Transgenic Strategies Towards Nutritional Enrichment of Crops

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A. Das, S. Thakur, K.R. Soren, S. Datta, and N P Singh

Abstract

All the essential nutrients in the human diet are ultimately derived from plants. However, most of the major food crops lack certain essential vitamins and minerals. Although a diversified diet regime provides adequate nutrition, majority of the population in developing countries relies on staple crops, such as rice, wheat, maize or cassava, which lacks full complement of essential nutrients. Malnutrition, thus, is a significant humanitarian issue in most of the developing world. A pertinent way to address this challenge is through *biofortification* of crops to increase their essential nutrient content. Transgenic approaches offer the most rapid and precise way to develop high-nutrient crops, thus complementing mineral fertilization and conventional breeding towards ameliorating the scourge.

Keywords

Biofortification • Minerals • Transgenic strategies

9.1 Introduction

Half of the human population worldwide has limited access to a healthy and fresh food. According to estimates for the period, 2011–2013, 842 million people worldwide (nearly one in eight people) suffered from chronic hunger (FAO 2014). In developing countries, the situation is further aggravated by limited fruit, vegetables, meat and fish, leading to reliance on a staple diet of cereals, such as rice, wheat, maize, cassava, etc. Milled cereal grains are poor sources of vitamins and minerals which are essential for normal growth and metabolism. Even in the developed countries, lifestyle choices

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A. Das (✉) • S. Thakur • K.R. Soren
Division of Plant Biotechnology, ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh 208 024, India
e-mail: alokbio@gmail.com; shallu.thakur85@gmail.com; sorenars@gmail.com

S. Datta
Division of Biotechnology, ICAR-Central Research Institute for Jute and Allied Fibres, Barrackpore, West Bengal 700 120, India
e-mail: subhojit@email.com

N P Singh
Director, ICAR-Indian Institute of Pulses Research, Kanpur, Uttar Pradesh 208 024, India
e-mail: npsingh.iipr@gmail.com

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and lack of education can lead to an improper diet, although this can be addressed to a certain degree by dietary supplements and fortification programs. Therefore, undernourishment/malnutrition and the deficiency diseases (e.g. anaemia, night blindness, beriberi, pellagra, scurvy, rickets, etc.) remain a significant public health challenge. With food security a major focus, it is timely to look at the strategies available to scientists to improve the nutritional value of food.

Dietary diversity and supplementation with vitamins and minerals are possibly the best ways to tackle malnutrition, but these appear to be impractical. An alternative approach is to tackle the problem of nutritionally poor crops at its source by increasing their nutritional qualities through a strategy known as 'biofortification', which should translate into improved diets. Several different strategies for biofortification have been advocated. The simplest strategy relies on an increase of the mineral content of plants through the addition of the appropriate mineral as an inorganic fertilizer. Though this has been reported to be successful in few instances, but it depends on the crop species and cultivar, the mineral itself and the quality and properties of the soil, making the strategy difficult to apply generally. Another strategy to improve the nutrient content of plants is by conventional breeding in combination with mutagenesis. The identification of genetically enriched varieties and the use of marker-assisted selection (MAS) to introgress such traits into widely cultivated, adapted germplasm have also been reported (e.g. golden sweet potato). Mutagenesis has also been used to enhance nutrient levels (e.g. lysine-enriched maize opaque-2 mutant). However, the strategies are slowed down by the time taken to identify useful traits and breed them into elite cultivars.

Transgenic strategies to enhance the nutritional value of crops are now being demonstrated (e.g. Golden Rice). Transgenic strategies differ from other approaches in that novel genetic information is introduced directly into the plant's genome, i.e. plants can be tailored to *green factories* for the synthesis of desired compounds. The approach depends on whether the nutritional compound is synthesized de novo by the plant or obtained from the environment. Organic molecules (amino acids, fatty acids and vitamins) are synthesized by the plant and hence increasing the nutritional value requires some form of *metabolic engineering* with the aim of increasing the amount of these desirable compounds, decreasing the amount of a competitive compound or even extending an existing metabolic pathway to generate a novel product. By contrast, mineral nutrients are obtained by the plant from the environment; therefore, mineral enhancement involves strategies to increase uptake, transport and/or accumulation in edible tissues. The following sections highlight reports of transgenic approaches utilized to enhance the nutrient content of crops.

9.2 Transgenic Approaches

9.2.1 Enrichment of Vitamins

Vitamins are small group of organic compounds that are required in the human diet. While considerable advances have been made in understanding vitamin metabolic pathways in plants, efforts to provide adequate levels are a grand challenge. In this section, two prominent examples of metabolic engineering of vitamins (vitamin A and vitamin E) are discussed.

Vitamin A (retinol) is an essential nutrient needed in small amounts by humans for the normal functioning of the visual system: growth and development, maintenance of epithelial cellular integrity, immune function and reproduction. Humans can synthesize vitamin A, if the precursor molecule β-carotene (also known as pro vitamin A), a pigment found in many plants but not in cereal grains, is provided. Therefore, a strategy was devised to introduce the correct metabolic steps into rice endosperm to facilitate β-carotene biosynthesis (Ye et al. 2000). An initial breakthrough was the development of a rice line expressing a daffodil (*Narcissus pseudonarcissus*) phytoene synthase, enabling the accumulation of the vitamin A precursor, phytoene in the endosperm, followed shortly thereafter by the original 'Golden Rice' variety, expressing two daffodil enzymes and one from *Erwinia uredovora*, which reconstituted the entire pathway enabling the rice endosperm to accumulate β-carotene, resulting in its eponymous golden colour (Fig. 9.1). In the best lines, the grain contained >1.5 mg of β-carotene per gram of dry weight. Subsequently, additional lines have been generated that contain only two recombinant enzymes (daffodil *phytoene synthase* and Erwinia *phytoene desaturase*). Later, 'Golden Rice II' variety was developed in which the daffodil *phytoene synthase* gene is replaced with its more efficient maize homolog, resulting in a 23-fold improvement in β-carotene content (up to 37 mg/g). This has led to similar progress in other crops, like '*super banana*', '*potato*', '*orange cauliflower*', carrots and tomatoes. Strategy for higher expression levels with the β-carotene metabolic pathway transferred to the plastids was also reported (Wurbs et al. 2007).

A recently developed potato variety containing the phytoene synthase, phytoene desaturase and lycopene β-cyclase from *Erwinia herbicola* contained 114 mg carotenoids per gram of dry weight and 47 mg β-carotene per gram of dry weight (Diretto et al. 2007). These studies showed that investigations into alternative gene sources and expression strategies can have a profound effect on achievable β-carotene levels. Although the original Golden Rice line was criticized because of the large amounts of rice that would need to be consumed, the latest fortified potato contains enough β-carotene to provide 50 % of the recommended daily allowance.

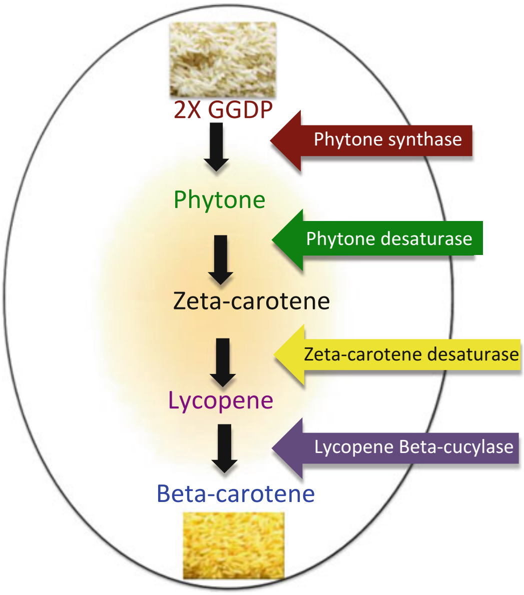

Fig. 9.1 Golden Rice for enrichment of vitamin A

Vitamin E is another group of extremely important compound. Vitamin E is a group of eight hydrophobic compounds, the most potent of which is α-tocopherol. Dietary vitamin E is obtained mainly from plant seeds and functions by preventing oxidation and polymerization of unsaturated fatty acids. Vitamin E deficiency leads to general wasting, kidney degeneration and infertility. In plants, tocopherol synthesis requires input from two metabolic pathways. Levels of vitamin E activity can be increased either by increasing the total amount of vitamin E or by shifting the metabolic flux towards α-tocopherol, as reported in *Arabidopsis*. Expression of *Synechocystis* PCC6803 and *Arabidopsis* genes encoding ϒ-tocopherol methyltransferase (ϒ-TMT) in *Arabidopsis* seeds, resulted in shift from ϒ/δ- to α/β-tocopherol, indicated that nutritional enhancement in plants was possible without altering total vitamin E levels (Shintani and Della-Penna 1998). However, the expression of *Arabidopsis* homogentisic acid prenyltransferase (HPT) produced twice the level of vitamin E found in normal seeds, whereas expression of the *Escherichia coli* tyrA gene, which encodes a dual-function enzyme (chorismate mutase and prephenate dehydrogenase), resulted in up to three times than the normal level of vitamin E (Savidge et al. 2002). Simultaneous expression of *Arabidopsis* genes encoding 2-methyl-6-phytylbenzoquinol (MPBQ) methyltransferase and ϒ-TMT in soybean showed a significant elevation in the total amount of vitamin E activity (fivefold greater than that of wild-type plants), which was attributable mainly to an eightfold increase in the levels of α-tocopherol, from its normal 10 % of total vitamin E to over 95 %.

Recently, plants have been engineered to accumulate several other vitamins like folate and ascorbate (vitamin C). Folate biofortification of lettuce by expression of chicken GTP cyclohydrolase I gene was reported (Nunes et al. 2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins (vitamin A, folate and ascorbate) representing three distinct metabolic pathways has also been reported (Naqvi et al. 2009).

9.2.2 Enrichment of Essential Amino Acids

Essential (indispensable) amino acids cannot be synthesized de novo by humans and therefore must be supplied by diet. The nine amino acids considered essential are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine and histidine. Most crops are deficient in certain essential amino acids like cereal grains, rice and wheat are deficient in lysine and threonine, whereas legumes such as peas and beans lack methionine and cysteine. Since majority of the world's population relies on cereal and legume combination as a staple diet, there is a great interest in developing novel varieties with increased essential amino acid content.

A simple approach is the expression of recombinant storage proteins with desirable amino acid profiles. Expression of pea (*Pisum sativum*) legumin (high lysine content), in rice and wheat grains, and the expression of sunflower seed albumin (rich in methionine), in the laboratory model lupin (Molvig et al. 1997), are some of the examples. Similarly, AmA1 from the Prince's feather (*Amaranthus hypochondriacus*), which encodes seed albumin, was expressed in potato and was shown to double the protein content and increase the levels of several essential amino acids (Chakroborty et al. 2000). However, this approach has limited success as shown by the expression of sunflower seed albumin in rice (Hagan et al. 2003) and chickpea (Chiaiese et al. 2004). There was little impact on the content of sulphur-containing amino acids, perhaps because of the regulatory mechanism that enables seed storage-protein composition to be adjusted in response to sulphur and nitrogen levels (Tabe et al. 2002). Synthetic proteins (i.e. proteins designed from first principles) can also be expressed to boost the levels of particular amino acids. For example, a synthetic protein matched to human amino acid requirements was expressed in cassava (Zhang et al. 2003). The inability of heterologous proteins to change abruptly and predictably the essential amino acid content of target crops probably reflects the limited free amino acid pool, which provides the substrates for protein synthesis.

As far as metabolic engineering is concerned, an understanding of biosynthetic pathway is very essential. In all higher plants, lysine, threonine and methionine are synthesized from aspartic acid via a highly branched pathway under complex feedback control mechanism. Two key enzymes are aspartate kinase (AK), which functions early in the pathway and is inhibited by both lysine and threonine, and dihydrodipicolinate synthase (DHPS), which functions in the lysine-specific branch and is inhibited by lysine alone. Feedback-insensitive homologs from bacterial system have been expressed in plants resulting in increase in the free lysine content of *Arabidopsis* seeds or by knocking out the lysine catabolism pathway, resulting in 12-fold or fivefold gain in lysine content. However, where both the transgene and knockout were combined in the line, surprisingly, increase of 80-fold over wild-type levels were achieved (Zhu and Galili 2003). Similarly, the expression of DHPS in maize increased the levels of free lysine with concomitant increases in threonine. Analogous approaches have increased the lysine levels in canola and soybean (Falco et al. 1995). Expression of a feedback-insensitive subunit of rice anthranilate synthase in rice resulted in twice accumulation of tryptophan as compared to the wild-type level in the grain (Wakasa et al. 2006). Thus, combination of the heterologous protein and amino acid pool approaches in a single plant may be pivotal to boost the levels of essential amino acids.

9.2.3 Enrichment of Essential and Polyunsaturated Fatty Acids

Essential fatty acids (EFAs) are fatty acids [alpha-linolenic acid (omega-3 fatty acid) and linolenic acid (omega-6 fatty acid)] that humans and other animals require for good health, but cannot synthesize them. Fatty acids are also target for biofortification because some of them are essential nutrients with diverse roles in metabolism, cardiovascular health, inflammatory responses, blood pressure regulation, etc. The metabolic pathway of fatty acid biosynthesis has been demystified (Wu et al. 2005), and transgenic strategies for the modification of oil and fat content in plants are reported, either by enhancing the level of the essential fatty acid linoleic acid and α-linolenic acid or to synthesize polyunsaturated fatty acids (PUFAs) like arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are usually sourced from fish.

Successful high level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids (EPA and DHA) was reported in oilseed crop, *Camelina sativa* (Ruiz-Lopez et al. 2014). The crop was transformed with five or seven genes for EPA and DHA biosynthesis: Δ6-desaturase gene from *O. tauri*, Δ6 fatty acid elongase gene from *P. patens*, Δ5-desaturase gene from *Thraustochytrium* spp., Δ12-desaturase gene from *P. sojae*, Δ3-desaturase from *P. infestans*, Δ5-fatty acid elongase gene from *O. tauri* and Δ5-desaturase gene from *E. huxleyi*. Genetically engineered seeds accumulate 12 % EPA and 14 % DHA, levels equivalent to those in fish oils, thus representing a sustainable source of fatty acids. However, the first breakthrough report of successful reconstitution of fatty acid synthesis pathway in *Arabidopsis* was obtained using the prokaryotic genes encoding D9-elongase, D8-desaturase and D5-desaturase (Abbadi et al. 2004). The elevated accumulation of VLC-PUFAs seemed to have no deleterious effect on plant growth. Another study exhibited lower enhancement by introducing genes from the conventional biosynthesis pathway under seed-specific promoter driven D6-desaturase, D6-elongase and D5-desaturase. This elevated the total level of D6-desaturated fatty acids, but EPA and ARA each accounted for <1 % of this fatty acid pool, which suggests a bottleneck during elongation; this bottleneck will be the target for future experiments.

Progress has also been made in the reconstitution of the DHA biosynthetic pathway in transgenic plants. *Arabidopsis* seeds with total fatty acids containing up to 0.5 % DHA were produced by expressing a bifunctional zebrafish D6/D5-desaturase and a dipteran D6-elongase in the seed, producing EPA for subsequent conversion into DHA by enzymes from the alga *Pavlova salina*. Utilizing similar strategy, DHA production in transgenic soybean seeds was obtained, but using a D6-elongase from the fungus *Mortierella alpina* and adding a v-3 microsomal desaturase from the fungus *Saprolegnia diclina* to maximize the accumulation of n-3 VLC-PUFAs. Another successful result has come from a study in which the VLC-PUFA biosynthetic pathway was reconstituted in *Brassica juncea* supplemented with a desaturase from *Phytophtora infestans*, a D12-desaturase from *Calendula officinalis* and an acyltransferase from *Thraustochytrium aureum*.

In each of these studies, there has been a striking increase in flux through the VLC-PUFA biosynthetic pathway, leading to the accumulation of EPA and DHA. Further investigation of pathway competition, feedback and branching will allow the development of plants with higher levels of DHA and other essential fatty acids in the future, presenting alternative to dwindling marine sources.

9.2.4 Enrichment of Minerals

Although *metabolic engineering* is the most suitable approach to enrich plants with organic nutrients, a different approach is required for minerals because they are not synthesized in the plant but obtained from the immediate environment. Transgenic strategies to increase the mineral content of crop plants have concentrated mainly on iron and zinc (which are most frequently deficient in human diets) and have used two distinct approaches, viz., increasing the efficiency of uptake and transport to harvestable tissues and increasing the amount of bioavailable mineral accumulating in the plant i.e. how much is accessible after digestion.

Iron, Fe (III), is the most abundant form of iron in the soil; unfortunately, plants cannot absorb iron in this state. Two different pathways are used to convert Fe (III) into Fe (II) for absorption: *Strategy I* involves the expression of Fe(III) reductases and the subsequent absorption of Fe (II) (all other plants), and *Strategy II* involves the secretion of chelating chemicals, called phytosiderophores, that bind to Fe(III) before absorption (graminaceous plants, i.e. grasses and cereals). Specific transport proteins are then used to absorb the minerals into the roots, and they are transported through the phloem to sink tissues, such as leaves in the form of complexes with nicotianamine, which specifically chelate Fe (II). The overexpression of these transport and chelating proteins promotes Fe accumulation. For example, efforts to increase iron uptake in roots by genetic modification have focused on the expression of iron transport proteins (Connolly et al. 2002). Alternatively, iron accumulation can be enhanced by the production of higher levels of phytosiderophores; for instance, the expression of the barley *naat*-A and *naat*-B genes, encoding nicotianamine aminotransferases (involved in phytosiderophore biosynthesis) in rice, resulted in increased iron uptake (Takahashi et al. 2001). Interestingly, there seems to be some crosstalk between the iron and zinc transport pathways because transgenic plants and mutants with overexpressed Fe (III) reductases and iron transporters also show increased zinc accumulation. This could reflect the enhanced synthesis of nicotianamine, which increases the mobilization of both metals in the vascular tissue. Thus, the overexpression of nicotianamine synthase also leads to iron and zinc accumulation; for example, the expression of barley *Hv*NAS1 in tobacco (*Nicotiana tabacum*) doubled the iron and zinc concentrations in leaves (Takahashi et al. 2003).

The second approach to mineral biofortification is to express recombinant proteins that enable minerals to be stored in a bioavailable form, for instance, overexpression of soybean ferritin in rice using an endosperm-specific promoter. Ferritin is an iron-storage protein. This produced rice grains with threefold levels of wild-type iron. Iron concentration was also measured in polished grains, because minerals are lost during polishing, but the levels of iron (and zinc) were still higher than in non-polished wild-type grains. By contrast, the use of a constitutive promoter to drive ferritin expression resulted in elevated iron levels in the leaves of transgenic rice plants, but not in grains, owing to higher expression levels of ferritin in vegetative tissues (Goto et al. 1999).

Another problem with mineral availability is its bioavailability, i.e. amount present in a form that can be utilized and absorbed by the human gut. Phytic acid (also known as phytate) is an anti-nutritional compound that chelates minerals and reduces their bioavailability in the gut. Therefore, a transgenic strategy has been developed that involves the expression of both ferritin and phytase (a fungal enzyme that breaks down phytate). Experiments with transgenic rice and maize indicated that the rice grains contained twice the wild-type amount of iron, and simulations of digestion and absorption using the maize kernels showed that the amount of bioavailable iron had also increased. The combined use of multiple strategies for iron fortification therefore provides the maximum levels of bioavailable iron (Drakakaki et al. 2000, 2005). Similar strategies can also be adopted for other minerals.

9.2.5 Improvement in Protein Quality in Pulses

Pulses are an important component of human nutrition because of high protein content (20–35 %). However, pulses are reported to have limited essential amino acids particularly methionine and cysteine. Strategies like metabolic engineering for increased methionine and cysteine content or/and expression of endogenous storage protein can provide potential alternative in this direction (Singh and Yadav 2010). Key regulatory proteins of essential amino acid biosynthesis pathway like cystathionine-γ-synthase (CGS), aspartate kinase (AK) and dihydrodipicolinate dehydrogenase (DHDPS) have already been engineered with insensitive homologs from other species, leading to increase in free Thr and Lys content (Fig. 9.2) (Zhu and Galili 2003). Alternatively, engineering of endogenous storage protein (like cereal *prolamins* and *glutamines*, legume *globulins*, etc.) by in vitro mutagenesis to mutate appropriate amino acid codons into essential amino acid codons (Met and Lys) or to insert stretches of additional codons of these amino acids appears promising. Inclusion of synthetic protein like ASP1 designed to have stable protein-like structure with desired amino acids profile can be another potential alternative. Single strategy or combination may be adopted for increased enrichment of pulses. However, potential biosafety (allergenicity) of the protein needs to be ascertained before such strategy is adopted.

9.3 Conclusion

Malnutrition is a big challenge particularly in the developing world, where measures like varied diet, fortification schemes and dietary supplements are difficult to realize. Transgenic biofortification strategies could help to alleviate malnutrition; however, regulatory and public perception issues need to be addressed. An extensive biosafety characterization of modified crops needs to be done addressing human health and allergenicity issues. Recent concepts of the use of *synthetic metabolons* could also greatly enhance the efficiency and increase the outcome of metabolic engineering in plants (Singleton et al. 2014). Engineering strategies have been implemented on a handful of plant species only and need to be transferred to highly consumed staple crops to maximally reach target population. Genetic engineering is not a silver bullet to eliminate malnutrition on its own; however, it can provide a significant component of integrated approaches towards alleviating malnutrition.

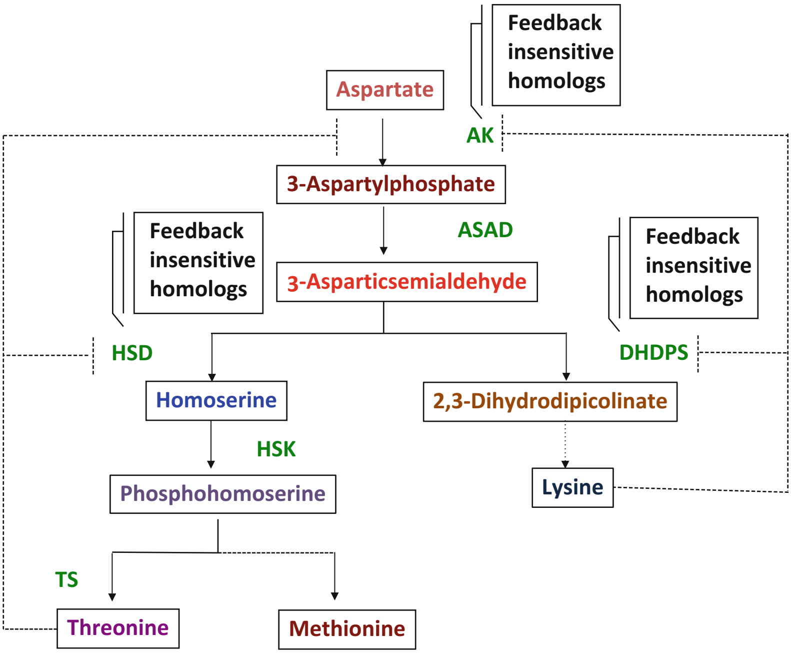

Fig. 9.2 Biosynthetic pathway for amino acid synthesis. Inclusions of feedback-insensitive homologs are the targets of metabolic engineering for enrichment of amino acids in plants

References

Abbadi A, Domergue F, Bauer J, Napier JA, Welti R, Zahringer U, Cirpus P, Heinz E (2004) Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. Plant Cell 16:2734–2748

Chakroborty S, Chakroborty N, Datta A (2000) Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from *Amaranthus hypochondriacus*. Proc Natl Acad Sci U S A 97:3724–3729

Chiaiese P, Ohkama-Ohtsu N, Molvig L, Godfree R, Dove H, Hocart C, Fuliwara T, Higgins TJ, Tabe LM (2004) Sulphur and nitrogen nutrition influence the response of chickpea seeds to an added, transgenic sink for organic sulphur. J Exp Bot 55:1889–1901

Connolly EL, Fett JP, Guerinot ML (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 14:1347–1357

Diretto G, Al-Babili S, Tavazza R, Papacchioli V, Beyer P, Giovanni G (2007) Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini pathway. PLoS One 2(4), e350

Drakakaki G, Christou P, Stoger E (2000) Constitutive expression of soybean ferritin cDNA in transgenic wheat and rice results in increased iron levels in vegetative tissues but not in seeds. Transgenic Res 9:445–452

Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fischer R, Christou P, Stoger E (2005) Endosperm-specific co-expression of recombinant soybean ferritin and *Aspergillus phytase* in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 59:869–880

Falco SC, Guida T, Locke M, Mauvais J, Sanders C, Ward RT, Webber P (1995) Transgenic canola and soybean seeds with increased lysine. Biotechnology (NY) 13 (6):577–582

FAO (2014) Food and Agriculture Organization of the United Nation. http://www.fao.org/

Goto F, Yoshihiro T, Shigemoto N, Toki S, Takaiwa F (1999) Iron fortification of rice seeds by the soybean ferritin gene. Nat Biotechnol 17:282–286

Hagan ND, Upadhyaya N, Tabe LM, Higgins TJ (2003) The redistribution of protein sulphur in transgenic rice expressing a gene for a foreign, sulphur-rich protein. Plant J 34:1–11

Molvig L, Tabe LM, Eggum BO, Moore AE, Craig S, Spencer D, Higgins TJV (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. Proc Natl Acad Sci U S A 94:8393–8398

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L et al (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. PNAS (USA) 106:7762–7767

Nunes AC, Kalkmann DC, Aragao FJ (2009) Folate biofortification of lettuce by expression of a codon optimized chicken GTP cyclohydrolase I gene. Transgenic Res 18:661–667

Ruiz-Lopez N, Haslam RP, Napier JA, Sayanova O (2014) Successful high level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. Plant J 77:198–208

Savidge B et al (2002) Isolation and characterization of homogentisate phytyltransferase genes from *Synechocystis* sp. PCC 6803 and Arabidopsis. Plant Physiol 129:321–332

Shintani D, Della-Penna D (1998) Elevating the vitamin E content of plants through metabolic engineering. Science 282:2098–2100

Singh NP, Yadav IS (2010) Strategy for improving the nutritional quality of grain legumes through genetic engineering. Trends Biosci 3:1–4

Singleton C, Howard TP, Smirnoff N (2014) Synthetic metabolons for metabolic engineering. J Exp Bot 65:1947–1954

Tabe L, Hagan N, Higgins TJ (2002) Plasticity of seed protein composition in response to nitrogen and sulphur availability. Curr Opin Plant Biol 5:212–217

Takahashi M, Nakanishi H, Kawasaki S, Nishizawa NK, Mori S (2001) Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. Nat Biotechnol 19:466–469

Takahashi M, Terada Y, Nakai I, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK (2003) Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. Plant Cell 15:1263–1280

Wakasa K, Hasegawa H, Nemoto H, Matsuda F, Miyazawa H (2006) High-level tryptophan accumulation in seeds of transgenic rice and its limited effects on agronomic traits and seed metabolite profile. J Exp Bot 57:3069–3078

Wu G, Truska M, Datla N, Vriten P, Bauer J, Zank T, Cirpus P, Heinz E, Qiu X (2005) Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants. Nat Biotechnol 23:1013–1017

Wurbs D, Ruf S, Bock R (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. Plant J 49:276–288

Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287(5451):303–305

Zhang P, Jaynes JM, Potrykus I, Gruissem W, Puonti-Kaerlas J (2003) Transfer and expression of an artificial storage protein (asp1) gene in cassava (*Manihot esculenta* Crantz). Transgenic Res 12:243–250

Zhu X, Galili G (2003) Increased lysine synthesis coupled with a knockout of its catabolism synergistically boosts lysine content and also *trans* regulates the metabolism of other amino acids in Arabidopsis seeds. Plant Cell 15:845–853