Response to Dr Grant Jacobs's article, "Is GM corn really different to non-GM corn?"

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Dr Grant Jacobs has written a blog post (http://sciblogs.co.nz/code-for-life/2016/12/31/gm-corn-really-different-non-gm-corn/) titled, "Is GM corn really different to non-GM corn?", in which he dismisses the significance of our findings (http://www.nature.com/articles/srep37855) that GM NK603 maize is not substantially equivalent to its non-GM counterpart (closest relative). In our state-of-the-art integrative multiomics analysis, we found significant differences in both protein and metabolite profiles in the GM maize. The alteration in protein profile indicated an imbalance in energy metabolism, whereas the most pronounced difference in metabolite profile was higher levels of polyamines, including the potential toxins putrescine and cadaverine.

Dr Jacobs implies that we should have first established the typical range of each protein across the totality of maize crops, so that we could see how the GM maize under test fitted into this range of natural variation. The aim here appears to be to minimize the importance of any changes seen in the GM maize by stating that they are within the range of natural variation.

However, Dr Jacobs misses the point of the study, which was to specifically ascertain the effect of the GM transformation process on the composition of this maize variety. As a result, the only scientifically valid comparator in this investigation is the non-GM closest (isogenic) relative . Comparisons with different varieties of maize that have been grown under different conditions would only serve to increase variation in the dataset and thus mask rather than highlight the effects of the GM transformation process, thereby negating the very purpose of the study.

Scientifically invalid comparisons

Dr Jacobs criticises us for not following the common regulatory practice of comparing the GM crop not only with the non-GM isogenic counterpart but also with "the range of what humans are already exposed to in their diet, as that may indicate the need for more testing. Therefore, you will see most substantial equivalence tests address not

only the non-transformed isogenic line and/or a null transformant, but also the range of known compositions for that crop."

It is correct that regulators look at the range of normal variation across many varieties of maize and see whether the GM crop falls within that range; if it does, they often consider it "substantially equivalent" in spite of large differences in composition of the GM crop compared with the non-GM isogenic crop. However, as mentioned above, this practice is scientifically invalid because it only serves to mask differences in the GM crop, even though it is the purpose of GMO regulation to highlight such differences and investigate their importance, if any.

We were not surprised to find significant proteome and metabolome differences between the GM NK603 maize and its non-GM counterpart. What is of interest is the quality and quantity of changes that we found. Such changes are part of the innate nature of transgenic technology and stem from a combination of transgene insertional mutagenesis, tissue culture-induced genome wide mutations, and the resulting novel combinations of gene functions.

The changes observed in the metabolome and proteome profiles reflect a mixture of effects and outcomes. Some of the changes are a consequence of the GM transformation process whilst others can be due to downstream interventions such as backcrossing or outcrossing of the initial GM event. However, our interactome analysis makes us confident that the majority of the changes observed can be attributed to a metabolic effect of the heterologous EPSPS-CP4 transgene (Figure 5 in our publication). The analysis of predicted interactions of biochemicals and proteins that might have a link to the transgene-associated EPSPS-CP4 pathway reveals that some proteins or metabolites altered in the NK603 GM maize are interacting with EPSPS-CP4. We are nonetheless aware that our study does not present an absolute truth. We are building on previously published studies to make progress in the use of omics methods in the investigation of GM crops. We acknowledge the potential limitations of our study in the discussion section and suggest ways forward. For instance, we stated that *"further experiments made under different environmental conditions would be needed to determine the full range of effects of the GM transformation process on NK603 phenotype*".

Major regulatory implications

We were aware that our multiomics analysis could not address the safety of this GM food product, unless it revealed something unexpectedly dramatic that was unequivocally harmful. As we state, the main purpose of our study was to see if the claims of NK603 being substantially equivalent to its non-GM counterpart stand up to an in-depth molecular profiling, rather than gross nutritional compositional analysis. Our results clearly show that they do not. And although we cannot make any definitive inferences regarding the safety of consuming this product, our findings nevertheless have major regulatory implications, since the starting point and indeed the foundation that underpins the safety evaluation of a GM food is whether it is "substantially equivalent" to its non-GM counterpart. If this is found to be the case (based on a crude nutritional compositional analysis only), then little or no further safety evaluation is usually required.

Our multiomics results clearly call into question and indeed challenge the claim that NK603 maize is substantially equivalent to its non-GM counterpart. This in turn implies that industry and regulatory assertions of the safety of this product based on substantial equivalence are unfounded. Thus our study suggests that the health risk assessment of this product should be revisited, to possibly include more generic safety testing based on long-term animal toxicity feeding.

We believe our study significantly builds on previous work demonstrating the value of using omics analyses to evaluate the effects of the GM transformation process on a crop. This approach can clearly provide initial insight into the safety of GM foods and can potentially inform more targeted toxicity follow-up studies in lab animals.

Science Media Centre "expert" response to our study

Dr Jacobs cites quotes (http://www.sciencemediacentre.org/expert-reaction-to-multiomics-analysis-of-nk603-gm-maize/) from the Science Media Centre website in support of his dismissal of our study. My response to these quotes is below.

Dr Dan MacLean, Head of Bioinformatics at The Sainsbury Laboratory, said:

"A big issue with this analysis is that materials were collected under potentially quite different conditions. Different parts of the same farm, potentially different chemical

makeups in the soil, different water contents, different elevations, exposures and temperatures. Under tight laboratory conditions the metabolome and proteome are very variable and the statistics presented here do not go anywhere near controlling for those factors.

“There are a huge amount of things that could be affecting the expression and levels of everything in those plants and no exploratory and controlling statistics are presented. The analysis just jumps straight into ‘everything is equal, let’s do tests’ and then uses underpowered ones. Much better statistical modelling of the variables is required to allow the workers to definitively ascribe any protein/metabolome changes to any of the experimental variables supposedly under test.

“This has the effect of making the decisions about what pathways are changing moot. No clear conclusions can be reached, and certainly not on the basis of p-values. Hence all downstream analyses could not be expected to show clearly any patterns because of considerable noise in the list of things that are changing.”

My response: Dan MacLean states that different growing conditions could account for the differences found between the GM and non-GM crops. However, this suggests that he has not read the paper in sufficient detail, since we state in the Materials and Methods section that all these factors were carefully controlled for, thus minimizing the possibility of their being significant contributors to the changes found in the GM crop. A typical soil type analysis of the growing areas is shown in the supplementary online data (Additional File 1). The plots of land on which the different crops were grown were similar and consisted of an imperfectly drained field with a coarse loam surface texture and fine loam subsoil.

We also minimized the possibility that different growing seasons may be responsible for the differences. As mentioned in our article, “the fold changes observed in the comparisons of the NK603 maize sprayed with Roundup, the unsprayed NK603 maize and the isogenic control corn were highly correlated between the two cultivations performed during two different growing seasons”.

However, even though our experimental design takes into account the effect of the growing season, further experiments conducted under different environmental conditions would be needed to determine the full range of effects of the GM transformation process on this maize type.

Dan MacLean takes issue with the statistical analytical methods used. However, these methods have been used for decades to explore the significance of differences seen in biomedical research. It is widely known that errors can be made by using underpowered statistics when a study is measuring multiple variables and this is why we adjusted the p-values using the Benjamini-Hochberg multi-test method for a high number of comparisons.

The investigation we have undertaken using these established molecular profiling and statistical analytical methods solidly establishes the biological differences between the GM maize and its non-GM counterpart by looking at:

1. The biochemical pathways affected in the plants
2. The two maize cultivations, and
3. Previously published studies. The results of these analyses were highly consistent and robust.

It is unclear which “experimental variables supposedly under test” Dan MacLean is referring to, because in fact only one experimental variable was under test – the effect of the GM process on this maize type.

Our experiment has established the biological differences between the GM and non-GM maize types tested, including elevated levels of two potentially toxic polyamines (putrescine and cadaverine) in the GM maize. However, the toxicological effects on the consumer are outside the scope of the study, as stated in the paper.

Dr Joe Perry, former Chair of the European Food Safety Authority GMO Panel, said:

“In contrast with compositional analysis, which is done for every application, and reported by EFSA, and which involves proper replicated field trials, this study appears to have been done with single, unreplicated plots.

“Therefore it is not possible to say with any certainty whether the differences reported are due to differences between the treatments or differences between the two fields (or two plots within the fields) used.

“In other words the basic tenets of experimental design seem not to have been followed. For that reason I could not yet describe this as a thorough piece of science.

“Further details about the conduct of the experiment would be useful to confirm or otherwise this initial impression.”

My response: Joe Perry is incorrect in claiming that our study was done with single, unreplicated plots. In reality there were two cultivations of maize over two growing seasons, and the results were consistent over both, as presented.

The compositional analyses of GM crops performed by the GMO producer companies and submitted to regulators in support of market authorisation are extremely superficial, looking at major elements such as total proteins, carbohydrates and fats. They do not examine the types of proteins or metabolites that are present, yet these factors can determine whether a GM crop is substantially equivalent to the non-GM crop and safe to eat.

As we explained in response to Dan MacLean, differences between the fields in which the maize varieties were grown were controlled for and thus were not a factor in explaining the differences in the GM maize.

Prof Johnjoe McFadden, Professor of Molecular Genetics at the University of Surrey, said:

“The science is good as far as it goes. But the analysis only emphasises the inadequacy of the ‘substantial equivalence principle’. How equivalent does it need to be? If you perform this detailed level of analysis on any perturbation of any organism you will detect this level of change – organisms are extraordinary sensitive and, for example, similar changes are produced when treated with e.g. pesticide or herbicides or when attacked by pests.

“I would expect that practically any perturbation to an organism will generate a response that can be detected by these powerful techniques – that is after all what life does.

“So all it shows is that GM, like pesticides, herbicides, drought, predation or even growing in a different field will produce a response by the organism. If GM was banned on these grounds then so would all herbicide pesticides and indeed anything that causes a change (which is everything).”

My response: Johnjoe McFadden seems to imply that our analysis is too detailed, but the methods employed are both cutting-edge and widely used in both research and diagnosis. If he is implying that differences in pesticide or herbicide use could be responsible for the changes in the GM maize, he is incorrect, since these factors were taken into account. Our analysis did indeed reveal an effect resulting from the application of Roundup herbicide on the GM Roundup-tolerant maize, but this factor did not make such a large difference as the GM process itself (see Figure 2 in our paper).

In addition, we conducted a detailed analysis of pesticide residues in the GM and non-GM maize (Additional File 2) and found there were none above levels of detection. There were no differences in pesticide or fertilizer use between the GM and non-GM maize, except that Roundup herbicide was applied to one cultivation of the GM maize, in accordance with how this type of maize is designed to be grown. Therefore the differences observed in the GM maize cannot be attributed to differences in pesticide use or presence.

However, even if the toxicological relevance of the differences remains unclear, what is clear is that the use of these molecular profiling tools allows a better understanding of the composition of GM plants and thus could improve the risk assessment of the non-target effects of genetic modification. In fact, genetic engineers cannot control or predict the effects of genetic engineering on plants and currently these are not measured at the molecular level.

Infected corn?

Dr Jacobs quotes “Mem_somerville” (Mary Mangan) as hypothesizing that the differences seen in the GM corn were likely to be due to fungal contamination. She wrote in a comments thread on the journal website:

“1. What is your explanation for the fact that top fold-change proteins in your data set are fungal proteins (and it’s a known maize pathogen)?

“2. Are you aware that fungal contamination could result in similar changes in regards to the pathway changes that you describe? Did you consider this at all? Why didn’t you address this in your paper?

Out of over 150 proteins that were significantly altered in the GM corn, a few were derived from fungal pathogens. This included two that matched a protein in Gibberella moniliformis, a corn pathogen.

However, our pathway analysis (Tables 1 and 2 in our paper) showed that these two proteins were not picked by the analysis and thus they were not over-represented in our dataset. Thus fungal contamination implied by the presence of these two proteins is highly unlikely to have contributed to the metabolic alterations found in the GM corn. In addition, it has also been suggested that the fungal infection could have caused the major metabolic alterations seen between GM and non-GM samples. This is incorrect for two reasons:

1. No other proteins related to pathogenic response have been observed in the entire dataset as expected, based on observations in other proteomic investigations looking at changes in plants in response to pathogen infection (Pechanova and Pechan, 2015).

2. In the case of a pathogen infection in control samples, these samples would be expected to show enzymes and metabolites related to an increase in oxidative stress or feedback enzymes to fight high oxidative cell environments. This is because the recognition of any invading pathogen results in a coordinated activation of plant defence mechanisms, including a strong and rapid oxidative response. However, we observed the opposite – our results suggest that the GM samples, not the control samples, had increased oxidative stress (Campo et al, 2004; Pechanova and Pechan, 2015).

Contrary to Mary Mangan's suggestion, we did consider the two pathogen proteins in our dataset. In fact, we raised concerns about any other mycotoxin contamination even before we performed the proteomics analysis. We performed a mycotoxin analysis but did not include this data in the manuscript because it was outside the scope of the work presented. This analysis revealed the presence of the fumonisin group of mycotoxins, but at only trace amounts (less than 200 µg/kg of fumonisin B1 and B2) in the corn samples. Levels of these fumonisins did not differ between varieties. The fumonisin class of mycotoxin is produced by G. moniliformis, also known as Fusarium verticillioides or F. moniliforme. Our analysis also revealed the presence of several other types of mycotoxins again all at trace amounts and within regulatory permitted limits. Therefore as only very low levels of the mycotoxins including fumonisins were found to be present this in turn implies very low levels of contamination with fungal pathogens.

There is indeed evidence that the presence of G. moniliformis/Fusarium verticillioides can change polyamine metabolism in plants. However, such changes occur at levels of

contamination, as measured by fumonisin levels, of at least 1,000 times higher than the levels we detected (Campos-Bermudez et al, 2013). Moreover, these studies show that the higher the levels of pathogen infection, the higher the levels of polyamine compounds. However, again, we observed the opposite. Although only semi-quantitative our proteomics analysis indicated higher levels of G. moniliformis/Fusarium verticillioides in the control (non-GM) corn than in GM corn and yet the non-GM corn had lower levels of polyamines. Therefore the elevated levels of polyamines in the GM corn samples were not caused by the presence of the fungal pathogen.

In a more recent comment posted on the *Scientific Reports* website, Mary Mangan argues that the detection of opposite changes in the levels of the same protein (B4G0K5_MAIZE) invalidates our results. However, this indicates a lack of familiarity with the basic principles of proteomics. In fact, opposite changes can be explained by different post-translational modifications on the same protein. For example, in the case of the activation of a signalling pathway, a phosphorylated protein will have its level increased whereas the unphosphorylated form will have its level decreased.

Thus Mary Mangan's theory is incorrect.

Summary and Conclusions

The presence of the two G. moniliformis-/Fusarium verticillioides-associated proteins, referred to by Mary Mangan and found in our dataset of more than 150 proteins whose levels were significantly altered in the GM compared to the non-GM isogenic corn samples, suggests fungal infestation. However, our mycotoxin results clearly show that the level of fungal contamination was extremely low and thus did not contribute to the observed metabolic changes. Hence we are confident that our molecular profiling results obtained by application of state-of-the-art proteomics and metabolomics analyses of NK603 GM corn and a non-GM isogenic counterpart unequivocally reveal that they are not substantially equivalent.

References

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