# Exploiting the Genetic Diversity of Maize using a Combined Metabolomic, Enzyme Activity Profiling, and Metabolic Modelling Approach to Link Leaf Physiology to Kernel Yield

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*Plant Cell. Advance Publication April 10, 2017; doi: 10.1105/tpc.16.00613*

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**Review timeline:**

| | | |
|---|---|---|
| **TPC2016-00613-LSB** | Submission received: | August 22, 2016 |
| | 1st Decision: | September 29, 2016 *revision requested* |
| **TPC2016-00613-LSBR1** | 1st Revision received: | January 2, 2017 |
| | 2nd Decision: | February 9, 2017 *accept with minor revision* |
| **TPC2016-00613-LSBR2** | 2nd Revision received: | March 7, 2017 |
| | 3rd Decision: | March 16, 2017 *acceptance pending, sent to science editor* |
| | Final acceptance: | April 10, 2017 |
| | Advance publication: | April 10, 2017 |

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

**TPC2016-00613-LSB 1st Editorial decision – *revision requested*** **September 29, 2016**

The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

We ask you to pay attention to the following points in preparing your revision.

The article provides a lot of data and analyses and the work has been solidly carried out, but the current version suffers from a lack of clear focus and message. It relies too much on being a data deposit and not enough attention in made to drawing clear conclusions of interest to TPC readers. Four specific issues need to be resolved:

1. The experimental set up itself is raised as a concern, especially by Rev 1. Specific factors in the design may directly affect conclusions about the genetic diversity of maize lines and this needs to be resolved.

2. Without the profile of single individuals and leaves it is noted that a lack of statistical power is failing to spot differences among the lines, so some power analysis to show what can be achieved or further metabolite measures are required.

3. More analysis needs to be done to identify specific examples of biological implications from your work and this needs to be clear in the text.

4. There were concerns raised by several reviewers over the labeling, fluxes and the use of in vitro activity maxima that need to be clarified and explained.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

Given the nature of the comments, we are offering you 60 days to complete the revision. If a revision is not returned within this time frame, and if you have not been granted an extension, we will withdraw the manuscript, which will

leave you free to submit the work elsewhere. If you need an extension, we encourage you to contact us at any point before submitting your revision.

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

[Reviewer comments shown below along with author responses]

**TPC2016-00613-LSBR1 1st Revision received January 2, 2017**

Reviewer comments and **author responses**:

**RESPONSE TO EDITOR: It is clear that all three reviewers have put a considerable amount of work into preparing their reports, for which we are very grateful.**

**Point 1. Additional information on the plant sampling has been provided. Full details on the leaf sampling procedure and dates of harvest are now provided.**

**Point 2. We have performed an additional metabolomic analysis of line B73 at the two stages that were examined. This experiment shows that, both at the V stage and 15DAS, for most of the identified metabolites, the three individual replicates in each of the three blocks are similar with a coefficient of variation of 35 % for the V stage and 28% for 15DAS.**

**Thus, pooling the three replicates in each block appears to be experimentally valid. Moreover, statistical analyses (including an ANOVA statistical test with significance at a P value ≤ 0.05, followed by a Bonferroni post-hoc test) were performed on the three replicates corresponding to each block, to identify metabolites that were significantly different between the 19 lines. This point has been clarified in the Material and Methods section (Statistical and hierarchical clustering analysis.**

**Point 3. A number of sections have been significantly shortened and headings and conclusions have been more clearly presented to provide specific examples of the biological implication of our work. In particular and as requested by reviewer #2, soluble leaf proteins, PEPC protein and kernel C and N contents have been included in the phenotypic analyses to strengthen the biological meaning of both the correlations and of the co-regulated module studies.**

**Point 4. The use of maximal enzyme activity has been justified throughout the manuscript.**

Reviewer #1:

The present manuscript by Cañes et al. is an ambitious project that uses a large data set obtained through high through-put omics-analyses together with a labelling study to disentangle genetic effects to distinguish 19 lines of *Zea mays*. The methodology that has been used is highly advanced and the analytical investment considerable. The maize lines belong to five subgroups mainly chosen (as it appears) on the basis of their origin. Two sampling events were performed and the main results from the study include developmental considerations. Further in the Abstract it is put forward that a maze "ideotype" of high grain yield potential is predicted to include low accumulation of amino acids and carbohydrates and express a high activity of the C4 pathway.

Point 1. Although the amount of work is large for this paper, I am skeptical as to how much new knowledge it presents. My worries start with the experimental set up. I had trouble understanding how the plants were grown and sampled and how the leaves were selected and pooled. After consulting with the supplementary material, I understood that leaves of ten individuals per line were pooled to make up three pooled samples, one per growing block, thus providing three pooled biological replicates per line for two sets of leaves. It was also difficult for me to understand the time line.

**RESPONSE: Additional information on the plant sampling has been provided. Full details on the leaf sampling procedure and dates of harvest are now provided. Dates of harvest for each line at 15DAS are indicated in the new Figure 2.**

Point 2. The seeds were sown on May 15th and leaves from vegetative individuals harvested (when?) and again 15 days after silking (15DAS) and this time point must be at least 15 days after the first line (or individual?) had started to silk, which happened on August 5th. If I am right about this, then I think that the questions addressed in the study are not possible to answer with the data material as it is presented here

**RESPONSE: More details on the harvesting dates are now provided. Plants were harvested at the V stage on July the 2nd. At 15DAS the leaf below the ear was harvested for each individual plant.**

Point 3. The complexity of the experimental set up makes it difficult to make conclusions about the genetic diversity of maize lines. The main problem as I see it is the pooling of the individuals of a line. Without the profile of single individuals and leaves, the authors lack statistical power to find differences among the lines, although they must exist based on the looks of Supplemental Figure 1.

**RESPONSE: I think there is confusion in the interpretation of Supplemental Figure 1. In this figure, the genetic relatedness between the different lines in the total S1P9 panel is presented. The profile of single individual leaves is now presented in the response to the next comment.**

Point 4. What I really miss is better resolution of the phenotypic variation within each line. The pooling of samples will not give that insight. Only a mean of expressions. Metabolomic analyses are not very expensive to perform and I wonder if there is powder left which could make it possible to make analyses the metabolic profile for single individuals and see how they are distributed relative to the mean values? This kind of detail would need to be included to properly investigate genetic or line dependent differences.

**RESPONSE: We do not fully agree with the statement "Metabolomic analyses are not very expensive". In our Institute the cost for one analysis is around 100 euros which makes 11,400 euros with the three replicates analysed in the present study. If we had used 9 replicates as requested by the reviewer the total cost would have been 34,200 euros.**

**Nevertheless, we have performed an additional metabolomic analysis with line B73 at the two stages that had been examined. This experiment shows that both at the V stage and 15DAS, for most of the identified metabolites, the three individual replicates in each of the three blocks are similar with a coefficient of variation of 35 % for the V stage and 28% for 15DAS.**

**Thus, pooling the three replicates in each block appears to be experimentally valid. Moreover, statistical analyses (including an ANOVA statistical test with a of significance at a P value ≤ 0.05, followed by a Bonferroni post-hoc test) were performed on the three replicates corresponding to each block to identify metabolites that were significantly different between the 19 lines. This point has been clarified in the Material and Methods section (Statistical and hierarchical clustering analysis).**

Point 5. Moreover, one of the most salient result of the paper appears to be that the two leaf types diverged, and I wonder if that would not be expected when leaves are sampled two months apart?

**RESPONSE: This point has been clarified in the Discussion. Moreover, we have performed leaf metabolomic studies during the entire maize life cycle. In this study, we observed that after the silking date and up to four to six weeks later the metabolomic profile remains practically unchanged (unpublished data available upon request of the reviewer) indicating that the plant physiological status is not markedly modified for approximately four weeks.**

<u>Reviewer #2:</u>

The authors are interested in systems-level comparison of maize lines, making use of a diverse set of omic tools and nineteen genetically distant maize lines. The goal in this study is to determine if the metabolite levels and enzyme activities can be used as selection markers for breeding better maize. The breadth of studies is striking; and the idea is good, with some support and significant summarizing of the big data that would be useful to the field; however more could be done to tie specific examples of biological implications. Also there are some concerns over the labeling, fluxes and activities and how they are attained and used that at a minimum need clarification.

Point 1. Can aconitate really be the most abundant organic acid, others have suggested malate is greatest with significant citrate, in some C4's, aconitate when measured is not so abundant.

**RESPONSE: Aconitate has been found to be the most important organic acid in maize leaves or in the phloem sap. Two references (Sicher and Barnaby 2012; Brauer and Teel, 1981) have been added to support our finding.**

**According to Sicher and Barnaby (2012) Both the *cis* and *trans* isomers of aconitate exist in maize leaves, and the trans configuration can attain concentrations in grass species exceeding 1% of the dry weight (Thompson et al 1997).**

**Thompson JF, Schaefer SC, Madison JT (1997) Role of aconitate isomerase in trans-aconitate accumulation in plants. J Agric Food Chem 45: 3684–3688.**

Point 2. Can you go further to link the observed variation to traits of specific lines that explain the variation phenotypically (e.g. do the altered amino acid activities or levels correspond with lines altered in protein? Does variation in α-ketoglutarate correspond to altered protein levels in leaves or amounts of exported/produced amino acids - given that this is the organic acid product from transamination of glutamate?) Does the variation in carbohydrates correspond with lines altered in cell wall or total biomass?

**RESPONSE: We agree that it would have interesting to extend the correlation studies to metabolites with the other measured traits. However, we think that with the leaf protein, the leaf PEPC protein, the kernel N and C contents (included in the revised version of the manuscript following your comment below: 359) it would have greatly increased the length of the manuscript with both results and discussion that will not be essential for an understanding of the paper.**

Point 3. What does it say that none of the activities have high coefficients of variation? Is this a reflection of the in vitro measurement, or does it speak to the ability of enzymes to act along their activity profile? When protein/enzymes are harvested and resuspended, the in planta concentration of substrates and products are no longer maintained.

**RESPONSE: Yes, indeed, *in vitro* activities have to be seen as an alternative for the amount of the corresponding active protein. There is no intention of considering that an activity measured under saturating conditions is an alternative for the flux of the reaction it catalyses. A sentence has been added to specify that maximal enzyme activities were measured, thus probably explaining the range of variation observed between the different lines.**

Point 4. Heat map-based descriptions (supplements 3-5) may be the best way to visually represent a significant amount of data but only allow one to speak in generalities. Clearly the purpose intended for Figure 2 is to show that clustered genetic lines have similarly clustered metabolite accumulation; which is then further analyzed by the PLS-DA. I think there might be better ways to represent the data of Figure 2, however I was able to follow the description.

**RESPONSE: For clarity, the figure has been simplified and modified as requested by reviewer # 1.**

Point 5. Figure 3 summarizes the metabolite differences, and the metabolites are named in the text; however, the list is not rationalized. How are we to interpret the metabolites that came up on the list? Many different phenomena could lead to an enhanced level of a particular metabolite or enzyme activity etc. A correlation if reproducible may be predictive in identifying genetic lines, but without a hypothesis or some understanding about the metabolites being used as markers, its utility is reduced.

**RESPONSE: The section describing Supplemental Figure 6 and Figure 6 has been modified to address this comment. Figure 3 has been modified to be consistent with the Results section. Two hypotheses on the accumulation of carbohydrates and chlorogenates have been proposed in the Discussion.**

Point 6. As suggested above, is it possible that the lack of correlation with enzyme activities is because the assays are in vitro and therefore do not make use of differences in metabolite concentrations that exist in vivo and that are part of the genetic signature? Most enzymes operate at less than the Km concentration, yet I suspect the individual Km's are not known and were not used which would have consequences on activity and the utility to help determine flux.

**RESPONSE: A sentence has been added page 10 (lines 307 to 309), to indicate that this result could be due to the fact that enzyme activities were measure *in vitro*.**

Point 6. What is the consequence on metabolism of using detached leaves? Also, would you expect differences along the leaf gradient that would be averaged by your approach (i.e. the leaf has source and sink cells, ones that are done dividing and others that are not)? The condition of floating a leaf in ammonium chloride even a low concentration may affect pH? - it is not described in the methods and would this affect metabolism? There is no description of controls that might address some of these concerns?

**RESPONSE: We have checked the pH of the nutrient solution after incubation with ammonium chloride. The pH remained stable with a value ranging from 5.6 to 5.8 from the beginning of the experiment to 8 hours ($^{15}$N-labelling experiment).**

**A similar experimental procedure has been used to monitor $^{15}$N distribution into amino acids using *in vivo* NMR measurements (Labboun et al. 2009). When [$^{15}$N]$H_4^+$ was provided to detached leaf segments, the incorporation of label into both the $\delta$-amido group of Gln and the $\alpha$-amino groups of Glu and Gln was observed. Even after 20-24h, the amount of label incorporated into the $\delta$-amido group of Gln was about twice as high as that incorporated into $\alpha$-Glx, indicating that the GS/GOGAT cycle was operating, meaning that the leaf tissue samples were in good physiological condition. Labboun et al., 2009. Resolving the role of plant glutamate dehydrogenase: I. *In vivo* real time nuclear magnetic resonance spectroscopy experiments. *Plant. Cell. Physiol.* 50 : 1761-1773**

**We agree that it would have been interesting to have performed an experiment along the leaf gradient. However, the main aim of the present study was to study the relationship between whole leaf metabolism and maize productivity. Thus, performing detailed metabolite and enzyme activity analyses in leaf sections, or in cellular compartments would not have been directly relevant and would have required a tremendous amount of additional work.**

Point 7. Did the ND36 line that accumulated the least 15N have the least protein in leaves? Does it produce less protein in the kernel? As asked above, can you link the measurement with a phenotype? Similarly in lines that had elevated amino acids, did they have higher protein content somewhere, or other N-containing compounds? C/N ratios are not as specific as total protein for example - I think you have information on at least the total soluble protein that could be used? The bigger question of your manuscript is: "Can you get past all of the grouping which is well done but then link particular phenotypes in lines with metabolites and activities". Some of the grouped descriptions are well-described in the discussion - thank you, however some come across as to generic and may provide a way to select, but do not seem to add to rational understanding of biology.

**RESPONSE: Leaf protein, total kernel C, N contents and relative amount of kernel C and N (C% and N%) have now been incorporated into the correlation studies (Figure 6, 7 and Supplemental Figure 8). These results are now presented and discussed to provide an improved integrated view of the biology of the lines with respect to assimilate accumulation and export both in sink and source leaves during the transition assimilation (V stage) to remobilization (grain filling stage 15DAS).**

Point 8. How are the enzyme activities being used? Given that activity is measured in vitro, it will not reflect the concentrations of substrates in vivo and therefore the lack of change in fluxes described elsewhere in the manuscript is possibly explained by the fact that the activity measurements cannot account for differences in concentrations of metabolites that change with genetic line. Also in vitro results avoid biochemical regulation that may be important in planta to final flux values. (See description of Km above). Finally, fluxes and activities do not correlate well - see for example Junker et al 2007 Phytochemistry. I presume this was not how activities were being used, but it is unclear from your description, and activities were not described in Simons etal 2014, rather that paper referred to levels to assign the active metabolic network (which would be fine), but not its flux or operation directly.

**RESPONSE: The enzyme activities were measured *in vivo*, indicating that the activities measured reflect their maximal activities. Therefore, the change in enzyme activities are being used to limit the maximal possible flux determined using only stoichiometric and thermodynamic constraints (i.e. the biomass level was not constrained here). This gives the largest stoichiometrically feasible flux range for each reaction associated with a measured enzyme. While other effects are expected to be factors in determining the metabolic fluxes, we expect that very large changes in enzyme activity can constrain the model, while small changes in the enzyme activity may not have an effect. This is captured by our approach. We have limited the upper bound of each flux range associated with a measured enzyme by the enzyme activity in the specific line divided by the maximal enzyme activity in all lines. This method does not directly imply the flux level based on the enzyme activity, but instead assumes that low levels of**

**enzymatic activity in one maize line will limit the flux through the reaction by approximating the restriction to the changes in the enzyme activity. From the analysis, we showed that only two enzymes result in an active constraint in the model. A similar approach has been used to apply the change in mRNA levels with success in predicting the metabolic state of *Mycobacterium tuberculosis* (Colijn et al., 2007). The publication by Junker et al directly compares the enzyme activities to the flux ranges derived by MFA and the qualitative trend does hold for 5/7 enzyme/flux comparisons.**

**Reference: Colijn C, Brandes A, Zucker J, Lun D, Weiner B, Farhat M, Cheng T, Moody D, Murray M, Galagan J (2009) Interpreting expression data with metabolic flux models: predicting Mycobacterium tuberculosis mycolic acid production. PLoS Comput Biol 5: e1000489**

Point 9. Why is the objective function - "the maximum rate of biomass production"? Leaves do not operate to produce biomass but rather export sucrose (assuming that the entire leaf is a source leaf, but in fact maize leaves contain a gradient of cells some that are dividing and growing and heterotrophic, others that are photosynthetic). Thus if the entire leaf is used it will be an average of many cellular fates, if the tip were used it would still operate with an objective function that is different from maximizing biomass. Probably this point should be considered in the discussion section at a minimum.

**RESPONSE: We agree with the reviewer's comment; we have changed the text to reflect the updated objective function of maximizing sucrose. However, in order to account for growth, as we have modeled the vegetative stage, we have set the biomass rate to be 35% of the maximum biomass based on growth rate at the time the sample was taken (at the 7-8 leaf stage) compared to the maximum growth rate observed in (Bender et al., 2013). Qualitative trends are very similar for the objective function of maximizing sucrose export compared to those results obtained from maximizing leaf biomass production.**

Point 10. It is not clear to me that examining enolase is useful because the source of PEP in c4 metabolism will be its recycling from pyruvate by PPDK, not generation de novo from enolase. Maybe the variation of PPDK should be considered here (and possibly other C4 enzymes - NADP-ME?).

**RESPONSE: Enolase enzyme activity was measured experimentally, with detectable expression in all maize lines suggesting that it is useful. In fact, Furbank and Leegood found that enolase and phosphoglycerate mutase were present in sufficient quantities and did not show any particular specialization compared to spinach leaves (Furbank and Leegood, 1984). Additionally, glycolysis is important at night and most glycolytic enzymes are stable across diel cycles (Missra et al., 2015). A similar discussion of PPDK has been added.**

**References: Furbank RT, Leegood RC (1984) Carbon metabolism and gas exchange in leaves of Zea mays L. : Interaction between the C3 and C 4 pathways during photosynthetic induction. Planta 162: 457-462**

**Missra A, Ernest B, Lohoff T, Jia Q, Satterlee J, Ke K, von Arnim AG (2015) The Circadian Clock Modulates Global Daily Cycles of mRNA Ribosome Loading. Plant Cell 27: 2582-2599**

Point 11. You indicate that network analysis was performed to uncover the mechanisms underlying the leaf physiology of different lines, but the description here is not mechanistic. Rather it is quite "descriptive". All of the correlations contribute value to your manuscript in describing big data, but specific examples that relate a line and measured values to generate a plausible relationship or mechanistic understanding or something more telling biologically would add to the value of the manuscript. For example, the description reads somewhat as a data dump. Though it may be "interesting" that tocopherol is correlated with TKW, or that 2-oxoglutarate and succinate correlate with GY, this has no meaning per se, how are they related?

**RESPONSE: We did not find any obvious correlations between the physiological function of α-tocopherol and succinate. We have thus modified the first sentence of the section: "To uncover the putative mechanisms…" instead of "To uncover the mechanisms …".**

Point 12. Could the lack of flux correlation be because of the in vitro activities that do not consider concentration or biochemical regulation?

**RESPONSE: It is possible that the lack of correlation here is a result of not considering the regulation or concentration; however, based on the other experimental evidence that does not provide clear correlations between genetic relatedness and fluxes, we do not expect that this will be the result. We have clarified in the Discussion that the metabolism is only constrained by the change in maximal enzyme activity levels.**

Point 13. Did you measure Rubisco levels - could it explain high N observation? This is an example of the biological insight that you could do more to validate or inspire with your data.

**RESPONSE: Additional experiments have been performed and the leaf proteins were separated using denaturing polyacrylamide gel electrophoresis. As previously observed, Rubisco is not present in large amounts compared to PEPC. Therefore, we have quantified the relative amounts of PEPC protein instead and included the data in the correlation analyses together with the total soluble protein content. Quantification of the relative amounts of PEPC is now included in Supplemental Data set 1.**

Point 14. Basing selection on one or two metabolic markers derived from correlative analysis and without mechanistic support as to why those metabolites are altered would be questionable because the altered level could be a result of many things, some of which would be desirable, but some???

**RESPONSE: A sentence has been added in the final conclusion in line with comment of reviewer 3. "However, modulating the level of these two markers using genetic techniques or performing association genetics studies will be required to fully validate their predictive value".**

Point 15. $^{15}N$ is described as highest in alanine, but yet glutamate and glutamine have rapid turnover - this implies that the latter should be higher labeled on a relative basis, unless there are pools that are not part of metabolism and therefore dilute the labeled glutamate and glutamine pools? Suppl dataset 5 shows $^{15}N$ absolute levels, but does not show remaining $^{14}N$ levels to evaluate the above considerations and the text does not suggest this possibility? Have I misunderstood something?

**RESPONSE: The quantification of $^{14}N$ amino acids is now included in Supplemental data set 5, and the results presented and discussed.**

Point 16. Nadp-me is higher later, yet other c4 assimilating were higher early?

**RESPONSE: Yes, but only in Corn Belt lines. In the other case, PEPCK is higher at 15DAS in all the lines except the Tropical ones. It was previously proposed that that maize is a plant operating with a NADP-ME type C4 pathway (Gutierrez et al., 1974, Planta 119:279-300). However, more recently this finding has been refined leading to the conclusion that maize C4 metabolism appears to be more complex than originally described (Pick et al. 2011. Plant Cell 23:4208-4220 and Wang et al. 2014. J Exp Bot. 65:3567-3578).**

Point 17. It seems odd that the positive correlations for 15DAS were all enzymes and negative were all metabolites? And for V stage there were no enzymes, only metabolites?

**RESPONSE: In this table, there is only a representative group of enzyme activities and metabolites with significant correlations with yield and its components. The complete set of metabolites and enzyme activities can be found in the Supplemental Data Sets 8 and 9. There are significant correlations for enzyme and metabolites in every case except for the negative correlations with metabolites at 15DAS when all the negative correlation belongs to metabolites. This can be explained as follows:**

**1.- All the measured enzyme activities are related to C and N assimilation and management which could lead to a bias in the correlation results.**

**2.- The accumulation of a number of metabolites in the leaves during grain filling suggests a low sink capacity of the kernels or problems with the metabolite transport capacity. Amino acids such as glutamine or sugars such as glucose must be transported and accumulated in the developing kernels rather than in the leaves during the kernel filling process.**

Reviewer #3:

The authors studied metabolic and enzyme activity profiles in a core collection of 19 maize lines of American and European origin. They related these parameters to the grouping of these maize lines into 5 groups based on genetic markers and to yield-related agronomic performance measures of the lines. This was further deepened by a $^{15}$N-labelling study in detached maize leaves and flux balance modelling for each of the lines in order to estimate flux ranges for metabolic enzymes. Finally, network analysis was employed to identify modules of intercorrelated metabolic traits and study their relationship to agronomic performance measures. The authors summarized their results by specifying which metabolic features, based on their findings, would contribute to creating a hypothetical "maize ideotype for optimal grain yield".

Point 1. Overall: While the article provides a lot of data and analyses and the work appears to have been solidly carried out, it unfortunately suffers from a lack of clear focus and message. It is generally very lengthy to read and not very accessible or engaging in style. In some cases, an excessive amount of details are described in the text. E.g. Description of details the specific response of individual metabolites or enzymes in individual maize lines could be omitted, unless a major statement is later derived from these observations. Same goes for individual correlation of specific eigengene modules with agronomic traits.

In my opinion it would be beneficial to clearly state at the beginning of each subchapter the aims of the described analysis in terms of contribution to the overall message. And then, to summarize results in a more concise form with a clear focus on results relevant to the stated aims and overall message and a clear narrative structure.

Message: The overall message is somewhat not very clearly defined, but in several instances the question is discussed, if the measured metabolite and enzyme activity profiles the maize lines can be used to assess the genetic similarity/dissimilarity of the lines.

**RESPONSE: A number of sections have been significantly shortened and headings and conclusions have been more clearly presented.**

**However, we believe that a description of individual correlations with the eigengene modules is necessary for a clear understanding of the analysis.**

Point 2. To me the principal question is how relevant this aim (i.e. classifying genetic similarity based metabolite/enzyme profiles) is. Considering that costs for genetic analyses have continuously gone down in recent years and can be expected to decrease even further, and that low amounts of tissue are sufficient for genetic analysis and do not require plants to be grown under defined conditions or to a certain developmental stage, it would seem more straightforward to analyze the genetics of lines directly than to take the detour over metabolic profiles. Perhaps it could be interesting to put more focus on the reversal of this question, i.e. can desirable metabolic traits/trait combinations ("the maize ideotype for optimal grain yield") be predicted based on genetic information, or directly the relationship of metabolic profiles and agronomic traits, and/or to generally focus and elaborate more on the "the maize ideotype for optimal grain yield", which seems an interesting concept.

**RESPONSE: We fully agree with this comment. It is now critically discussed at the end of the conclusion section.**

Point 3. Various correlation analyses: It is not detailed, neither in the text, nor methods nor figure legends whether multiple testing correction was conducted for p-values, to correct for the rather large total number of correlation analyses conducted.

**RESPONSE: Bonferroni corrections have been calculated and the new data are shown. The text has been modified accordingly (Results and Material and Methods).**

Point 4. The term "eigengene module" can be somewhat misleading, since these modules do not contain genes but rather metabolic traits. Perhaps consider changing the term.

**RESPONSE: Eigengene has been removed throughout the text and only module was kept.**

Point 5. There is lot of detailed discussion of the relationship of eigengene modules (referred by their color) to agronomic traits. However, information how these modules relate to actual metabolic classes or functions or how they could be applied is scares and it remains thus hard to connect this information to actual relevant relationships.

**RESPONSE: A brief description of the module composition has been added, including the new studied traits (leaf proteins, PEPC proteins, kernel C and N contents, request from reviewer 2).**

Point 6. Conclusion that C accumulation is detrimental to yield is based on observation that C/N ratio is negatively correlated to yield parameters. It should be a t least mentioned that C on its own is negatively correlated to KN. Because if only the ratio is negatively correlated this could be as much due to the positive correlation between N and yield parameters.

**RESPONSE: The sentence has been modified.**

Point 7. Figure 4: It is stated in the text and figure legend that HCA was performed using euclidian phenotypic distance and genetic distance as parameters. How did the genetic distance come into this analysis, considering that only enzyme activity traits are clustered? (I know MEV software, perhaps you could specify for me which steps were performed in the software).

**RESPONSE: The figure legend has been modified to explain more clearly how the HCA analyses were performed."Two hierarchical clustering analyses (HCA) were performed to group the lines and the enzymatic pathways according to their genetic distances based on molecular markers (A_IBD, see Material and Methods) and according to their Euclidean phenotypic distance based on enzyme activities, respectively".**

| **TPC2016-00613-LSBR1 2nd Editorial decision – *accept with minor revision*** | **February 9, 2017** |
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On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance however is contingent on revision based on the new comments of our reviewers. In particular, please consider the following:

1. requests for further clarification of your statistical treatment in the methods section (required)

2.requests to modify the order of one section that does make sense from a readership perspective (please consider carefully)

3. request for clarification of statements and reworking of several slightly ambiguous statements or lack of referencing of appropriate findings (required)

4. There are also a range of minor points noted that require your attention.

5. The figures are very pixelated, please make sure that high resolution images are provided. Figure 8, the fonts are too small to read. Please re-work the figure. The supplemental materials need to be in Arial or Helvetica -- here, please pay attention to consistent formatting for all supplemental datasets. The tables need legends to explain the column headings. It would be advisable to use freeze panes option (either for header or for first column) for long lists. Finally, please pay attention to display of significant figures for all values in all tables. Only the first uncertain digit should be shown for measurements (if operations are performed on measurements -- e.g. ratios, then the rules for displaying significant figures should be followed). There is an option in Excel to avoid displaying meaningless significant figures.).

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

[Reviewer comments shown below along with author responses]

| **TPC2016-00613-LSBR2 2nd Revision received** | **March 7, 2017** |
|---|---|

Reviewer comments and **author responses**:

**<u>RESPONSE TO EDITOR:</u> On behalf of all the authors, I would like to thank the two reviewers for the second round of helpful on our manuscript submitted to The Plant Cell. It is clear that reviewer 3 has again put a considerable**

**amount of work into preparing their reports, for which we are very grateful.**

**We have now carried out changes to the manuscript along the lines recommend by reviewers 2 and 3 and we have answered their comments point by point. In your letter, you also stated that four specific issues regarding the resolution of the Figures and the presentation of the supplemental data sets need to be resolved. We have indicated our responses below:**

**Point 1. Further information on the statistical treatments notably the various ANOVA tests has been provided.**

**Point 2. The order of this section has been modified, according to the suggestion of reviewer 3.**

**Point 3. We have modified the text concerning a number of statements and described more precisely some of our findings, notably concerning the description of the module elements.**

**Point 4. The minor points have been corrected or modified according to the comments and suggestions of reviewer 3.**

**Point 5. The figures, notably Figure 8, the supplemental Data Sets and the supplemental Figures have been modified to meet the requirements of The Plant Cell.**

Reviewer #2:

The authors have addressed the list of specific comments by reviewers and provided a comprehensive list of activities and levels that contribute significantly to the breadth of knowledge on metabolism in maize. It would appear from the data presented that phenotypic traits may indeed serve as markers for genetic diversity in maize, and that metabolites and enzymes could serve in aiding breeding efforts, at least in some cases, though as the authors admit more extensive tests are necessary.

Point 1. The higher labeling in serine probably reflects that there are multiple pools of glycine, only 1 that is highly labeled and used in photorespiration - thus it is more labeled than the serine and it is also diluted by other glycine pools that are unlabeled or less labeled, causing the appearance of glycine that is less labeled than serine.

**RESPONSE: This point is now discussed as follows:**

**The elevated labeling in serine may reflect that there are multiple pools of glycine, only one of which is highly labeled and involved in photorespiration. The other glycine pools, containing low or zero $^{15}$N-label, would be able to dilute out the photorespiratory glycine, giving the appearance of glycine that is less labeled than serine.**

Reviewer #3:

On overall, many of the reviewer comments have been taken into account and the manuscript has been somewhat shortened and focused, which is a definite improvement. I still have a number of comments for various sections of the manuscript:

Point 1. "Such a low correlation.." - But would genetically related lines not also possibly have similar capacities?

**RESPONSE: This point is now discussed. The sentence "The low genetic relatedness of the 19 lines could also explain why the relationship between their genetic distance and their enzyme activities was also very low, suggesting that genetically related lines have similar enzymatic capacities" has been added.**

Point 2. The use of ANOVA to assess repeatability: I assume a model is fitted which estimates genotype effect and replicate effect and the repeatability is 1-replicate effect, or something like this? Perhaps you can add more explanation in the methods part?

**RESPONSE: This method is explained in considerable detail in the Methods section, with a relevant reference. A description of the Student-Newman-Keuls test is also provided in the Methods; with relevance to Figure 5.**

Point 3. Use of Pearson correlation: Might be nice to specify here why Person correlation is applicable (i.e. normality assumptions fulfilled..)

**RESPONSE: We have indicated that following a Spapiro-Wilk test the variables used for Pearson coefficient calculation followed a normal distribution.**

Point 4. "[..] no significant correlation between GY and kernel physiological traits and thus any modules [..]" - Sentence very unclear. Modules contain leaf metabolic/enzyme traits, not kernel traits, right? So why does information about GY-kernel trait correlation allow any conclusion about correlation in modules.? What is meant by "correlation in modules"? - within each module? Between modules? Between modules and GY or other traits?

**RESPONSE: It is a mistake in the sentence. There is no correlation between GY and the Modules at V stage. The sentence has been modified. "At the V stage, there was no significant correlation between GY and any of the identified modules."**

Point 5. Also, the whole sectopn could be potentially still shortened and focused. Maybe it would be sufficient to about module elements and module correlations in exemplary form, when they lead to qualitative statements or conclusions which are discussed further.

**RESPONSE: We understand that the section describing the module/trait correlations could be shortened. However, we believe that this section is important because the correlations between individual module members and the traits are weighted correlations. Moreover, the weighted correlations take into account the module membership. Please see the following comment in relation to WGCNA.**

Point 6. Could be nice to explain why WGCNA was chosen, what is the difference or advantage compared to regular pairwise correlation analysis .

**RESPONSE: The WGCNA method uses pairwise correlations in the network construction and is a way to make clustering of the variables (genes, metabolites, etc) grouping them in modules. The weighted correlations used to study the relationships between the agronomics traits and the physiologic/enzymatic traits (module components) take into account the module membership and amplify correlations of the members of outcome-related modules.**

**See Langfelder P, Horvath S (2008) WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 2008, 9:559.**

**A sentence has been added to explain why WGCNA was chosen.**

Point 7. Discussion: Could still be significantly shortened. Don't repeat detailed data unless discussion is directly built upon it.

**RESPONSE: The discussion has been significantly shortened.**

Point 8. Some examples for photorespiration/glycolate metabolism in C4 plants. I think there are also examples from maize itself (e.g. Zelich et al, 2009, Plant physiology)

**RESPONSE: The sentence "In addition, studies with mutants of Amaranthus edulis, have also indicated that there can be an active photorespiratory glycolate pathway operating in C4 plants (Lacuesta et al. 1997; Wingler et al., 1999).**

**Has been replaced by:**

**"In addition, studies with mutants of Amaranthus edulis, (Lacuesta et al. 1997; Wingler et al., 1999) and later with maize (Zelitch et al 2009) have also indicated that there can be an active photorespiratory glycolate pathway operating in C4 plants."**

Point 9. It is not completely clear to me if proper multiple testing correction was now applied to all analyses or only Figure 7/ Suppl. Dataset 7. Please generally state for all datasets if and how they were multiple testing corrected (e.g. in the supplemental tables "Bonferroni adjusted p-values" instead of "p-values").

**RESPONSE: We have checked this and it is true that the adjusted *P*-values for correlations between modules and agronomic traits (Fig 7, Supp Fig 8, Supp Data Set 10) have not been previously calculated. Now, we have calculated the Bonferroni adjusted p-values and we have modified the text and Fig 7, Supplemental Fig 8 and Supplemental Data Set 10.**

**For the weighted correlations, the False Discovery Rate was used. See in the methods section: "The *q*-values (False Discovery Rates) were then calculated (Storey et al., 2004)."**

**TPC2016-00613-LSBR2 3rd Editorial decision – *acceptance pending*** **March 16, 2017**

We are pleased to inform you that your paper entitled "Exploiting the genetic diversity of maize (*Zea mays* L.) using a combined metabolomic, enzyme activity profiling and metabolic modelling approach for linking leaf physiology to kernel yield" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

**Final acceptance from Science Editor** **April 10, 2017**