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Letter to the Editors

HOW SPECIFIC IS CRISPR-CAS9?

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Dear editors

In contrast to conventional breeding methods, genetic engineering is often considered very precise because it involves adding or removing specific genes. In food plants the main uses so far have been in introducing genes for production of herbicide resistance (Glyphosphate, Roundup™) and *Bacillus thuringiensis* (Bt) protein for pest resistance. In 2020 Emmanuelle Charpentier and Jennifer Doudna were awarded the Nobel Prize in Chemistry for their discovery called Cripsr-Cas9, a method of making specific and precise changes to DNA. In common with conventional breeding methods, genetic engineering used to change food plant characteristics will change gene products and resource allocation to many biosynthetic pathways; new unpredicted products may also be obtained. The complexity of plant (and microbial) natural product pathways makes it inevitable that any genetic changes may cause unexpected alterations in secondary products.

We all know about the diversity of chemicals produced by plants and micro-organisms and their importance to health, both positive effects and potentially harmful. What isn't known for many of these chemicals is how they are produced and what genes are involved in their production. There are complex interactions between gene products leading to modifications of compounds and so one gene group does not necessarily lead to one product. Molecular biologists have now mapped the genes of many organisms but this genome analysis usually does not adequately explain the phenotype, e.g. humans and chimpanzees share over 95% of their genes and it is the gene products and their interactions that explain the differences in the species more than the genes themselves (Suntsova and Buzdin, 2020). In plants there are also interactions with micro-organisms (endophytes) to consider as they may cause post-translational changes and in humans the microbiome is also seen as important for modifications of chemicals.

Many of the plant foods we eat were probably selected on the combined observations of good effects on health and survival as much as taste. Those species that could be grown and stored most easily have become the staple foods such as rice, wheat, barley and potatoes. Plant selection and conventional breeding methods have allowed 'improvements' in many food plants resulting in better yields, early cropping, pest resistance or drought tolerance etc. The result now is that there are hundreds of varieties of many food plant species with different properties, *e.g.,* 40,000 varieties of rice. Some chemical components will have been selected such as oil, protein content, colour and flavour. Over recent decades shelf-life, appearance and stability in transport for some fruits and vegetables have become important characteristics and often more so than taste. While common nutritional analyses measure protein, fats, carbohydrates, vitamins, for example, there are almost always unknown, unseen and unmeasured chemical components changing in the selection process. Standard analyses of plant composition are likely to miss chemicals which are not known to occur in those plants or which have chemical characteristics very similar to common major components such as sugars or amino acids. These unmeasured chemical components will in some cases make certain varieties 'healthier' than others and this variation needs to be considered when selecting a healthy diet. I will use some examples of this chemical diversity in varieties of food plant that we have studied.

There are around 4,000 varieties of potato (*Solanum tuberosum*). While starch, carotenoids, anthocyanins, amino acids and toxic glycoalkaloids were well studied components it was surprising to find in 1993 that they all also contain nor-tropane calystegine alkaloids and each variety has a distinctive ratio of them. Previously it had been considered that Solanum unlike most Solanaceae could not produce the often highly toxic tropane alkaloids (such as atropine and hyoscyamine) because they did not have the genes necessary. What made it even more surprising that they had been missed by other workers is that they had a strong biological activity with potent glucosidase and galactosidase inhibitory activity. These glycosidase inhibitions are probably intended to reduce the digestibility of the starch by herbivores and no doubt also make assessments of the carbohydrate content of potatoes misleading when it comes to nutritional value. Calystegines have been reported in many food plants other than potatoes including sweet and chili peppers, eggplants, tomatoes, Physalis fruits, sweet potatoes, and the completely unrelated mulberries. More recently these calystegines are also reported to have additional biological activities involving reducing inflammation and oxidative stress (Kowalczuk et al., 2022). Remarkably the calystegines are stable in the body on consumption and are eventually excreted in urine (Beckman et al., 2020). While in the body calystegines are potent competitive inhibitors of bovine, human, and rat beta-glucosidase and alpha-galactosidase activities and human beta-xylosidase (Asano et al., 1997). A number of reports propose that intermediates in normal tropane alkaloid production such as tropinone and pseudotropine are metabolites in the biosynthetic pathway of calystegines in bindweeds (Scholl et al., 2003) but this doesn't explain why potatoes and mulberry would always remove the N-methyl to accumulate only the nor-tropanes. It seems probable we do not know the full distribution of tropane and nor-tropane alkaloids because there are many possible structures and calystegines, for example, do not behave as the better-known tropanes in being more water soluble and not reacting with common alkaloid reagents such as Dragendorff's and Iodoplatinate reagents. The same is true for pyrrolizidine alkaloids, often considered to be liver poisons, which are far more widely distributed than previously thought and probably have more than one biosynthetic route. Pyrrolizidine alkaloids with a carbon 3 substituent (e.g. the hyacinthacines and casuarines (Nash et al., 2011) are often present in high concentrations in plant genera and families not considered producers of pyrrolizidines as they are not reactive in the same way as the classic pyrrolizidines.

The calystegines are a good example of why we should look more closely at food plant chemistry to understand what the health implications of producing new varieties might be. The chances are that potatoes and other calystegine-producing foods have other surprises in their biologically active components. Scientists must be encouraged to delve deeper into food chemistry and the health impacts and not just doing simplistic superficial nutritional analyses. In 1974 Renwick et al. suggested that blight-infected or stored potatoes might have unknown chemicals related to spina bifida and their claims were dismissed but clearly they were correct about then unknown biologically-active components that we still do not fully understand. Given what we know now about the biological activities of calystegines of potatoes and the wide range of other secondary chemicals they contain, the claim that genetically modified potatoes had adverse effects on rats by Árpád Pusztai also seems more credible (Krimsky et al., 2015). Pusztai claimed that genetically modified potatoes with *Galanthus nivalis* protein introduced gave stunted growth and repressed the immune system of rats while thickening their gut mucosa. Pusztai and co-workers showed the introduced protein itself was not responsible for the toxicity and proposed that perhaps by introducing a gene you will activate or silence other genes in the plant as well (Ewen and Puztai, 1999). There are similar arguments around Glyphosphate resistance and Bt protein where the herbicide or introduced gene products do not appear to be toxic themselves but what else is changed? Stegelmeier et al. (2008) reported that mice given calystegine A_3 developed mild hepatic changes with increased pit cells (specialised NK cells). Calystegines in a recent study showed potential health benefits possessing the ability to protect Human antibody secreting cells against hyperglycaemia-induced cellular dysfunction, and it evidenced that the observed effects are associated to the promotion of PI3K/AKT/mTOR pathway (Kowalczuk et al., 2022).

Calystegines are present in several common foods and considerable amounts could be eaten daily in potato, pepper, sweet potato and aubergine (egg plant) but the ones present change and so will their activities as each one is different in biological activity. Other common foods also contain similar compounds that are not monitored and could be affecting health. Rice for example can contain piperidine alkaloids such as fagomine (first known in Buckwheat) but they are not in all rice varieties and the biosynthetic pathway in rice is unknown (Nash et al., 2014). Certain cucumber varieties can also contain an iduronic acid analogue (idoBR1) that is anti-inflammatory, and the biosynthetic pathway and genes involved are also not known (Oleide et al., 2022 and references therein). Fagomine and idoBR1 are probably beneficial to health but unknowingly modifying the genes of these plants might lead either to less of the beneficial compounds or more of biosynthetically related compounds that might be harmful. Currently these highly active molecules are not monitored as they are not easy to detect amongst high concentrations of similar primary metabolites and remain relatively unstudied. Fagomine is a weak glucosidase and galactosidase inhibitor and but also seems to have a beneficial effect on the gut microflora and counteracts sucrose-induced steatosis and hypertension (Ramos-Romero et al., 2020). Fagomine attenuated high-fat-induced visceral fat, incipient insulin resistance, and liver inflammation in rats (Hereu et al., 2019). Although fagomine clearly can have beneficial effects, as it is a glycosidase inhibitor, if a variety high in fagomine is given in a famine situation it could perhaps be less beneficial than a variety without it. Some rice varieties show a much wider range of glycosidase inhibitions suggesting a wider range of related compounds that could not be fully identified (nash et al., 2014). The Cucurbitaceae also contributes many other important food plants (e.g. squash, marrow, gourd, pumpkin) and so the relatively new discovery of a highly active iminosugar amino acid (idoBR1) in certain cucumbers opens up the possibility of there being other unknown active secondary compounds in the family and our foods.

In conclusion, we still have a lot to learn about plant secondary chemistry and this is highlighted by the new active compounds being found in common food plants. Such compounds may be beneficial to health or be detrimental perhaps in some cases. Plant breeding has already resulted in distinct chemotypes of crop plants. This ought to be considered more in food choices because which variety is eaten will change what effects the plant has on health. We have probably also taken some herbal medicine preparations well away from the chemistry related to the traditional uses by selection of varieties. New genetic manipulation methods such as Crispr may appear to enable fast and easy specific gene editing but what effects that might have on plant secondary chemistry may not be so specific or predictable and it will rely on natural product chemists to study what changes and biochemists/pharmacologists to determine benefits or adverse effects to health. Such important follow-up work will take time and resources.

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