Tansley review

Medicine is not health care, food is health care: plant metabolic engineering, diet and human health

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Summary

Plants make substantial contributions to our health through our diets, providing macronutrients for energy and growth as well as essential vitamins and phytonutrients that protect us from chronic diseases. Imbalances in our food can lead to deficiency diseases or obesity and associated metabolic disorders, increased risk of cardiovascular diseases and cancer. Nutritional security is now a global challenge which can be addressed, at least in part, through plant metabolic engineering for nutritional improvement of foods that are accessible to and eaten by many. We review the progress that has been made in nutritional enhancement of foods, both improvements through breeding and through biotechnology and the engineering principles on which increased phytonutrient levels are based. We also consider the evidence, where available, that such foods do enhance health and protect against chronic diseases.

I. Introduction

There is an uncredited slogan on the internet stating: ‘Medicine is not health care, food is health care. Medicine is sick care’ which carries with it an important philosophical message reflecting how our ideas on protecting our health have increasingly emphasised medical solutions. Yet, plants play very important roles in relation to our health, as components of our diet. Plants provide us not only with macronutrients (carbohydrates, lipids and proteins), but also most essential micronutrients (vitamins A, B, C, some vitamin D, E and K) and most of our essential minerals, as well as fibre. In addition, they provide phytonutrients which are compounds in plant-based foods that play beneficial roles in the prevention of disease. Phytonutrients include polyphenols (flavonoids, hydroxycinnamates and stilbenoids), carotenoids, plant sterols and polyunsaturated fatty acids (PUFAs). There is increasing evidence that the consumption of plant-based foods, particularly fresh fruit and vegetables, is inversely associated with the risk of major chronic diseases, particularly cardiovascular disease (Bradbury et al., 2014), metabolic diseases such as obesity and Type 2 diabetes (Mozaffarian et al., 2011) and certain types of cancer (Doll & Peto, 1981; Martin et al., 2011, 2013; Parkin et al., 2011; Traka & Mithen, 2011; Liu, 2013a,b; Norat et al., 2014; Wang et al., 2014a,b).
II. The two faces of nutritional enhancement

An unforeseen consequence of the green revolution, was the increasing dependence of rural communities in developing countries on a few, calorie-rich but nutritionally poor, staple crops. Deficiency diseases and mortality resulting from inadequate consumption of vitamins and/or minerals have been termed ‘hidden hunger’. Enormous financial and social effort has been invested in biofortification of staple crops to replace supplies of essential vitamins and minerals that have been lost as diets have become less diverse. Plant biotechnology has played and can play an important role in engineering metabolism to biofortify staple crops so that they supply adequate essential vitamins to prevent the occurrence of deficiency diseases.

In addition, in developed countries and in urbanized societies in developing countries, despite the clear benefits of consuming plant-based foods, public information campaigns have proved singularly unsuccessful at changing dietary choices (Rekhy & McConchie, 2014). The strength and pervasiveness of fast-food industries, the relative weakness of horticultural sectors, the increasing costs of fruit and vegetables relative to fast foods and sweet, oily processed snacks and the addictive nature of soft, palatable diets (Gearhardt et al., 2009, 2011) may all have contributed to a general decline in the nutritional value of diets, and the paucity of fruit and vegetables in modern, Western diets.

In this review we will focus on the role of plants in the diet for protection from and amelioration of chronic diseases including; vitamin deficiency diseases, cancer, cardiovascular disease, metabolic diseases, and the contributions of metabolic engineering to solutions that can promote health. We will consider the contribution that plant biotechnology has made and can make to nutritional enhancement. We will focus on the roles of phytochemicals in food, because although there is a general assumption that supplements might be used to address the problems of a lack of fruit and vegetables in the diet, the lack of regulatory oversight in this sector means that supplements very often do not contain the phytochemicals they claim (e.g. Tokyo Japan Health Food and Nutrition Association 2009 Bulletin No 58). In addition, there is increasing evidence that purified compounds or extracts do not have the same efficacy as those compounds within a defined food matrix (Eberhardt et al., 2000; Liu, 2003, 2004, 2013a,b; Goldman, 2004; de Kok et al., 2008; Prior et al., 2008; Titta et al., 2010). In larger intervention studies, adverse effects of some vitamins and phytonutrients have been observed, but only when they were supplied as supplements, far exceeding normal levels of consumption (Bowry & Stocker, 1993; Albanes et al., 1996; Rapola et al., 1997; The Myeloma Beacon, 2010).

III. Biofortification to address deficiency diseases

Mainstream programs for biofortification of staple crops have focussed on enhancing concentrations of provitamin A, iron and zinc in rice, cassava and maize, but other programs have had substantial success, for example the Orange Sweet Potato (OSP) project in promoting consumption of orange sweet potato as a major source of provitamin A. Perhaps the OSP project has been particularly successful, because sweet potato is not classified as a staple crop, and consequently consumer choice has been important in driving its success. Where natural variation is available, as for sweet potato, biofortification breeding programmes have been more effective at enhancing the nutritional value of staple crops than genetically engineered (GE) crops, but not necessarily for scientific reasons. Although genetic engineering often offers the quickest, most effective and cost-efficient route to biofortifying staple crops in essential minerals and vitamins, opposition to GE crops has provided risk-averse regulators with the cover to adopt overly precautionary policies for approving them, especially when compared to the approval processes for biofortified crops generated by conventional breeding (Miller & Kershen, 2013). Rice biofortification in provitamin A is, of course, the prime example of this. However, the alternative of conventional breeding of biofortified rice has had little success for iron and folate, and no success with enrichment of provitamin A, although zinc-enriched varieties of rice have been released (Lee & Krimsky, 2016).

1. Biofortification of essential vitamins: provitamin A

Improving the provitamin A content of staple crops has focussed on enhancing the concentrations of β-carotene in the parts of the crop that people consume, either endosperm of rice, sorghum or maize seeds, or the tubers of cassava. β-carotene can be cleaved, once consumed, to form two molecules of retinol (vitamin A), and consequently is considered a better source of vitamin A than α-carotene or β-cryptoxanthin, which can also be metabolised to retinol following digestion. β-carotene concentrations can be enhanced by increasing synthesis (a ‘pull’ strategy), increasing storage or reducing catabolism in the target tissues (‘protect’ strategies) (Fig. 1). In fact, almost all biotechnological biofortification strategies have initially pursued the ‘pull’ strategy of increased synthesis, because this can be effective in tissues such as rice endosperm where no β-carotene is normally synthesized. For Golden Rice this involved expressing the gene encoding phytene synthase (PSY) from daffodil and the gene encoding the multifunctional enzyme carotene desaturase, Crf1, from the bacterium Erwinia uredovora, which is capable of performing all the desaturation and isomerization reactions necessary to form lycopene. Activity of lycopene β-cyclase was found to be unnecessary for the formation of β-carotene in rice endosperm, probably due to endogenous activity of this enzyme (Fig. 1; Ye et al., 2000). The result was Golden Rice 1 which had a maximum carotenoid concentration of 1.6 μg g⁻¹ DW, with 50% of this as β-carotene (Ye et al., 2000). Further optimization of synthesis involved replacing the daffodil PSY with the gene encoding the more efficient PSY enzyme from maize together with the Crf1 gene from Erwinia uredovora, both driven by the endosperm-specific rice glutelin promoter, to produce Golden Rice 2 which maximally accumulated 37 μg total carotenoids per g of grain, of which >80% was β-carotene, a concentration conservatively estimated to supply 50% of the recommended daily allowance (RDA) for provitamin A in 100 g of rice (Paine et al., 2005).

Sorghum is the second most important cereal crop in Africa with 300 million people dependent on it as a staple, and it is seriously deficient in provitamin A, iron and zinc (Che et al., 2016). In
Fig. 1 The carotenoid biosynthetic pathway in plants. DXS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; GGPPS, geranyl-geranyl diphosphate (GGPP) synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; Z-ISO, ζ-carotene isomerase; CRTISO, carotenoid isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYD-B, β-Carotene hydroxylases. Redrawn from Zhu et al. (2010).
sorghum, use of the same strategy as for Golden Rice 2 resulted in poor accumulation of β-carotene (<2 μg g⁻¹), not due to a failure in synthesis, but due to degradation of β-carotene by oxidation in older, mature seed. This was significant in sorghum because this crop is traditionally stored, post-harvest, for several months before consumption. The expression of the maize PSY gene under the control of the endosperm-specific promoter in sorghum declined to zero by seed maturation, meaning that no more β-carotene was synthesised, post-harvest. Concentrations of β-carotene could be enhanced by reducing oxidative loss (a ‘protect’ strategy), by increasing the synthesis of tocotrienol and tocopherol (vitamin E) lipophilic antioxidants. This was achieved by expressing the gene encoding homogentisate geranylgeranyl transferase (HGGT), which catalyses the first step committed to tocotrienol biosynthesis (Fig. 2: involving the condensation of homogentisic acid, HGA and geranylgeranyl diphosphate, GGDP), under the control of an endosperm-specific promoter from barley in sorghum. Concentrations of α-tocotrienol, α-tocopherol and γ-tocopherol, were increased 27.3-, 1.8- and 1.7-fold, respectively, and consequently, concentrations of β-carotene were increased to 7–12 μg g⁻¹ DW, as a result of reduced loss of β-carotene through oxidation (Che et al., 2016).

In cassava a strategy of expressing PSY in roots gave increases of 10- to 20-fold in total carotenoids, whereas high-level expression of the gene encoding 1-Deoxy-d-Xylos-5-phosphate Synthase (DXS; encoding the first step in the non-mevalonate pathway for isopenoid synthesis) together with PSY (Fig. 1) gave 15- to 30-fold higher carotenoid concentrations in roots than those in storage roots from nontransformed plants, achieving concentrations >50 μg g⁻¹ DW (Sayre et al., 2011). Although concentrations were reported to be stable in field trials, these varieties have not been taken forward due to the availability of β-carotene enriched, ‘golden cassava’ germplasm for breeding (Welsh et al., 2010).

In staple crops where there is significant natural variation in β-carotene concentrations, the ‘protect’ strategy of reducing catabolism has proven most effective in breeding for high β-carotene varieties. Thus, selection of weaker alleles of lycopene ε-cyclase (LCYE), an enzyme which converts all-trans lycopene to α-carotene (Fig. 1), has been shown to enhance the levels of accumulation of β-carotene in maize kernels (Harjes et al., 2008). Similarly rare, weak alleles of crtRBl, which encodes Crtβ-carotene hydroxylase1 that converts β-carotene to β-cryptoxanthin and zeaxanthin (Fig. 1), have been associated with higher concentrations of β-carotene in maize, and have been proposed for use in selecting new varieties of maize associated with yet higher concentrations of provitamin A (Yan et al., 2010).

2. Biofortification of B vitamins

Beyond the mainstream biofortification programmes for provitamin A, other studies have looked at biofortification in B vitamins. Vitamin B6 deficiency results in neurological and inflammatory pathologies involving increased risk of Parkinson’s Disease, cancer, immune deficiencies, cardiovascular disease and high blood pressure (Fudge et al., 2017). Estimates of dietary deficiency for vitamin B6 run at 24% of US citizens, but 54% in Sudan and 64% in Uganda, perhaps because of the dietary dependency in Africa on vitamin B6-deficient staple crops such as rice and maize. The RDA of vitamin B6 is 1.3–2 mg g⁻¹, although excessive consumption (9.6 mg g⁻¹) has been associated with acute neuropathy (Fudge et al., 2017).

All attempts at crop biofortification with vitamin B6 have targeted increased synthesis. Vitamin B6 includes six vitamins, pyridoxal, pyridoxamine, pyridoxine and their 5’ phosphorylated esters. Pyridoxal 5’phosphate (PLP) vitamins were enhanced by expression of two biosynthetic genes, encoding a PLP synthase (PDX1) and a glutaminase (PDX2) in Arabidopsis leaves (Fig. 3). The best concentrations of B6 vitamins (four-fold increase) were obtained by combining overexpression of PDX1 and PDX2 in Arabidopsis seeds (Raschke et al., 2011). More encouraging effects were observed in cassava with a 15-fold increase in vitamin B6 concentrations in uncooked leaves using both PDX1 and PDX2 driven by the CaMV 35S promoter, and a six-fold enhancement in tubers when the genes were expressed under the control of a tuber-specific promoter, concentrations that were stable in field trials (Li et al., 2015). These values were reduced to nine-fold in leaves and four-fold in tubers following cooking. However, the predominant vitamer produced in cassava was pyridoxine glycoside (PN-Gly), which has only 50% bioavailability in humans (Nakano et al., 1997).

Folate deficiency gives rise to anaemia and slow growth rates in children. For pregnant women, dietary deficiency in folate increases the risk of low birth weight, premature babies and neural defects. Folate (vitamin B9) accumulation has been engineered successfully in rice endosperm by overexpressing genes encoding two enzymes involved in folate biosynthesis: aminodeoxychorismate synthase (ADCS, which catalyzes the first step in the paraaminobenzoate (pABA) branch of folate biosynthesis) and GTP cyclohydrolase I (GTPCHI, which catalyzes the first step in the pteridin branch of folate biosynthesis; Fig. 4) (Storozhenko et al., 2007). Enhancements of up to 100-fold were reported, reflecting the very low concentrations of folate that accumulate naturally in rice endosperm. At concentrations of folate up to 17 μg g⁻¹, a serving of 100 g of the biofortified rice could supply between two and three times the RDA for folate (400–600 μg d⁻¹). However, folates disappear on storage of rice, concentrations declining to 50% after 4 months and to 40% after 8 months storage (Blankquaert et al., 2015). In rice, folate stability was improved by the protective strategy of coexpression of folate binding proteins in rice endosperm, which prevented loss of folates during storage, although the bioavailability of the bound folates remains to be confirmed.

3. Enhancing vitamin C (ascorbate) concentrations in food crops

Vitamin C is an essential nutrient for humans where it acts as an antioxidant by protecting the body against oxidative stress (Padayatty et al., 2003) impacting cardiovascular disease, hypertension, chronic inflammatory diseases and diabetes (Kelly, 1998; Tak et al., 2000; Goodyear-Bruch & Pierce, 2002; Mayne, 2003). Humans, unusually amongst mammals, cannot synthesise their own ascorbate (vitamin C). Severe deficiency causes scurvy, a disease frequent in the past amongst sailors who lacked fresh fruit and vegetables in their diets. Scurvy is associated with other
deficiency diseases, caused by heavy dependency on staple crops. Although rare in developed countries, the incidence has risen recently, blamed on reduced consumption of fresh fruit and vegetables, and vitamin C deficiency has been reported to affect between 10% and 14% of US adults (Velandia et al., 2008). The symptoms of scurvy include joint pain, swollen gums and skin lesions, resulting from inadequate collagen synthesis, which requires ascorbate as an essential co-factor. A recommended dose of 90–100 mg vitamin C daily is required to protect against cancer, heart disease and cataracts, in contrast to 45 mg daily required to prevent scurvy (Carr & Frei, 1999).

The major pathway for the synthesis of ascorbate in plants is the Man/Gal pathway which utilizes D-fructose-6P or D-mannose-6P and converts these to L-ascorbate via eight or seven enzymatic steps (Fig. 5). Additional routes from myo-inositol, L-gulose and D-galacturonic acid operate, often in specialized tissues. There is strong evidence that transcription of GGP/vtc2 (encoding GDP-L-galactose phosphorylase/guanylyltransferase) in the Man/Gal pathway controls ascorbate accumulation in Arabidopsis (Smirnoff, 2011), and this is likely to be the case for other plants, because this is the first step in the pathway committed to ascorbate production. Enhancement strategies have focused on increasing the
activity of key biosynthetic enzymes, and elevation of the activity of GGP has had the greatest effect on concentrations of vitamin C giving 6.2-fold more in tomato fruit (up to 111 mg g\(^{-1}\) FW), 3.0-fold more in potato tubers (up to 36 mg g\(^{-1}\) FW), 2.1-fold more in strawberries (up to 131 mg g\(^{-1}\) FW) (Bulley et al., 2012) and 2.5-fold more in rice (Zhang et al., 2015a). Combined expression of \(GGP/\text{vtec2}\) and the gene encoding GDP-mannose epimerase (\(\text{GME/\text{vte4}}\)) has been attempted to overcome the problem of GME becoming rate-limiting in plants with high GGP activity, but without very strong effects on ascorbate concentrations in leaves (Imai et al., 2012; Macknight et al., 2017). Recent results showing post-transcriptional regulation of GGP production via an untranslated open reading frame (ORF) in the 5\(^{'}\) untranslated region of the \(GGP/\text{vte2}\) gene in response to ascorbate concentrations, suggest that removal of this homeostatic regulatory unit (by mutation or genome editing) could elevate ascorbate synthesis substantially (Laing et al., 2015). Transient assays of deregulated GGP and GME in \(\text{Nicotiana benthamiana}\) leaves suggest that increases in ascorbate concentrations of the order of 12-fold (up to 300 mg g\(^{-1}\) FW) might be achievable (Laing et al., 2015; Macknight et al., 2017).

### 4. Enhancing the activity of vitamin E in food crops

There are two groups of vitamin E vitamers (tocopherols and tocotrienols) collectively termed tocochromanols. Bioactivity of tocochromanols is determined by their affinity for the \(\alpha\)-tocopherol transporter in the liver of humans, which in turn, determines bioavailability, and for which \(\alpha\)-tocopherol has the highest affinity. Consequently, \(\alpha\)-tocopherol is the most biologically active form of vitamin E, vitamers of which are lipid-soluble antioxidants protecting against LDL and PUFA oxidation (Herrera & Barbas, 2001; Packer et al., 2001). Suboptimal concentrations of vitamin E are associated with cardiovascular disease (Stampfer et al., 1993; Knekt et al., 1994; Glynn et al., 2007), some cancers (Bostick et al., 1993; Heinonen et al., 1998; Jacobs et al., 2002) and impaired immune function (Kowdley et al., 1992). The dietary reference intake is 15 mg d\(^{-1}\) but only a minority of people, even in developed countries, consume these amounts (Gao et al., 2006). Although mainstream biofortification programs have not included increasing vitamin E activity, higher levels of consumption could protect consumers at least by improving immune functionality.

Tocochromanols are synthesized in the plastids of plants, from homogentisate and a long-chain isoprenoid which is either fully saturated (tocopherols) or contains three \(\alpha\) unsaturated double bonds (tocotrienols; Fig. 2). Tyrosine is used to make the homogentisate which forms the head group of the tocochromanols, and the plastidial deoxyxylose 5-phosphate pathway produces the hydrophobic isoprenoid tail groups (Fig. 2). It has long been thought that tocochromanol concentrations are dependent on the supply of homogentisate from tyrosine, which is, in turn, limited by feedback regulation of its own synthesis, in plants. Confirming this, experiments that overcame the feedback regulation of tyrosine production, using a gene encoding a prephenate dehydrogenase from bacteria (which is not subject to metabolite regulation) resulted in 10-fold enhanced concentrations of tocotrienols in leaves of \(\text{Arabidopsis}\) and tobacco (Zhang et al., 2013). However, a similar ‘push’ strategy in oil seeds increased tocochromanol concentrations by only 140% in canola seeds and by 160% in soya beans (Karunananda et al., 2005), suggesting that the supply of HGA is less limiting for tocochromanol production in oil seeds than in leaves.

Other ‘pull’ strategies have involved overexpression of homogentisate prenyl transferase (\(\text{HPT; vte2}\)) which is the first enzyme committed to tocopherol synthesis (Fig. 2) but this strategy had only modest impact on tocopherol concentrations in seeds (Falk...
et al., 2003; Zhang et al., 2013) compared to overexpression of HGGT which significantly increased the concentrations of tocotrienols in maize and sorghum seeds (Cahoon et al., 2003; Dolde & Wang, 2011; Che et al., 2016). Increasing the flux through the mehtylerythritol 4-phosphate isoprenoid (MEP) pathway to provide more GGPP or PPP for tocotrienol and tocopherol biosynthesis, respectively, resulted in small, but measurable increases in tocochromanol concentrations (Estévez et al., 2001; Vom Dorp et al., 2015).

By far the greatest enhancement of vitamin E activity in crops has been achieved by altering the balance between vitamin E vitamers in oil seeds. γ-tocopherol has only 10% of the vitamin E activity of α-tocopherol, so elevated expression of the gene encoding γ-tocopherol methyl transferase (γ TMT; vte4) which converts γ-tocopherol to α-tocopherol, enhanced the vitamin E activity of seed oil from Arabidopsis (Shintani & DellaPenna, 1998), soya bean (Van Eenennaam et al., 2003; Tavva et al., 2007), mustard (Yusuf & Sarin, 2007) and maize (Zhang et al., 2013). A similar strategy has been adopted in breeding for vitamin E-enhanced rice (Shammugasamy et al., 2015).

Protection strategies may also prove effective in selecting for varieties of oil crops with enhanced vitamin E activity. A mutant of soya bean affected in the seed-specific activity of homogentisate dioxygenase (which breaks down homogentisate) has two-fold elevated concentrations of γ- and β-tocopherols, four-fold higher concentrations of α-tocopherol and 27-fold higher concentrations of tocotrienols compared to near isogenic controls (Stacey et al., 2016). This analysis showed that tocotrienol synthesis is more dependent on homogentisate concentrations than tocopherol synthesis in oil seeds. This mutant, or perhaps similar mutations engineered by genome editing of other oilseed crops, could be used to enhance the vitamin E activity of oil, further. However, despite the production of several oil crops with enhanced vitamin E activity (usually measured as higher α-tocopherol concentrations), there are no reports of these offering greater protection of health to consumers. Perhaps the results of larger studies with vitamin E
supplements which reported pro-oxidant toxicity associated with high vitamin E consumption, have discouraged similar trials of vitamin E-enriched foods (Bowry & Stocker, 1993; Albanes et al., 1996; Rapola et al., 1997).

Efforts to biofortify crops in micronutrients such as iron and zinc strictly fall outside the remit of plant metabolic engineering, and have been well reviewed recently (Bouis & Saltzman, 2017; Díaz-Gómez et al., 2017; Finkelstein et al., 2017; Vasconcelos et al., 2017).

IV. How successful has metabolic engineering been in biofortification?

The success of metabolic engineering to biofortify crops in essential vitamins depends very much on the baseline concentrations that have been the starting point for engineering. Although Golden Rice 2 provided maximally 37 μg total carotenoids g⁻¹ of polished rice, it started from zero in unmodified rice. Where there have been enhancements of existing concentrations, such as for vitamin B6 in...
cassava or provitamin A in maize, the fold-enhancement achieved by metabolic engineering of the biosynthetic pathway has usually been modest, in the range of two- to four-fold. This is because the ‘pull’ engineering strategy is often based on improving the activity of key ‘rate-limiting’ steps, such as PSY in β-carotene biosynthesis. In fact, flux control theory suggests that control is frequently vested in many steps in metabolic pathways and, by improving the effectiveness of one step that may be limiting flux in any particular tissue, another will rapidly become limiting, so restricting the maximum rates of synthesis achievable (Martin, 1996). In addition, it is becoming very clear that biofortification should be restricted to the edible tissues of the plant, to avoid yield penalties associated with disturbing metabolism and its homeostatic control, outside the target tissues. Thus, the importance of driving biosynthetic gene expression with promoters specific to the targeted tissues has become increasingly apparent during the development of different biotechnological biofortification programs.

Measuring the impact of biofortification of essential vitamins on health is facilitated by the existence of RDA. These offer an absolute measure of the concentrations required to meet dietary needs if the staple crop is the only or main source of the vitamin, such as Golden Rice for provitamin A. However, for most vitamins these values are complicated by the fact that several vitamers are made in plants, which may have different bioactivities in the human body as well as different bioavailabilities. For example, β-carotene, α-carotene and β-cryptoxanthin can all be converted to retinol, but with different efficiencies. Biofortification levels are usually assessed as provitamin A carotenoids (PVAC) rather than β-carotene concentrations alone. Furthermore, PVAC concentrations may change in foods with processing, cooking, and storing, as with other vitamins, leading to the necessity of estimating stability and taking measures to improve it in biofortified crops, as described above.

All biofortified crops, whether produced by conventional breeding or by genetic engineering, need to pass bioavailability assessments to establish the extent to which they can supply the essential vitamin to healthy humans. Harvest Plus has reported on the efficacy of orange sweet potato (OSP) in improving vitamin A status in 26,000 people. They showed an increase in vitamin A status in children consuming OSP, including a decrease of 9% in the prevalence of low serum retinol, compared to children not consuming OSP and a decline in the incidence of marginal vitamin A deficiency in women (Hotz et al., 2012a,b). Consumption of orange-β-carotene-enriched maize has been compared directly with white maize and showed improved stores of vitamin A and visual acuity in Zambian children (Gannon et al., 2014; Palmer et al., 2016). Consumption of orange cassava showed a small but significant increase in retinol and β-carotene concentrations in plasma of Kenyan children compared to controls eating white cassava (Talsma et al., 2016). Attempts at undertaking similar trials for Golden Rice have met with voracious criticism, which even led to retraction of the paper on which the claims of bioefficacy were based (Tang et al., 2012; Lee & Krinsky, 2016).

These data on bioefficacy of provitamin A-biofortified crops are largely from healthy subjects. In fact, for subjects already suffering from provitamin A deficiency, the effectiveness of provitamin A-biofortified crops may be substantially lower because many children with provitamin A deficiency suffer from protein deficiencies and their intestinal infections interfere with the absorption of β-carotene and its conversion to retinol. Studies with provitamin A-deficient children are required to test the efficacy of biofortified crops, but until then biofortified crops must be seen as a means of protection against deficiency diseases, rather than any cure for them.

V. Improving the concentrations of phytonutrients in foods

Humans are genetically adapted to the environment of their ancestors (Cordain et al., 2005). Diet and nutrition constitute important components of environment which underwent profound changes with the introduction of agriculture and animal husbandry c. 10 000 yr ago, and with the food processing techniques that have been applied over the past 100 yr. The human genome evolved when our ancestors were hunter-gatherers; diets were rich in fruits, vegetables and protein, and low in fats and starches (Eaton & Konner, 1985). The 10 000 yr since the first cultivation of cereals, and particularly the last 30 yr of the predominance of highly processed foods, have not been long enough for the human genome to adapt to starchy diets based on cereals, with much lower contents of fresh fruit and vegetables. Chronic diseases may result from an evolutionary discordance with modern diets (Simopoulos, 2002). Concentrations of phytonutrients in the diet have dropped significantly, due to reductions in the amount of plants that we eat (currently only 17 plant species are consumed as 90% of the global human diet) and due to selective breeding focused on yield (Willett, 2010). Thus, humankind is now experiencing a rising incidence of chronic disease based, in part, on the low concentrations of phytonutrients in diets. Dietary improvement in phytonutrients has the potential to lead to significant reductions in the incidence and progression of chronic diseases, and health care costs, and improvements in the quality of life of those living in more developed countries and urban societies in low and middle income countries (Martin et al., 2013; Wang et al., 2014a,b).

The compounds in these plant-based foods which underpin their beneficial roles in the prevention of disease and the promotion of health have been termed phytonutrients. These include polyphenols (flavonoids, stilbenes), carotenoids, plant sterols and PUFAs. However, the constituents in plants that promote health through diet have proved difficult to identify with certainty and, because many are nonessential dietary constituents, it has also proven difficult to persuade people, at all levels, of their nutritional value. These issues have, in turn, confounded both the precision of dietary intake recommendations for the consumption of phytonutrients and attempts to develop foods enriched in phytonutrients that offer consumers better protection against chronic diseases.

1. Polyunsaturated fatty acids (PUFAs)

There has been considerable confusion about which polyunsaturated fatty acids have been definitively associated with health benefits, starting from the opinions that were issued in the 1950s, that saturated fatty acids (derived mostly from animal fats)
increased the risk of cardiovascular disease (CVD), and that polyunsaturated fats should be consumed instead (Davignon, 1978; Ramsden et al., 2009). More recent evaluations of epidemiological data suggest that the association between consumption of saturated fats, cholesterol/LDL concentrations in serum and mortality from CVD may not be as strong as originally argued. Of course, no specific health benefits have been claimed for saturated fats, but oleic acid, a mono-unsaturated medium chain fatty acid, is present in substantial amounts in ‘Mediterranean diets’ and mono-unsaturated fatty acids have consequently been suggested as ‘healthy fats’ (Kromhout et al., 1995; Ramsden et al., 2009; Martin et al., 2013).

PUFAs can be divided into two classes: medium chain and very long chain (VLC). Dietary medium-chain PUFAs are derived largely from plants, and both ω-3 and ω-6 medium-chain PUFAs (α-linolenic acid and linoleic acid) are essential in the human diet (Burr & Burr, 1929, 1930; Burr et al., 1932). By contrast, ω-3 VLC PUFAs are derived almost exclusively from marine fish and seafood. They, in turn, acquire high concentrations of ω-3 VLC PUFAs from the algae on which they feed. Many dietary campaigns have recommended replacement of saturated fats with medium-chain PUFAs such as linoleic acid (a medium-chain ω-6 PUFA) and on average, linoleic acid now supplies c. 6–7% total energy intake in the modern US diet and 8–12% in some modern European diets. However, traditional Mediterranean diets contain low concentrations of linoleic acid and are accompanied by lower incidence of Coronary Heart Disease (CHD). A long-term intervention study that reduced linoleic acid to concentrations similar to those in a ‘Mediterranean diet’ reduced CHD events and mortality by 70% (de Lorgeril et al., 1994; de Lorgeril & Salen, 2012). Consequently, the health benefits of linoleic acid itself are unclear. As a medium-chain ω-6 PUFA, linoleic acid can be extended, once absorbed, to form ω-6 VLC PUFAs such as arachidonic acid (ARA).

ω-3 VLC PUFAs are generally associated with anti-inflammatory effects, whereas ω-6 VLC PUFAs promote inflammation and induce autoimmune responses. The cause of the deleterious effects of ω-6 VLC PUFAs is believed to be because they are used to form signalling molecules, particularly prostaglandins, and other proinflammatory eicosanoids, whereas ω-3 VLC PUFAs are used to form anti-inflammatory eicosanoids (Simopoulos, 2002). Animals and humans metabolize dietary linoleic acid to form arachidonic acid (ARA, an ω-6 VLC PUFA) and α-linolenic acid to form eicosapentaenoic acid (EPA, an ω-3 VLC PUFA) and docosahexaenoic acid (DHA, an ω-3 VLC PUFA) although the conversion of essential fatty acids to ARA, EPA and DHA is inefficient (< 5%). Mammals, including humans, are unable to convert ω-6 to ω-3 PUFAs. Consequently, tissue concentrations of the ω-6 and ω-3 PUFAs and their corresponding eicosanoid metabolites are linked directly to the amounts of ω-6 vs ω-3 VLC PUFAs consumed in the diet. VLC PUFAs are not technically ‘essential fatty acids’ unlike their medium-chain precursors, because vegetarians often have better risk markers for chronic diseases, especially CVD, than those consuming ω-3 VLC PUFAs from fish and shellfish, although as vegetarians they are dependent on self-synthesis of EPA and DHA from medium-length ω-3 PUFAs (Harris, 2014). Considerable data from cell, animal, epidemiological and intervention studies suggest that higher ω-3 : ω-6 ratios prevent or reduce chronic diseases of different types, probably owing to the promotion of anti-inflammatory activity by ω-3 VLC PUFAs (He et al., 2002; Maillard et al., 2002; Erkkila et al., 2003) and it is the ratio of ω-3 : ω-6 VLC PUFAs that impacts health, in the form of susceptibility to cancers, CVD and neurodegeneration, rather than the absolute concentrations of ω-3 VLC PUFAs.

There has been considerable effort, and recently significant success, in engineering VLC PUFA production in plants, ostensibly driven by concerns over the sustainability of fish supplies for ω-3 VLC PUFAs to maintain human health. Because the pathway from α-linolenic acid (ALA) to the VLC PUFAs, EPA and DHA, is not present in higher plants, such engineering efforts of necessity involve biotechnological solutions and have focussed on introducing the biosynthetic pathways (‘pull’ strategies) from a range of lower eukaryotes including diatoms, algae, yeast and oomycetes. This pathway engineering has involved expression of a number of genes encoding fatty acid desaturases and elongases (Fig. 6).

Initial steps focused on enhancing the production of stearidonic acid (SDA) and γ-linolenic acid (GLA) from ALA using a Δ-6 desaturase from either higher plant or fungal sources (Haslam et al., 2013). Some plants, like borage, can synthesize Δ6-desaturated fatty acids like SDA and GLA, but the activity of plant Δ-6 desaturase is low and it is often inefficiently expressed (Sayanova et al., 2012). Higher concentrations of SDA (ω-3) have been achieved in oilseed crops by co-expression of a Δ-6 desaturase with a Δ-15 desaturase which reduces the concentrations of GLA (ω-6) relative to SDA (ω-3) by improving the relative availability of ALA as substrate (Ursin, 2003; Eckert et al., 2006). A soybean variety with 15–30% SDA and 5–8% GLA was approved for agricultural release but has not yet been commercialized. Consonption of this oil showed efficacy in raising serum EPA amounts in clinical trials (16.6% of the efficacy of EPA; Harris et al., 2008; Lemke et al., 2010). A high-GLA oil was also developed in safflower, and recently received regulatory approval for Arcadia Biosciences. The GLA in this oil is claimed to support heart and eye health, when used in combination with the ω-3 fatty acids EPA and DHA, although no experimental data to support this claim for the GLA-rich oil has been published. More recently linseed was engineered with the ALA-specific Δ-6 desaturase from Primula vialii, which produces oil with 13% SDA and ω-6 GLA (Ruiz-Lopez et al., 2009). Although these SDA-enriched oils may enhance EPA concentrations by providing more precursor SDA, they are still dependent on the relatively inefficient endogenous conversion of medium chain PUFAs to VLC PUFAs for beneficial effects. In addition, the concomitant enhancement of ω-6 PUFAs through enhanced concentrations of GLA in some oil products, and the failure to impact concentrations of DHA, may limit the uptake of these products for their health benefits, especially in competition with newer oils that make EPA and DHA at concentrations comparable to those in fish oil.

One of the major problems encountered in engineering EPA and DHA in oil crops has been that the pathways are different in different lower eukaryotes, and early attempts using a mixture of Δ-6 and Δ-5 desaturases and Δ-6 elongases (Abbadi et al., 2004)
showed inefficient synthesis of VLC PUFAs. This was explained by the substrate preferences of the elongases being for acyl-CoA-linked fatty acid substrates and those for the desaturases being for phospholipid-linked fatty acids (Napier, 2007), and has been largely resolved by selection of genes encoding enzymes that draw their substrates from the same, acyl-CoA-linked fatty acid pools. The use of an acyl-CoA-dependent Δ-6 desaturase not only elevated flux to EPA, but also reduced the production of unwanted C-18 intermediates such as GLA (Cheng et al., 2010; Sayanova & Napier, 2011; Ruiz-Lopez et al., 2013).

Production of DHA from EPA requires addition of at least two more enzymic steps, a Δ-5 elongase and a Δ-4 desaturase (Fig. 5), but is desirable because the neuroprotective and certain cardioprotective effects of fish oils have been associated particularly with DHA. The activity of the Δ-5 elongase has been suggested as particularly crucial in achieving high concentrations of DHA (Petrie et al., 2012) and selection of genes encoding relatively efficient enzymes has proved important in the successful development of oil seed crops producing fish oil-like concentrations of EPA and DHA (Petrie et al., 2014; Ruiz-Lopez et al., 2014). Several other issues have been crucial to the engineering of target concentrations of ω-3 VLC PUFAs in oil seed crops including targeting oil crops with already appreciable concentrations of medium chain ω-3 PUFAs (ALA rather than LA) to avoid the undesired production of ω-6 PUFAs. Seed-specific expression of biosynthetic genes is important, and further success has been achieved by avoiding the use of the same promoter, multiple times, to reduce gene-silencing (Petrie et al., 2014). It has also been suggested that promoter choice should be targeted for sequential steps in the biosynthetic genes in the pathway, so that later steps are not active before earlier steps and consequently have no substrate to work on (Haslam et al., 2013; Petrie et al., 2014). Also important is ensuring that as much as possible of the ω-3 VLC PUFAs are incorporated into triglycerides, and, for this, production of acyl-CoA linked fatty acids has been key, because these intermediates can be incorporated into triacylglyceride (TAG) relatively directly by the Kennedy pathway (Kennedy & Weiss, 1956). Acyl-CoA: diacylglycerol acyltransferase (DGAT) is the only enzyme that is solely committed to TAG biosynthesis as its activity diverts the flux of diacylglycerides (DAG) from phospholipid synthesis towards TAG synthesis. DGAT activity is presumably adequate in oilseeds, but if oils are to be accumulated in other tissues, enhancing the activity of DGAT may be important (Liu et al., 2017). Phosphatidylcholine-linked fatty acids are incorporated into TAG by a more indirect route, meaning that this route is probably less efficient (Bates & Browse, 2012; Ruiz-Lopez et al., 2014).

New, health-beneficial triglyceride concentrations could be improved further by protection strategies to prevent triglyceride loss during storage of oil seeds. Suppression of members of the SDP1 gene family, which encode seed-specific lipases, during seed development enhanced seed oil up to 30% in Brassica napus (Kelly et al., 2013). In addition, oil content can be enhanced by the use of a ‘push’ strategy such that larger amounts of fatty acids are synthesized in addition to being ‘pulled’ to produce specific types of fatty acids and triacylglycerides containing them. A useful tool for this type of engineering is the WRINKLED 1 (WRI1) gene of Arabidopsis, which encodes a transcription factor (TF) belonging to the APetala 2/Ethylene Response Factor (AP2/ERF) family of TFs. WRI1 and closely related TFs regulate the expression of genes involved in fatty acid biosynthesis, a function broadly conserved in Angiosperms (To et al., 2012), including genes encoding acetyl-CoA carboxylase (BCCP2), acyl carrier protein (ACP1), and ketoacyl-acyl carrier protein synthase (KAS1) and the later steps of glycolysis, as direct targets (Maeco et al., 2009). Expression of genes for the synthesis of TAGs and their packaging into oil bodies in the
endoplasmic reticulum in developing seeds also requires WRI1, but the induction of these genes is likely an indirect effect of WRI1 (Maeo et al., 2009). WRI1 also regulates the gene encoding the post-transcriptional regulator PII, which integrates signals to report the cellular carbon/nitrogen status of the cells (Hsieh et al., 1998). The PII protein forms a homotrimeric structure capable of binding ATP and 2-oxoglutarate (Uhrig et al., 2009), thus integrating information on the energetic and C status of the cell and influencing the flux from central metabolism to fatty acid biosynthesis (Baud et al., 2010; Marchive et al., 2014).

The ability of WRI1 and its close relatives to increase the activity of the enzymes of fatty acid biosynthesis as well as the supply of carbon and energy from primary metabolism has made it an effective tool (a ‘push’ strategy) in producing higher amounts of oil (> 40%) in maize kernels (Shen et al., 2010; Pouvreau et al., 2011) as well as in new tissues such as leaves and potato tubers (Vanhercke et al., 2013, 2014; Liu et al., 2017). However, ‘push’ strategies to enhance ω-3 VLC PUFA concentrations in plant oils further, using WRI1 have not been adopted, yet (Marchive et al., 2014).

Despite the engineering to produce VLC PUFAs for health-promoting plant oils being challenging, considerable progress has been made. The production of Camelina oils with concentrations of ω-3 PUFAs equivalent to those in fish oils has been a landmark in plant metabolic engineering, although there has not yet been a substantial drive to provide these for human consumption. Rather, the emphasis has been to develop such oils as food for farmed fish, to improve the sustainability of ω-3 VLC PUFA availability for fish consumption. Although this has been justified in terms of the general public’s rejection of genetically engineered food products, in the light of the RDA of 450 mg d⁻¹ for ω-3 VLC PUFAs, and estimates that the average human consumption is actually less than half this value (Haslam et al., 2013), it would seem that an important opportunity to improve the intake of ω-3 VLC PUFAs and consequently to improve health of consumers, might be being missed. It would be particularly interesting to see randomized, controlled trials that compare consumption of unmodified camelina oil to ω-3 enriched camelina oil, to establish whether the health of the consumers of the ω-3 VLC PUFA-enriched oil is better protected or, at least, whether their internal ω-3 PUFA concentrations are enhanced and inflammatory markers lowered, relative to controls, as would be predicted for these products.

2. Polyphenols

There is increasing interest in dietary polyphenol phytonutrients which are present at high concentrations in some plant-based foods and beverages (tea, coffee, chocolate, berry fruits). Where such foods are consumed in reasonable quantities (c. 1 g d⁻¹), they have been reported to have a range of biological activities expected to deliver protection against the development of chronic diseases (CVD, Type 2 diabetes and certain cancers).

Amongst the bioactive polyphenols, anthocyanins have received a lot of recent attention. They are brightly coloured polyphenols providing the red-purple-blue colours of fruits and vegetables such as blackberries, blueberries, red cabbages and aubergines. Reports from large cohort studies show that subjects consuming the most dietary anthocyanins have the lowest risk of developing CVD (Mink et al., 2007; Cassidy et al., 2013; Jennings et al., 2014). These observed associations between anthocyanin intakes and CVD risk are consistent with several recent reports showing that dietary interventions with anthocyanin-rich foods or extracts result in favourable changes in plasma lipid and lipoprotein profiles and reductions in markers of CVD risk (Alvarez-Suarez et al., 2014; Basu et al., 2014; Soltani et al., 2014). The reported effects are substantial, with total cholesterol reductions in the 10–20% range. However, a number of factors such as lack of suitable control foods, the reputedly poor bioavailability of anthocyanins, the lack of plausible mechanisms, and the absence of official RDAs, limit the translation of these observations into current dietary recommendations. Often increased intake is recommended, involving consumption of anthocyanin-rich fruits such as blueberries or cranberries although consumption of such foods with large amounts of sugar confounds the positive effects of dietary anthocyanins. Recent data have suggested that daily anthocyanin consumption is much lower than previously thought (median consumption 27.5 mg d⁻¹; Rizzi et al., 2016), probably as a result of more accurate recent measurements of anthocyanin contents of foods. These studies showed an inverse relationship between anthocyanin consumption and some markers for risk of CVD, although there were stronger associations to total phenol consumption. There were also marked differences between individuals in their risk factor responses to high-anthocyanin diets, suggesting a strong association with genotype (Rizzi et al., 2016). Supplementation studies suggest beneficial effects of anthocyanins on CVD and inflammatory markers when consumed at levels of between 200 and 300 mg anthocyanins d⁻¹ (Pojer et al., 2013), although many available, commercial supplements fall far below their claimed contents of anthocyanins (Tokyo Japan Health Food and Nutrition Association 2009 Bulletin No 58).

High concentrations of flavonols, another group of flavonoids, are present in vegetables such as onion and fruits like apple. Dietary flavonols inhibit LDL oxidation, the primary risk factor for atherosclerosis and related diseases. Animal studies are supported by human epidemiological studies, which show inverse correlations between CVD and the consumption of flavonol-rich diets (Neyrinck et al., 2013). Isoflavonoids are polyphenols produced almost exclusively by members of the legume family. Inclusion of isoflavonoids in the diet is linked to reduced incidence of CVD, breast and prostate cancers. Anti-cancer effects are thought to be due to the interactions of isoflavonoids with some members of the estrogen receptor family of proteins, which may explain the significantly lower incidence of steroid-hormone responsive cancers (25% lower incidence of prostate cancer and 10% lower incidence of breast cancer) in Asian communities consuming high-soy diets (rich in genistein and daidzein isoflavones).

Resveratrol is a stilbene phytoalexin produced by specific plant species in response to biotic and abiotic challenges. It is thought to be one of the principal agents in the health-promoting effects of red wine, particularly in reducing the mortality from CHD (Baur & Sinclair, 2006). Resveratrol is also reported to have both anticarcinogenic and anti-inflammatory effects, as well as the ability to reverse obesity,
attenuate Type 2 diabetes, improve cardio-protection and increase life span (Sanghyun et al., 2017).

Epicatechins (which are flavan-3-ols) are the major polyphenolic compounds in green tea, and the most significant active component is thought to be epigallocatechin gallate (EGCG). Epidemiological, clinical and experimental studies have established an inverse correlation between green tea/epicatechin consumption and CVD (Bose et al., 2008). Flavan-3-ols, particularly catechins, are abundant in teas derived from the tea plant Camellia sinensis and other fruits and vegetables as well as in chocolate (made from the seeds of Theobroma cacao). The antioxidant, anti-inflammatory and anti-thrombogenic activities of flavan-3-ols make them excellent dietary constituents for preventing CVD. Preclinical studies also suggest they serve important roles as anti-carcinogens. This action is suggested to be due both to their antioxidant scavenging of free radicals and their regulation of the signal transduction pathways controlling cell growth and proliferation. Proanthocyanidins are polymers of catechin and epicatechin and have been suggested to be the principal vasoactive polyphenols in red wine (Corder et al., 2001). There are many reports in the literature claiming that proanthocyanins, such as those from red wine and grape seed offer protection against CVD (Corder et al., 2001; Caton et al., 2010; Gresele et al., 2011).

Polyphenols are made via the phenylpropanoid pathway and polyphenol bioactives are predominantly synthesized by the flavonoid pathway, although hydroxycinnamnic esters, such chlorogenic acids, are synthesized from a branch of the general phenylpropanoid pathway and resveratrol is synthesized using a specialized polyketide synthase (stilbene synthase) found in some, but by no means all, Angiosperm species (Fig. 7). Initial attempts to engineer polyphenol metabolism using key rate-limiting steps were either unsuccessful (Napoli et al., 1990) or very modest in their effects (e.g. Niggeweg et al., 2004). However, genes encoding TFs regulating different branches of phenylpropanoid metabolism have been known for 30 yr, and these have proved to be very effective tools for enhancing the polyphenol content of food crops, either by selective breeding or by genetic engineering.

In maize the coexpression of genes encoding the transcription factors R (encoding a bHLH TF; Ludwig et al., 1989) and C1 (encoding an R2R3 MYB TF; Paz-Ares et al., 1987) was shown to be sufficient to activate anthocyanin biosynthesis and a second gene, P1, encoding another R2R3MYB TF, could activate flavone biosynthesis in maize cells (Grotewold et al., 1999). Near-isogenic lines either producing (RR, C1C1) or not producing (rr, c1c1) anthocyanins in kernels were used to prepare flour to supplement rodent chow at 20% w/w. The damage to heart tissue caused by myocardial infarction of rats fed for 8 wk on the different diets was tested using a Langerdorff model. Diets supplemented with purple corn flour showed significant cardio-protection following ex vivo ischaemia/reperfusion, compared to heart tissue of the animals fed the diet supplemented with yellow corn (Toufektsian et al., 2008).

In addition, the purple corn diet was associated with higher serum concentrations of ω-3 VLC PUFAs (Toufektsian et al., 2011). Strong alleles of genes functionally equivalent to R, called B1, and functionally equivalent to C1, called P1, have subsequently been used to select lines producing highly pigmented kernels, enriched in different anthocyanins and flavonoids (Petroni et al., 2014). Concentrations of anthocyanins in kernels were measured as higher than in blueberries. These new lines have been used to develop new, ‘healthier’ corn products, including polenta, purple sweet corn and purple popcorn (Lago et al., 2013, 2014). Anthocyanin-rich extracts from the pigmented corn varieties have also been produced commercially for addition to mineral water for ‘healthy’ beverages (Petroni et al., 2014).

Using genetic engineering to express the R2R3MYB protein MdMYB10 constitutively in apple, (Espley et al., 2007), red fleshed apples with high concentrations of anthocyanins were produced. Trials on healthy mice showed that consumption of the red-fleshed apple was associated with decreases in some inflammation markers and changes in gut microbiota (Espley et al., 2014) compared to those animals consuming untransformed control apples. As a result of the publicity surrounding the health benefits of red-fleshed apples, breeders in several countries have announced they are developing or reintroducing red-fleshed apple varieties, often derived from traditional, local varieties or bred afresh from wild apples from Kazakhstan.

In tomato, expression of two anthocyanin-regulating TFs, Delila (bHLH) and Rosea1 (R2R3MYB) from Antirrhinum majus, under the control of a fruit-specific promoter led to high-level anthocyanin production, of the order of 20–30 mg g⁻¹ DW. Inclusion of the high-anthocyanin tomatoes as a 10% supplement in the diet of cancer-prone (p53 knock-out) mice led to the animals surviving, on average, 30% longer than animals on the control, red tomato-supplemented diet, or those on the standard diet (Butelli et al., 2008). It is worth noting that the concentrations of anthocyanins achieved in the purple tomatoes were significantly higher than those in the red-fleshed apples or in many other plant tissues engineered to produce anthocyanins by overexpression of R2R3 MYB anthocyanin regulators, alone. The red-fleshed apples contained maximally 380 μg g⁻¹ DW of cyanidin 3-galactoside, whereas the purple tomatoes contained maximally 28 000 ± 5000 μg g⁻¹ DW of delphinidin or petunidin 3-coumaroyl rutinoside, 5-glucoside. The substantially higher concentrations of anthocyanins achieved in tomato were due to the fact that the bHLH partner (Delila) in the Myb-basic-Helix-Loop-Helix-WD-repeat (MBW) regulatory complex was coexpressed with the R2R3MYB TF (Rosea 1). In the apple experiments, ectopically expressed MdMYB10 was dependent on the availability of the endogenous bHLH protein in apples to co-activate transcription, and this may have limited the concentrations of anthocyanin produced in the apples (Ramsay & Glover, 2005; Espley et al., 2007; Butelli et al., 2008).

Very high concentrations of flavonols (rutin and kaempferol 3-rutinoside), in the order of 100 000 μg g⁻¹ DW, have been engineered in tomato using the AtMYB12 gene which encodes an R2R3 MYB TF that controls flavonol biosynthesis. AtMYB12 is structurally related to the P1 regulator of flavone and phlobaphene biosynthesis in maize (Luo et al., 2008). The high concentrations of flavonols produced in fruit were the consequence of AtMYB12 being able to activate expression of genes encoding enzymes of primary and intermediary metabolism, as well as those involved in flavonol metabolism (Zhang et al., 2015a,b). The ability of AtMYB12 to supply additional carbon skeletons, energy and
reducing power for flavonoid biosynthesis, means that this TF can be used to enhance flavonoid production using a ‘push’ strategy. Thus, coexpression of AtMYB12 with Delila and Rosea 1 in tomato resulted in three-fold higher concentrations of flavonols (120 mg g\(^{-1}\) DW) compared to tomatoes expressing AtMYB12 alone (40 mg g\(^{-1}\) DW), and almost double the concentrations of anthocyanins (5.8 mg g\(^{-1}\) DW) compared to fruit expressing Delila and Rosea 1 (3.0 mg g\(^{-1}\) DW; Zhang et al., 2015a,b). Similarly, expression of AtMYB12 together with isoflavone synthase (IFS) for the synthesis of genistin in tomato, elevated...
the concentrations achievable in fruit from 1.6 μg g\(^{-1}\) DW (with IFS alone) to c. 15 mg g\(^{-1}\) DW (for IFS plus AtMYB12) (Shih et al., 2008; Zhang et al., 2015a,b). Concentrations of genistin could be enhanced further in tomato fruit by blocking the flow of flavonoid intermediates to flavonols by introducing a mutation in the gene encoding flavanone 3-hydroxylase, resulting in genistin concentrations of up to 80 mg g\(^{-1}\) DW. These concentrations of isoflavones are 100-fold greater than those in soya products such as tofu (Zhang et al., 2015a,b). The power of incorporating a regulator that can improve the supply of not only carbon in the form of phenylalanine precursors, but also energy and reducing power from primary metabolism (glycolysis, the TCA cycle and the oxidative pentose phosphate pathway), was also demonstrated for the production of resveratrol in tomato, the concentrations of stilbenoids in fruit rising from 0.5 mg g\(^{-1}\) DW (with grape stilbene synthase gene alone) to 5–6 mg g\(^{-1}\) DW when fruit-specific expression of AtMYB12 was combined with the expression of the gene encoding stilbene synthase from grape (Ingrosso et al., 2011; Zhang et al., 2015a,b).

None of these polyphenol-enriched tomato varieties, apart from the purple high-anthocyanin tomatoes, have yet been tested for health protection or promotion, but the development of varieties enriched in different polyphenols at high, and roughly equivalent levels, offers the opportunity for comparing the effects of different polyphenol phytonutrients in a common food matrix for protection against different diseases (Martin et al., 2011). This type of comparative nutrition analysis could be conducted through preclinical trials with a range of disease models such that the relative efficacy of each polyphenol type against different types of chronic diseases could be measured (for example using the ApoE mouse model for atherosclerosis, the High Fat Diet or ob/ob mouse models for obesity and MMTV-PyMT, MMTV-Neu, PTEN mouse models for specific types of cancer). Similar approaches could be used to establish any synergistic interactions between polyphenolic phytonutrients, which should help to give greater clarity for dietary recommendations using approved conversion calculations for dosages between animals and humans (Reagan-Shaw et al., 2007). The results from such comparisons could then form the foundation for randomized, controlled human intervention studies.

Some fresh tomato varieties have been bred by introgression to produce anthocyanins in the peel and sub-epidermal layers of the fruit, examples being Indigo Rose and Sunblack varieties. These have been commercialized with claims that they have been ‘specially bred for extra nutrition’. They contain the same antioxidants as blueberries. These tomatoes contain a special antioxidant called anthocyanin which is responsible for fighting diseases like diabetes and obesity. Although these statements are true, they are also misleading, because they take no account of the concentrations of anthocyanins present in these new tomato varieties. Their anthocyanins (which are exactly the same as those in the GE purple tomatoes reported by Butelli et al., 2008; Tohge et al., 2015; Zhang et al., 2015a,b) are not present in the tomato flesh and consequently the amounts are between 20- and 1000-fold less than in the GE tomatoes, and are therefore unlikely to confer any substantial health benefits to consumers, beyond their benefits as regular tomatoes.

### 3. Carotenoids

Out of > 700 carotenoids produced by plants, six (α-carotene, β-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin) seem to be important for human health. These also represent the most abundant carotenoids found in humans (Maiani et al., 2009). More than 40 carotenoids are taken up from a typical human diet, most of which are obtained from fruit and vegetables (Mangels et al., 1993; Johnson, 2002). Carotenoids are produced in chloroplasts which are abundant in leafy vegetables and chromoplasts (abundant in red, orange and yellow fruit and vegetables). The composition of carotenoids in chromoplasts is fairly constant but the carotenoid composition of chloroplasts differs widely between different plant species. Lycopene is a 40-carbon open-chain hydrocarbon carotenoid, with high lipophilic antioxidant capacity. Dietary sources rich in lycopene are watermelon, pink grapefruit, pink guava and papaya but, importantly, it is the red compound that accumulates in ripe tomatoes which accounts for c. 85% of the lycopene found in the human diet (Canene-Adams et al., 2005). At the red-ripe stage of tomato fruit development, lycopene accumulates to high concentrations due to induced expression of genes encoding specific isoforms of the enzymes involved in the synthesis of lycopene (Fig. 1). Interestingly, in tomato, genes encoding enzymes that metabolise lycopene to α-carotene and β-carotene are also switched off to facilitate lycopene accumulation in fruit (Hirschberg, 2001).

Lycopene can protect against the development of Type 2 diabetes and reduce the risk of prostate cancer (Etminan et al., 2004; Stacewicz-Sapuntzakis & Bowen, 2005). Lutein and zeaxanthin, which are richest in green leafy vegetables (such as spinach, broccoli, peas and lettuce), protect against the development of age-related macular degeneration (AMD), due to their selective accumulation in the macula of the retina of the eye (Bone & Landrum, 1992).

Because they are lipophilic, the bioavailability of carotenoids can be influenced by a wide range of factors. The bioavailabilities of β-carotene and lycopene from papaya have been reported to be approximately three-fold higher than the same compounds from tomatoes (Schweiggert et al., 2014). Even within the same species, natural variation and different cultivation practices can affect the composition and content of carotenoids in crops (Pott et al., 2003; Lenucci et al., 2006), which influence their bioavailability indirectly. Each step of food processing, from postharvest storage and thermal processing to product storage, may also affect carotenoid stability and bioavailability (Maiani et al., 2009).

Engineering carotenoid biosynthesis has been successfully achieved in several fruits and vegetables, in addition to the biofortification programs for β-carotene (provitamin A), described earlier. Massive accumulation of carotenoids in plants is associated with a very active endogenous isoprenoid biosynthetic pathway in plastids. The main approach to engineer the carotenoid pathway, as for β-carotene biofortification, has been to overexpress one or multiple biosynthetic genes (from either plants or bacteria) or combinations of genes from the two sources. PSY is thought to catalyse the key step in carotenoid biosynthesis (Cunningham & Gantt, 1998), which has made it the primary target for metabolic
engineering. Overexpression of a bacterial CrtB (PSY) in canola (B. napus), driven by a seed-specific, napin promoter and fused to a plastid-targeting peptide, achieved visibly orange seeds with up to 50-fold increase in carotenoid content (Shewmaker et al., 1999). Plastid-targeted expression of the CrtB (PSY)–CrtI (PDS)–CrtY (LYCB) bacterial mini-pathway in canola seeds led to increased β-carotene concentrations and higher β- to α-carotene ratios (Ravanello et al., 2003). Engineering several genes encoding biosynthetic enzymes has also been used in soybean, tobacco, lettuce and other species to increase specific carotenoid concentrations, in targeted tissues (Hasunuma et al., 2008; Kim et al., 2012; Harada et al., 2014).

Tomato ripening involves the visible breakdown of chlorophyll and build-up of carotenoids, with massive accumulation of antioxidant components such as lycopene and β-carotene within the chromoplasts (Egea et al., 2010). Different approaches combining conventional breeding and genetic engineering have been used to increase carotenoid content in tomato fruit (Zamir, 2001; Fraser et al., 2009). Although PSY1 has been shown to be a key regulator of carotenoid accumulation in tomato fruit, overexpression of CrtB (PSY) from Erwinia under the control of a fruit-specific promoter, did not increase lycopene content, but resulted in higher phytoene and β-carotene with higher total carotenoid concentrations. Transgenic tomatoes with overexpressed CrtI (PDS) had an increased content of β-carotene, amounting to up to 45% total carotenoids, at the expense of reduced lycopene content (Romer et al., 2000). In these two cases, unchanged or decreased lycopene content together with higher β-carotene accumulation suggested the limitations of adopting a ‘pull strategy’ alone, especially when the target compound (in these cases, lycopene) is not the end product of the pathway. By contrast, silencing of LCYB using antisense technology gave elevated lycopene concentrations in tomato fruit (a ‘protect strategy’) (Rosati et al., 2000).

Several studies have described regulation of carotenoid biosynthesis at the molecular level in plants (Cunningham & Gantt, 1998; Hirschberg, 2001; Liu et al., 2004b). Different types of regulatory mechanisms, operating at the transcriptional and post-transcriptional levels, have been suggested to be involved in accumulation of specific carotenoids (Sauret-Gueto et al., 2006; Lee et al., 2012). Overexpression of the Orange (Or) gene from cauliflower, in potato plants triggered β-carotene accumulation in tubers, by increasing PSY stability and chloroplast generation (Li et al., 2012). However, the regulatory mechanisms that control carotenoid accumulation remain poorly understood. Metabolic engineering of carotenoid biosynthesis would benefit enormously from the identification of regulatory mechanisms, particularly transcription factors specifically controlling carotenoid biosynthesis, to overcome the limitations on flux in particular tissues that are consumed as foods.

VI. Conclusions

In taking the area of nutritional enhancement forward, we need to start thinking about biofortification and nutritional enhancement of foods as parts of the same problem of global nutritional security. Improvements in the nutritional value of foods are likely to impact many more people positively than providing phytoneutrants as supplements or medicines. However, food is very complex. Understanding how it works to prevent and protect against disease is exceptionally challenging because of the diversity of what we eat, the large differences in the digestibility of what we eat, the role of the microbiome in the digestion of food, the influence of food composition on the constitution of an individual’s microbiome and the impact of an individual’s genome on their microbiome. Confounding our understanding further are differences in absorption and bioavailability of phytoneutrants, differences in the metabolism of nutrients by different individuals and differences in the mechanisms of action of different phytoneutrants.

Plant metabolic engineering can be used to investigate some of this complexity as well as to improve our understanding of the relationship between what we eat and our health. Plant metabolic engineering could also provide us with new foods with enhanced concentrations of phytoneutrants and essential vitamins, in the near future. Nutritional improvement can be achieved using not only genetic engineering, but also selection of appropriate natural variation and new breeding technologies, especially genome editing, or combinations of different technologies. Most nutritional enhancements of crops have, to date, focused on improving synthesis, and in the majority of cases, such improvements have been relatively modest (usually between two- to four-fold higher concentrations of the target phytoneutrant). However, in the case of polyphenols, the availability of transcription factors has allowed much larger changes in content. The identification of regulatory mechanisms, particularly the transcriptional control of biosynthesis of more phytoneutrants (ascorbate/vitamin C, tocochromanols, carotenoids) must therefore be a clear priority for the near future, both as loci for selection and for metabolic engineering of nutritionally improved varieties.

Even if nutritionally enhanced crops are produced and accepted by the public, real nutritional improvements for consumers will not result unless farmers can be persuaded to grow such crops in preference to established varieties. Consequently, nutritional improvements should not impact yield negatively, and although such ‘consumer traits’ may have boutique value, cultivation of these crops will not be sustained unless their nutritional traits can be bundled with improved agronomic or producer traits, for widespread adoption by growers. This point is particularly pertinent for biofortified crops and underpins current interest in combining resistance to major diseases with improved nutritional quality in staple crops. Alternatively, state aid or public investment may be necessary to move these crops from proof-of-principle to products, to ensure that their health benefits are available to consumers, even if the free market approach fails to deliver.

In the longer term, we should aim not only to improve the nutritional value of the crops that people eat in significant amounts, but also to provide adequate access by all to diverse foods, to avoid the scourge of dependency on staples and consequent hidden hunger, as well as reconsidering the benefits of traditional diets and dietary practices to promote global health and to protect from chronic disease.
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