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Plant synthetic biology as a tool to help eliminate hidden hunger

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Agricultural systems are under increasing pressure from declining environmental conditions, a growing population, and changes in consumer preferences, resulting in widespread malnutrition-related illnesses. Improving plant nutritional content through biotechnology techniques such as synthetic biology is a promising strategy to help combat hidden hunger caused by the lack of affordable and healthy foods in human diets. Production of compounds usually found in animal-rich diets, such as vitamin D or omega-3 fatty acids, has been recently demonstrated *in planta*. Here, we review recent biotechnological approaches to biofortifying plants with vitamins, minerals, and other metabolites, and summarise synthetic biology advances that offer the opportunity to build on these early biofortification efforts.

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Introduction

Hidden hunger, or micronutrient deficiency, can be defined as a state in which a person’s diet is calorically fulfilled, but nutritionally deficient. This definition covers a range of conditions, from minor deficiencies with few symptoms to extreme deficiencies resulting in mortality. Hidden hunger is a global phenomenon, with the latest global analysis estimating that half of pre-school-aged children are affected [1]. Without innovations in crop yield, nutritional quality, and climate resilience, we may fail to meet the nutritional needs of the future population [2]. A recent factor contributing to this issue is the rise in popularity of plant-based diets, which typically do not provide some essential nutrients such as vitamin B_{12}, omega-3 fatty acids (ω3 FA), or vitamin D, and contain lower concentrations of iron and protein [3].

The major strategies currently employed to combat micronutrient deficiency include supplementation, fortification, and increasing dietary diversity, though these solutions are not universally implementable due to, for example, a lack of resources or the capacity to implement them [4]. Another solution in the toolbox is biofortification, the practice of increasing micronutrient content in plants before they are processed into food, chiefly by soil supplementation or through the use of gene technology (e.g. selective breeding, genetic modification, or gene editing [GE]; Table 1). This solution is not without flaws, though it may help to address the issue of malnutrition in situations where the other solutions fail, for example, in remote or developing regions without regular access to supplements, fortified foods, or the climate to grow a varied diet. In these cases, a genetically modified (GM) crop that targets one or two of the most prevalent micronutrient deficiencies could be distributed to eliminate potentially devastating states of malnutrition.

Adoption of GM crops has not been easy, however, as exemplified by the struggles described in this opinion piece [5]. Some of these struggles may be overcome through the development of genetic tools such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9-mediated gene GE, which are considered more akin to traditional selective breeding rather than GM in many countries. Indeed, greater advances and availability of synthetic biology (SB) tools over the past decade, especially in GE and plant transformation technology, have made it far easier to create engineered plants. As a result, there has been a dramatic increase in literature describing the creation of GM plants that aim to tackle malnutrition-related illnesses. Regulation seems to be catching up with technology; however, plans for the European Parliament to

Table 1

A glossary of terms used in this review to describe different types of genetic technologies and practices used to achieve biofortification.

Term	Definition
Genetic modification/genetically modified (GM)	A broad term that describes the modification of an organism's genome (distinct from natural processes such as sexual recombination and selective breeding) through the use of genetic technologies such as the insertion of cisgenic or transgenic DNA into a genome by transformation or small insertions, deletions, or base edits.
Synthetic biology (SB)	The creation and use of cisgenic, transgenic, or synthetic standardised biological parts to modify biological systems to our advantage, especially in the context of utilising the engineering principles of the design-build-test-learn cycle.
Metabolic engineering (ME)	A method of genetic modification that involves the overexpression, knock-down, or knockout of one or many genes in a metabolic pathway to modify the outputs of that pathway in a predictable manner.
Gene editing (GE)	Precise modification of an organism's genome, using biological machinery such as site-directed nucleases with or without the use of a guide to repair the DNA cleavage site in a specific manner.

vote on the deregulation of some genetic technologies used in crops later this year [6] may be delayed due to elections and important amendments and debates surrounding patent usage.

In this review, we describe a selection of recent GM approaches to the biofortification of vitamins, minerals, and other metabolites in plants, in addition to summarising key developments in plant SB techniques that allow for more advanced approaches to plant biofortification.

Vitamins

At the turn of the century, 'Golden Rice' was developed with the goal of preventing blindness and mortality associated with vitamin A deficiency, particularly in countries where staple foods did not provide adequate amounts, and supplementation by nutraceuticals was rare. The initial iteration of the crop was created in 2001 and was one of the first examples of a GM plant that aimed to provide major nutritional benefits. It was achieved simply by introducing two genes that were missing from the ß-carotene biosynthesis pathway in rice [7]. The overproduction of vitamins in plants has now been widely explored using a variety of approaches and has helped develop SB principles and technologies to open a world of possibilities.

Genetic approaches to increase the content of vitamins or other valuable products in plants fall under four major categories: 'push' or 'pull' strategies, which aim to increase the amount of upstream precursors and the target molecule; 'block' strategies, which prevent the formation of downstream products; strategies that increase the bioaccessibility or stability of the target molecule; and finally, strategies that increase the metabolic sink of the product [8]. The latter strategies supplement the first two strategies, which often run into inhibitory roadblocks that decrease the capacity fro product accumulation and utilisation; however, they often require a much deeper knowledge of the metabolic pathways involved.

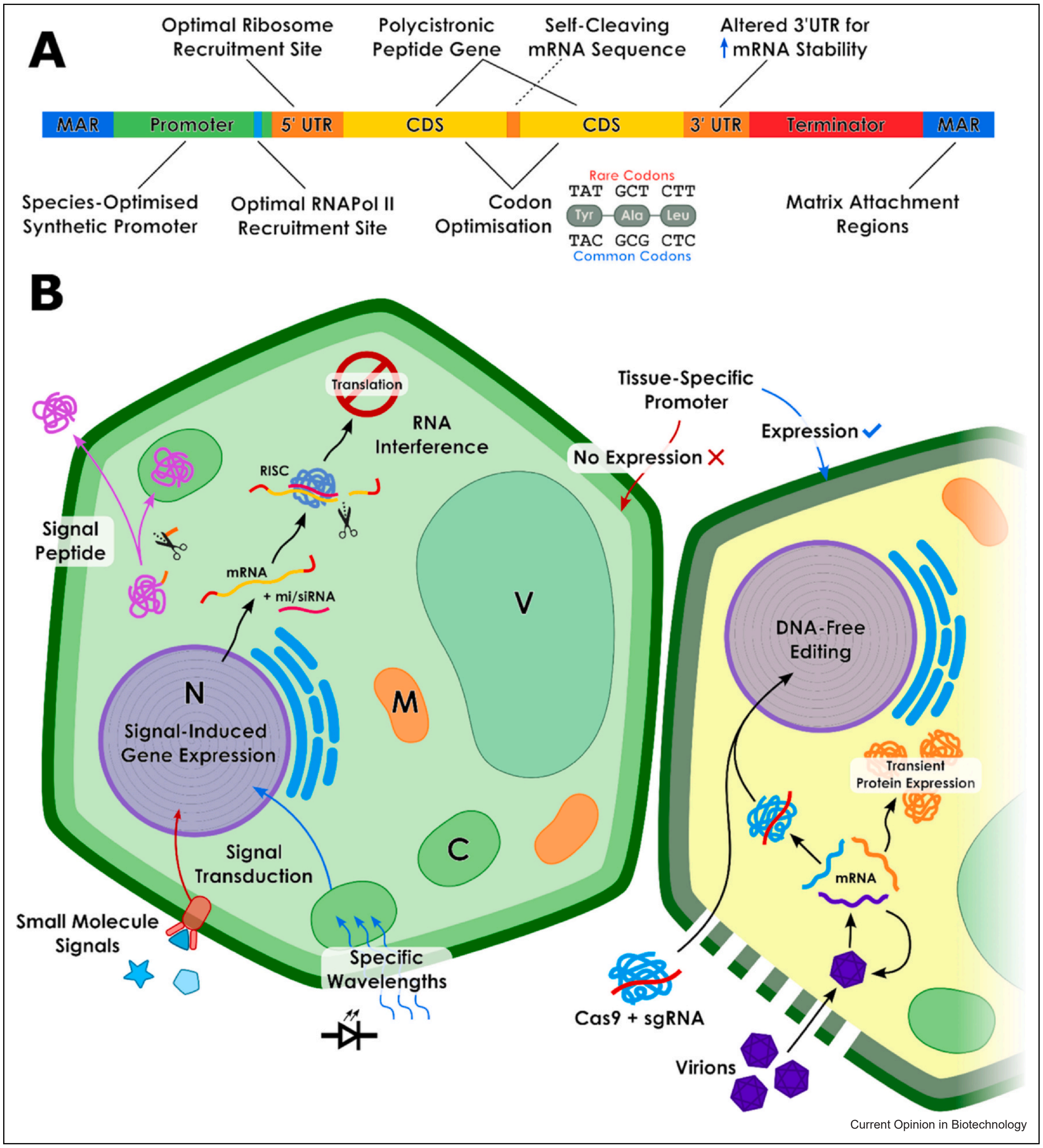
These generalised strategies have been supplemented by a wide variety of technologies that exploit biology in creative ways, as can be seen in Figure 1. Modifications to nontranscribed regions of the DNA, such as promoters that are more efficient, signal-activated, or tissue-specific, have been characterised, as well as modifications within the transcribed regions that result in improved translation rates. Base-editing or mutation-inducing systems have allowed for the efficient modification or knockout of native genes, with some popularised methods like the CRISPR-Cas9 system being considered for non-GM regulation status by governments. Tools that avoid the introduction of DNA to the germline for temporary or permanent changes, such as the direct delivery of editing complexes, grafting with transgenic donors, or viral-induced expression, are now being developed to overcome the original concerns that regulation was imposed for. These technologies are explored in greater detail in the following reviews [9–13].

Vitamin C

Whilst vitamin C is not scarce in diets due to its ubiquity in plants and animals, deficiency is still prevalent worldwide [14]. Many genetic approaches for the overproduction of vitamin C have been trialled, with the major approach being ME by ubiquitous overexpression of vitamin C biosynthesis genes. The most targeted genes for this approach have been *GDP-D-MANNOSE PYROPHOSPHORYLASE* (*GMP*), *GDP-D-MANNOSE-3,5-EPIMERASE* (*GME*), *GDP-L-GALACTOSE PHOSPHORYLASE* (*GGP*), and *L-GALACTONO-1,4-LACTONE DEHYDROGENASE* (*GLDH*) in the L-Galactose pathway [15,16]. Results from this approach can be found in Figure 2, showing significant variation, from sevenfold increases in ascorbate concentration to none at all, indicating that our understanding of vitamin C biosynthesis regulation in plants may be lacking [16].

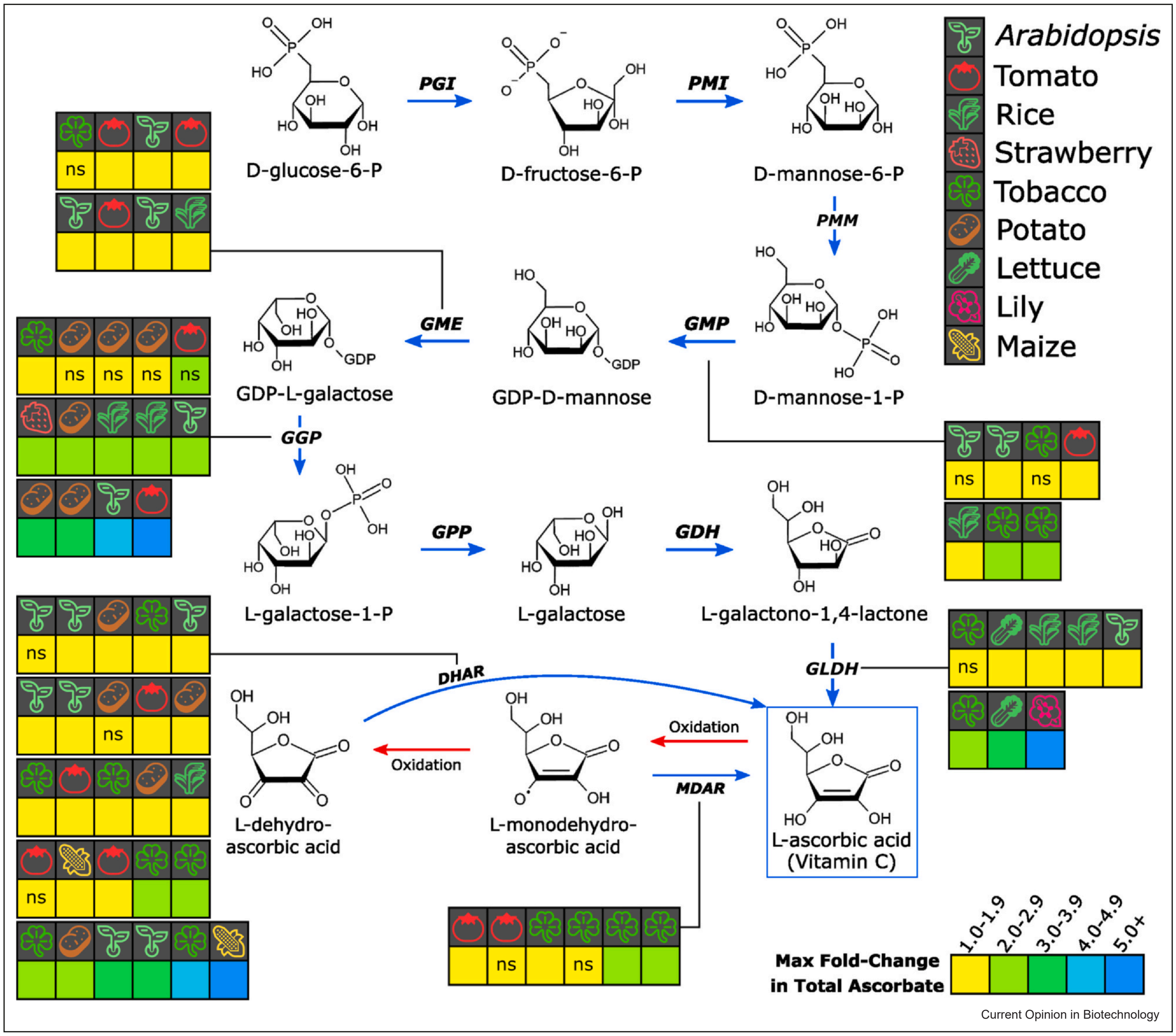
In a recent gene-pyramiding study, combinations of four key pathway genes, *L-GALACTOSE-1-P PHOSPHATASE* (*GPP*), *GME*, *GMP*, and *GGP*, were expressed constitutively, with results ranging from some constructs

Figure 1



An oviewview of some approaches available to produce valuable products in plants **(a)** A schematic diagram showcasing some of the features of a gene that can be modified for optimal expression in plants. Promoter and 5'UTR sequences that are optimised for the target species can increase transcription by enhancing the recruitment of the required proteins for these processes. Polycistronic peptide genes allow for a single promoter to drive the expression of multiple genes, reducing construct size. Codon optimisation increases the translation rate of proteins by removing 'rare' codons that delay or even abort the translation process. The 3'UTR can be modified to include motifs that recruit proteins to increase or decrease the stability of the mRNA. Matrix attachment regions provide an anchor point to the nuclear matrix at each end of the construct, which helps to minimise chromatin silencing. **(b)** A diagram showcasing a selection of technologies that can be used to regulate gene expression and product accumulation. The use of signal peptides can direct proteins to designated cellular compartments, where, for example, the substrate they interact with exists. RNA interference can decrease or modulate the level of mRNA translation. Small molecules, specific light wavelengths, and other signals can be used to drive synthetic promoters to control gene expression on demand. Tissue-specific promoters found in nature allow for spatial, and also often temporal, control of gene expression. Transient changes in gene expression can be induced by infection of plants by viruses that have had their harmful properties removed. DNA-free editing techniques, in which the editing effectors are not expressed by the target plant, can be achieved by the uptake of site-directed nucleases and guide RNAs. This is not an exhaustive list of genetic technologies used in plant biofortification, and it is important to note that due to the complexity of biological systems, the application of such technologies can result in unexpected outcomes. Abbreviations: C, Chloroplast; CDS, Coding sequence; M, Mitochondria; MAR, Matrix attachment regions; N, Nucleus; RISC, RNA-induced silencing complex; UTR, Untranslated region; V, Vacuole.

Figure 2



Summary of single-gene overexpression lines in the L-Galactose pathway of vitamin C synthesis as reviewed in reference [16], illustrating how the choice of plant species or step in the pathway can shape the outcome. Each icon describes a single study, and the legend indicates which plant species was transformed in that study, with the success measured by maximum fold change in total ascorbate below it. ns = no statistically significant difference compared to wild-type control. Abbreviations: PGI: *PHOSPHOGLUCOSE ISOMERASE*, PMI: *PHOSPHOMANNOSE ISOMERASE*, PMM: *PHOSPHOMANNOSE MUTASE*, GMP: *GDP-D-MANNOSE PYROPHOSPHORYLASE*, GME: *GDP-D-MANNOSE-3,5-EPIMERASE*, GGP1/GGP2: *GDP-L-GALACTOSE PHOSPHORYLASE 1/2*, GPP: *L-GALACTOSE-1-P PHOSPHATASE*, *GDH: L-GALACTOSE DEHYDROGENASE*, GLDH: *L-GALACTONO-1,4-LACTONE DEHYDROGENASE*, MDAR: *MONODEHYDROASCORBIC ACID REDUCTASE*, DHAR: *DEHYDROASCORBIC ACID REDUCTASE*.

showing no improvements in total ascorbate in fruits to the doubling of total ascorbate in leaves [17]. The study found that there was a limit to the amount of vitamin C able to be accumulated in each tissue type, and this limit was reached when overexpressing just two of the four genes, indicating that feedback inhibition loops or other regulatory methods were creating a 'ceiling' for vitamin C accumulation [17]. Moving forward, the transcriptome and metabolome of cells must be better understood to be able to bypass these regulatory hurdles.

It is important to note that whilst these strategies may be successful in modifying the nutritional content of the plant, they may not contribute to realised nutritional benefits in the consumer. These can be defined as measurable advantages directly derived from a food's nutritional content, such as the reversal of symptoms caused by deficiency or the improvement of traits such as bone density or immune system strength. Benefits like these must be ascertained through dietary studies in animals and humans [18], an expensive and time-consuming process, although it has been reported for a small but growing number of GM crop examples [19–21].

Vitamin D

Vitamin D_3 is an important prehormone for the musculoskeletal and immune systems and is produced as a byproduct of the cholesterol synthesis pathway in skin cells exposed to the UV-B fraction of sunlight. Because of this reliance on sunlight, its biosynthesis is affected by uncontrollable factors such as a person's skin tone or the latitude at which they live. Vitamin D is also scarce in diets, typically found in animal products, such as fish, egg yolk, and red meat [22], which makes it an ideal target for biofortification in plants.

Recently, vitamin D_3 was successfully produced in tomato (*Solanum lycopersicum*) using a simple genetic knockout. The method by Li et al. [23] aimed to accumulate the precursor to both vitamin D_3 and cholesterol, 7-dehydrocholesterol (7-DHC), and then expose the plant to UV-B light to convert it into vitamin D_3. This was achieved by knocking out *7-DEHYDROCHOLESTEROL REDUCTASE 2*, the gene responsible for converting 7-DHC into cholesterol. After exposing the gene-edited line to UV-B light, ~200 and ~0.25 $\mu g\, g^{-1}$ dry weight of vitamin D_3 was produced in the leaves and fruit, respectively [23]. Although the amounts produced in fruit are relatively low compared to daily sufficiency requirements of 20 μg [24], this is a positive example of how GE crops can potentially assist with nutritional deficiencies of those with restricted diets. Future prospects in this area could include modification of the cholesterol pathway to further increase the amount of 7-DHC available for vitamin D_3 conversion. This could include the upregulation of cholesterol synthesis genes or knockout of branching pathways, both of which have been shown to increase cholesterol concentration in plants [25,26].

Minerals

Minerals are essential in human health and must be sourced from diet. Mineral malnutrition is a global issue, with the most prevalent deficiency, iron, affecting nearly one-third of women globally [27]. This problem is exacerbated by reductions in world soil quality and how that affects plant yield and nutritional quality [28]. Concurrently, plant uptake of toxic minerals in soil is also a major issue, though, with GM solutions being explored [29,30]. To help address mineral nutrition, future crops will need to be increasingly efficient at providing beneficial minerals through improvements in both concentration and bioaccessibility while avoiding toxicity concerns.

Traditionally, mineral fortification has been achieved through soil supplementation with mineral-rich fertilisers or by 'spiking' downstream food products with minerals [31]. Both strategies, however, require additional investment by food producers at the farm or factory. Whilst this is achievable, and perhaps favourable as companies are able to advertise a healthier product with a better value proposition, this kind of investment is unlikely to occur in developing regions. A biotechnological approach may be more attractive, whereby recently developed GM plants with improved bioaccessibility and concentration of a range of minerals are produced instead.

Iron and zinc

A range of strategies to increase the iron and zinc concentration in cereal crops have been tested, with many focusing on modifying the expression of mineral uptake, storage, and trafficking proteins. A recent strategy in bread wheat (*Triticum aestivum*) examined the effect of overexpressing two key genes. One, *NICOTIANAMINE SYNTHASE 2* (*NAS2*), encodes a metal-chelating peptide capable of binding iron and zinc, and this was coupled with endosperm-specific expression of *VACUOLAR IRON TRANSPORTER 2* (*VIT2*), which encodes a peptide responsible for iron transport into the more commonly consumed starchy endosperm fraction [32]. This resulted in a twofold increase in grain zinc concentration, and similar increases in flour concentrations of iron and zinc [32]. Whilst these results are promising, future attempts could stack these approaches with strategies that address the issues presented by plant antinutritive chelating compounds that effectively 'lock' minerals in a conformation that is inaccessible to our digestive system.

Antinutritive chelators

The major antinutritive chelating compounds present in plants are oxalate, which binds calcium and can contribute to kidney stone formation; phytate, which binds a range of divalent metal ions excluding them from diet; and glucosinolate, which binds iodine altering thyroid function [33]. Efforts to negate these effects in foods have focussed on preparation techniques such as soaking, boiling, germinating, and fermenting [34]; however, some of these techniques have their own drawbacks, such as the degradation or leaching of vitamin content [35], leaving consumers to 'pick their poison' when preparing food.

Attempts at reducing antinutrient chelator content by GM strategies have been made, but a lack of knowledge about the genetic pathways and regulation involved in their production has hindered their utilisation. As a result of this deficit, most genetic technology strategies in crops have focussed on the overexpression of enzymes that degrade these chelators rather than preventing their biosynthesis [36,37]. A simple example of this strategy was the overexpression of a transgenic oxalate decarboxylase in soybean (*Glycine max*), which resulted in the halving of oxalate concentrations in most transformants and twofold (or more) increases in calcium, magnesium, iron, zinc, and manganese in the next generation seeds [38].

Future attempts to help alleviate mineral-related malnutrition through SB should combine the discussed strategies of mineral concentration, localisation, and bioaccessibility. Other strategies to explore could include the modification of regulatory elements that affect mineral uptake, transit, and storage, though this will require a deeper understanding of the science.

Macronutrients

The importance of micronutrients to human health has been highlighted above, but plants can also be an outstanding source of proteins, carbohydrates, and lipids. Because these three components cover a huge spectrum of independent biosynthetic pathways, progress in the modification of macronutrient profiles is scattered. Most studies focus on one macronutrient at a time with single gene overexpression or knockdown lines, and we have not been able to identify any comprehensive literature reviews that link their modification together. The scope of this paper does not allow for such a review; however, we will provide a brief overview of the main research foci, including how the nutritional quality of plant-based proteins and carbohydrates is being improved and how animal-based lipids are being introduced into plants.

Proteins

In the face of modern environmental challenges, plant-based alternatives to animal proteins are garnering interest, and a great deal of research is being placed into improving their nutritional and sensory properties [39]. Crops such as legumes are a major source of plant-based protein in the market, but one drawback to their adoption is that they typically fail to provide sufficient levels of sulphur-containing amino acids (SAA) methionine and cysteine [40]. Improving sulphate capture and enhancing SAA assimilation in proteins by genetic technology has been the primary approach to solve this issue [40]. For example, the overexpression of a modified ATP sulfurylase 1 in soybean (*Glycine max*) effectively captured free sulphate in the cytoplasm and, as a result, increased the methionine and cysteine content of the cells by 15–19% and 37–52%, respectively [41]. These kinds of improvements in quality will be essential if the adoption of plant-based proteins continues to grow as expected.

Carbohydrates

The structure of carbohydrates is important to their function in plants and nutrition in humans. Carbohydrates like starch are digested promptly to provide energy, while dietary fibres or resistant starches (RS) are difficult to degrade due to their linear crystalline structures and are functionally important to human gut health [42]. Biofortification of these carbohydrates in staple crops like wheat has been used as a strategy to maximise the health benefits of derivative foods. The main approaches for RS accumulation have been to employ block strategies to suppress the branching of carbohydrates or push/pull strategies to increase the proportion of linear starch [43]. Recently, one study utilised the branching suppression strategy in cassava (*Manihot esculenta*) by RNAi knockdown of two branching enzymes, *STARCH BRANCHING ENZYME 1 & 2* (*SBE1/SBE2*) [44]. They showed that some double knockdown lines had both increased their apparent amylose content by over 100% and increased their RS content from ∼0.4% in wild type to 18.3–25.2% in those lines.

Another type of carbohydrate product that has been associated with several health benefits, such as lower cholesterol, cardiovascular, and diabetic risk, is mixed linkage (1,3;1,4)-β-glucan (MLG) [45]. Classed as a dietary fibre, it is a polysaccharide formed from D-glucose subunits and is present in the cell walls of cereals such as barley (*Hordeum vulgare)* and oat (*Avena sativa*). Biofortification of MLG has been achieved mainly by overexpression of cellulose synthase-like (CSL) genes. The endosperm-specific overexpression of *HvCSLF6* in barley, for example, more than doubled MLG content paired with decreased starch content [46]. The optimisation and application of these approaches to carbohydrate-rich diet staples could assist in cases of energy-rich nutrient-poor diets.

Lipids

Genetic modification of lipids in plants is not a recent endeavour; however, efforts have chiefly focused on increasing the amount of lipid-biofuels rather than nutritionally beneficial lipids [47]. Much of the work has been concentrated in oilseed crops, though there are successful examples of lipid modification outside of this group [48]. Nutritionally beneficial lipids such as ω3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are vital for health and development; however, deficiency is common, fish sources are constrained by overfishing, and plant-based diets lack EPA and DHA, providing an opportunity for plant SB to contribute [49]. Significant efforts have been made to address this and improve the quality of plant-based oils, mainly through altering seed oil profile and introducing non-native fatty acid biosynthesis pathways [50].

A study by Han et al. [51], in which the SB design-build-test-learn cycle was followed, has built on previous success in engineering the pathway for EPA and DHA in camelina (*Camelina sativa*) [52]. The study carried out systematic step omissions, additions, and ortholog substitutions in order to test which parts of their construct design could be improved upon. Both indoor controlled environment and outdoor field trials of their transgenic lines revealed that constructs made with transgenes from a mix of species showed greater accumulation of the health-promoting ω3 FAs [51]. The work reported in this study and subsequent animal and human dietary studies [21,53] is a good example of what is required to translate a GM plant into a product with real-world value. It is also exemplary of the promise that following SB principles holds for the development of other biofortified crops.

Future perspectives

Although solutions such as micronutrient supplements or fortified foods are readily available in the market, hidden hunger persists. Affordability, accessibility, and education regarding healthy foods must improve. Advances in SB continue to enable the improvement of plant-derived nutrition, but there are several factors to consider for future research to be more effective. The first is to better understand the regulation and responses of target pathways to develop creative and efficient strategies of biofortification. Second, further investigation into improving both the bioaccessibility of targets and their localisation to the edible portions of the plant is vital to ensure that any nutritional changes achieved are passed onto the consumer. Finally, dietary studies and field trials must be utilised to assess the nutritional benefits and real-world value that GM crops can provide. Without clear value propositions, the justification to expand the regulatory boundaries of our gene technology toolkits into SB and beyond will be challenged. In the future, assessment of risk should be based on both the extent of the modification, with higher regulatory hurdles reserved for the insertion of synthetic and transgenic DNA, and the risk profile of the introduced modification. As standardised SB parts become more prevalent, as understanding of plant metabolism improves, and with the cost of 'omic technologies to monitor changes continuing to decline, it is hoped that the better predictability of these engineering approaches will reduce the cost and time to market new crops. This in turn will enable a wider range of companies to utilise the technology and engage the SB sector.

The encouraging progress of SB in plant-derived nutrition is evidence for the need to expand this science into areas other than those highlighted here. Other emerging use cases in plants include the production of pharmaceutical products, robust materials, and energy-dense biofuels [54]. With a snowballing global population and depleting natural resources, applications like these will become increasingly vital. Plant SB is still in its infancy and will not be the magic bullet to meet all such needs, but its promise to provide renewable and sustainable alternatives is reason enough to watch on with excitement.

CRediT authorship contribution statement

Ryan A Edwards: Conceptualization, Writing – original draft, Writing – review & editing. **Xiao Y Ng:** Writing – original draft. **Matthew R Tucker:** Writing – review & editing. **Jenny C Mortimer:** Conceptualization, Writing - review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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