for our subsequent research. The issue you raised regarding the spatial separation between the cytoplasmic localization of $NIR2^{T2}$ and its compatible electron donor also puzzled us initially. In fact, alternative splicing is a widespread post-transcriptional regulatory mechanism in plants. Beyond the primary transcript, one or more alternative transcripts are often generated through this process. As described in this work, NIR2 undergoes alternative splicing to produce two transcripts. Compared to $NIR2^{T1}$, $NIR2^{T2}$ lacks the N-terminal chloroplast transit peptide, thereby preventing its entry into chloroplasts. Several studies have reported that alternative splicing can alter the subcellular localization of proteins（Kashkan 2022）.

Some protein isoforms encoded by alternative transcripts may exhibit non-functional characteristics, while others can influence plant growth. Research on the BR signaling pathway transcription factor *AtBES1* initially focused on the classic

short isoform, BES1-S. Subsequent studies, however, revealed that BES1 also produces a novel long isoform, BES1-L. Overexpression of BES1-S did not result in an obvious enhanced BR phenotype, and its localization was observed in both the nucleus and cytoplasm. In contrast, overexpression of BES1-L led to elongated, curled leaves and accelerated flowering time, with localization observed exclusively in the nucleus. Further investigation showed that BES1-L contains an additional nuclear localization signal at its N-terminus, which confines it to the nucleus and enhances its activity in promoting BR signaling compared to the classic short isoform BES1-S (Jiang 2015). In potato, the TCP transcription factor *BRC1a* generates two protein isoforms, $BRC1a^{Long}$ and $BRC1a^{Short}$, through alternative splicing (Nicolas 2015). The C-terminal region of $BRC1a^{Long}$ contains a strong transcriptional activation domain, enabling it to function as a transcriptional activator in the nucleus. In contrast, the absence of this activation domain in $BRC1a^{Short}$ affects its nuclear localization, resulting in predominant cytoplasmic localization and loss of transcriptional activation activity. Importantly, the ratio of these two transcripts changes in response to various environmental stimuli, including light conditions, decapitation, and hormonal treatment (Nicolas 2015).

Given that the cytoplasm lacks the requisite enzymatic conditions, such as compatible electron donors, the $NIR2^{T2}$ transcript should theoretically encode a non-functional protein. To further validate the function of $NIR2^{T2}$, we overexpressed $NIR2^{T1}$ and $NIR2^{T2}$ separately within the same genetic background. The results showed that $NIR2^{T1}$ overexpression lines exhibited increased biomass and plant height, whereas $NIR2^{T2}$ overexpression lines did not display significant differences from the wild type, confirming our hypothesis (Extended Data Fig.17 d-g). Nonetheless, the underlying reasons for the generation of the two *NIR2* transcripts via alternative splicing require further exploration. The preference for $NIR2^{T1}$ vs. $NIR2^{T2}$ among different materials essentially reflects a redistribution of isoform usage at this locus across varying genetic backgrounds. We detected haplotype differences significantly associated with transcript proportions near the gene, suggesting that this variation is likely primarily driven by cis-regulatory variations (affecting the efficiency and

preference of transcript selection)（Zhang *et al.*,2024）.

References Involved：

Kashkan, I., Timofeyenko, K. & Růžička, K. How alternative splicing changes the properties of plant proteins. *Quant Plant Biol* **3**, e14 (2022).

Jiang, J., Zhang, C. & Wang, X. A recently evolved isoform of the transcription factor BES1 promotes brassinosteroid signaling and development in Arabidopsis thaliana. *Plant Cell* **27**, 361–374 (2015).

Nicolas, M., Rodríguez-Buey, M. L., Franco-Zorrilla, J. M. & Cubas, P. A Recently Evolved Alternative Splice Site in the BRANCHED1a Gene Controls Potato Plant Architecture. *Curr Biol* **25**, 1799–1809 (2015).

Zhang, H. *et al.* Population-level exploration of alternative splicing and its unique role in controlling agronomic traits of rice. *Plant Cell* **36**, 4372–4387 (2024).

6) In the section dedicated to the functional validation of the PG-localized NIRT1, the appropriate control should be the NIRT2 overexpressors. It would be advisable to indicate the nitrogen (N) concentrations (LN, NN, and HN) directly in the text, rather than only in the legend of Figure 5.

Building on the previous question, our results show that overexpression of $NIR2^{T1}$ leads to increased biomass and plant height, while overexpression of $NIR2^{T2}$ exhibits no significant difference from the wild type. To further explore the nitrogen use efficiency (NUE) potential of $NIR2^{T1}$ for practical agricultural applications, we conducted the experiments presented in Fig. 5. You are absolutely correct that control selection should be as rigorous as possible. However, in the practical context of varietal improvement, we typically compare modified lines directly with the unmodified parental background. Additionally, since $NIR2^{T2}$ did not show clear functional effects, our focus remained primarily on assessing the NUE potential of $NIR2^{T1}$.

Furthermore, we have thoroughly reviewed the manuscript and specified the exact nitrogen concentrations throughout the text to ensure clarity for readers.

7) The discussion emphasizes the importance of cellular compartmentalization in the control of primary nitrogen (N) assimilation. However, the study is limited to events

occurring in the chloroplast, without considering that maize leaves contain two types of cells and thus two types of chloroplasts, each with distinct metabolic and physiological functions. While it has been clearly demonstrated that plastoglobules play a key role in controlling nitrite reduction and ammonia assimilation within one type of chloroplast (probably in mesophyll cells), the broader physiological implications at the leaf level and potentially at the whole-plant level remain poorly understood. The text also mentions compartmentalization mechanisms, but these will need to be further assessed using a combination of metabolic analyses and metabolic modeling approaches.

Response to the Reviewer’ s Comment:

We highly value your expert comments regarding the cellular compartmentalization of nitrogen assimilation. In the revised manuscript, we have supplemented multiple relevant experimental results to address the previous gaps in this aspect of our study. Furthermore, we have carefully revised the “Introduction” and “Discussion” section, with a particular focus on the nitrogen assimilation model within the cellular compartments of C_4 plants. Building on this foundation, we have further elaborated on the contributions and advances of this study in the field.

In summary, the technical and scientific aspects of the work are both novel and excellent. However, the plant biochemistry and physiology, along with their interpretation, are insufficiently supported by the available data. This limitation leads to an overestimation of the potential breeding and agronomic applications.

Response to the Reviewer

We sincerely thank you for this critical and constructive summary. We particularly appreciate the recognition of the novelty and excellence of our technical and scientific approach.

We have taken your concerns regarding the empirical support for our physiological and agronomic interpretations very seriously. During this five-month revision period, we have focused our efforts on providing the "missing links" to

ensure that every claim is backed by robust, multi-dimensional evidence. Specifically:

(1) The integrated investigation combining the cellular compartmentalization model with the subcellular PG compartment represents a significant theoretical advancement of this study. Given the Kranz anatomy unique to C_4 plants, research on carbon and nitrogen metabolism necessitates careful differentiation of the structural characteristics and functional distinctions between mesophyll cells and bundle sheath cells. The revisions you highlighted concerning this aspect have been crucial for enhancing the professionalism and rigor of our work. In the revised manuscript, we have provided key experimental evidence based on cellular compartmentalization, refining the working model of the subcellular PG compartment specifically to mesophyll cells.

(2) In the revised manuscript, we have supplemented additional genetic evidence, including transcriptomic data from over 40 teosinte accessions, genetic evidence comparing the functional differences between the two *NIR2* transcripts, and field data from inbred lines subjected to differential nitrogen treatments. This evidence serves to demonstrate the application potential of the nitrogen metabolic enzyme compartmentalization mechanism for enhancing crop nitrogen use efficiency. We hope this substantially reinforces confidence in the relevance of this mechanism for breeding and agronomic applications.

(3) Through updates to the “Introduction” and “Discussion sections”, this study is now positioned within a broader physiological and biochemical context of nitrogen metabolic enzymes. The evidence presented not only corroborates existing research conclusions from a new perspective but also offers innovative insights into the regulation of enzyme metabolism via subcellular compartmentalization, thereby further expanding the scope of nitrogen metabolism research. However, exploring the numerous open questions regarding the physiological mechanisms of nitrogen metabolic enzymes represents a key direction for our future efforts.

We are deeply grateful for your rigorous standards, which have been instrumental in helping us refine our interpretations and provide the robust empirical support necessary to substantiate the study’s broader implications. We believe the

manuscript is now significantly strengthened and more accurately reflects the biological and agricultural significance of our findings.

Response to the Reviewer

We would like to express our sincere gratitude to the reviewers for their time, effort, and constructive feedback throughout the review process. Their insightful comments have been instrumental in improving the quality, clarity, and scientific rigor of our manuscript. We are delighted that the reviewers now support the publication of our work in Nature. We have carefully addressed all remaining minor points raised by the reviewers in this final version.

Referee #1 (Remarks to the Author):

I now fully endorse this study. The revised manuscript is a major improvement on the original version. The authors have addressed all of my comments in their 75 page rebuttal. They have done so in a specific and very satisfactory manner, having carried out a substantial amount of additional experimentation in the process. Overall, the manuscript is novel and comprehensive, covering everything from biochemistry to field testing. It provides a blueprint for engineering plastoglobules (PGs) for metabolic channelling in crop species.

Improvements regarding my comments:

1) Questions surrounding the localisation of NIR2 and GLN1 have been answered, and the separate sequences required for chloroplast targeting and plastoglobule association have been experimentally identified.

2) A clear rationale has been provided for the association of NIR2 and GLN1 with PG.

3) The relationship between PG content and NUE has also been experimentally addressed.

4) Questions regarding the semi-in vivo CO-IP have been answered.

5) The discussion has been significantly enhanced.

Response to the Reviewer

We are very grateful for your positive assessment and for your endorsement of our study. We are pleased that you find the revised manuscript to be a significant improvement and that our additional experiments have satisfactorily addressed your concerns. Thank you for recognizing the novelty and comprehensive nature of our work.

Referee #2 (Remarks to the Author):

The authors have thoroughly addressed all my concerns and the manuscript has been significantly improved. I am satisfied with the current version and strongly support its publication in Nature.

This study represents an important contribution to the field of plant and crop science. It provides new insights into the spatial regulation of nitrogen metabolism at the intracellular level and introduces an innovative framework that goes beyond conventional gene discovery approaches.

The newly added data are of high quality, and the statistical analyses are appropriate and well presented.

Only a few minor issues remain before publication:

1. In Fig. 1a, the capitalization of the first letter appears inconsistent. Please check and ensure consistent formatting across all figures.

2. The titles of Extended Data Fig. 6 (“Gene structure of nir and gln mutants”) and Extended Data Fig. 9 and 1 (“Different nitrogen concentration treatments on nir/gln mutants”) could be revised for clarity.

3. The manuscript currently uses both WT and CK to denote controls. For consistency, it may be preferable to use WT throughout, with figure legends indicating different genetic backgrounds if necessary.

Response to the Reviewer

Thank you for your strong support and for your encouraging words regarding the contribution of our study to the field of plant and crop science. We have addressed your minor suggestions as follows:

Figure formatting: We have carefully reviewed all figures (including Fig. 1a) and ensured consistent capitalization and formatting throughout the manuscript.

Figure titles: We have revised the titles of Extended Data Fig. 6 and Extended Data Fig. 9/10 to ensure they are clear and descriptive.

Terminology consistency: We have standardized the use of "WT" throughout the manuscript to denote controls, ensuring consistency across all text and figure legends.

Referee #3 (Remarks to the Author):

The authors have thoroughly addressed all scientific concerns, either through improved reasoning or, in many cases, by presenting new data. The PDB report has

also been significantly improved through improvements to the 3D density and the model fitting. I particularly appreciate the re-examination of the contradictory data from Jia et al. (Nature Plants, 2025), which has improved the quality and significance of the study

All minor issues regarding typos and labelling have been corrected. However, the authors are submitting a heavily revised manuscript (substantial textual changes). I advise the authors to carefully check their discussion for grammar and typos, should they be considered for print.

Overall, I have no remaining major points and recommend publication.

Response to the Reviewer

We appreciate your thorough review and your positive comments regarding the improvements made to the 3D density and model fitting of our cryo-EM data. We are also glad that our re-examination of the data from Jia et al. (2025) has strengthened the significance of our study. As you suggested, we have conducted a final, careful proofreading of the entire manuscript to ensure the highest standard of grammar and clarity.

Referee #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Response to the Reviewer

We thank you and your colleague for your time and effort in reviewing our manuscript. We appreciate your constructive input, which has contributed to the final version of this paper.

Referee #5 (Remarks to the Author):

I am fully satisfied with the revisions made to the manuscript and the additional experiments conducted, particularly regarding the dual compartmentation of nitrogen (N) assimilation in maize leaves. Overall, this is excellent work that significantly advances our understanding of nitrogen use efficiency in maize and its regulatory mechanisms, a research field in which very few major breakthroughs have been made recently. However, I still disagree with two statements made in response to one of my previous comments. These two minor points listed below can easily be addressed in the final revised version of the manuscript.

1) “Cytosolic GS1 serves as the predominant active form in roots, responsible for assimilating root-derived NH4+to prevent ammonium toxicity under high concentrations,”

This statement correctly reflects the predominance of GS1 in roots. However, it is highly unlikely that a toxic build up of ammonium occurs in the soil, and consequently in roots, under standard physiological or agronomic conditions, unless maize is grown in soils or habitats where ammonium naturally accumulates.

2) “chloroplast-localized GS2 is expressed in green tissues and represents the most

abundant isoform in leaves, where it functions to reassimilate ammonium released

during nitrate reduction in plastids and during photorespiration (Moreira et al., 2022).”

I strongly disagree with this statement. A thorough review of the literature clearly shows that the proportion of GS1 and GS2 activity in maize leaves remains very similar in both mesophyll cells (MC) and bundle sheath cells (BSC), although some variations may occur depending on plant developmental stage, leaf age, and nitrogen nutrition mode. This primarily concerns two cytosolic GS isoenzymes, which are referred to in several older studies, sometimes under different nomenclatures: for example GS1.3 (or GS112/1B) original gene accession number (d14577x65928) and GS1.4 (or GS107/1A), (d14576x65929). Furthermore, it is well established that maize belongs to the group of plants that predominantly assimilate nitrogen in the leaves.

Response to the Reviewer

We are honored by your positive remarks regarding our work on the dual compartmentation of nitrogen assimilation. We appreciate your expert guidance on the physiological nuances of nitrogen metabolism.

We have carefully read your comments and the relevant literature. The unreasonable statements in our previous response to the reviewers' comments have been deleted. We once again thank you for your rigorous academic attitude.