Plant Synthetic Metabolic Engineering for Enhancing Crop Nutritional Quality

Qinlong Zhu1,2, Bin Wang1, Jiantao Tan1, Taoli Liu1, Li L13,4 and Yao-Guang Liu1,2,*

1State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources; College of Life Sciences, South China Agricultural University, Guangzhou 510642, China
2Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510642, China
3Robert W. Holley Center for Agriculture and Health, USDA-ARS, Cornell University, Ithaca, NY 14850, USA
4Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14850, USA

*Correspondence: Yao-Guang Liu (ygliu@scau.edu.cn)
https://doi.org/10.1016/j.xplc.2019.100017

ABSTRACT

Nutrient deficiencies in crops are a serious threat to human health, especially for populations in poor areas. To overcome this problem, the development of crops with nutrient-enhanced traits is imperative. Biofortification of crops to improve nutritional quality helps combat nutrient deficiencies by increasing the levels of specific nutrient components. Compared with agronomic practices and conventional plant breeding, plant metabolic engineering and synthetic biology strategies are more effective and accurate in synthesizing specific micronutrients, phytonutrients, and/or bioactive components in crops. In this review, we discuss recent progress in the field of plant synthetic metabolic engineering, specifically in terms of research strategies of multigene stacking tools and engineering complex metabolic pathways, with a focus on improving traits related to micronutrients, phytonutrients, and bioactive components. Advances and innovations in plant synthetic metabolic engineering would facilitate the development of nutrient-enriched crops to meet the nutritional needs of humans.

Key words: plant metabolic engineering, synthetic biology, transgene stacking, crop biofortification

Zhu Q., Wang B., Tan J., Liu T., Li L., and Liu Y.-G. (2020). Plant Synthetic Metabolic Engineering for Enhancing Crop Nutritional Quality. Plant Comm. 1, 100017.

INTRODUCTION

From the beginning of crop domestication, humans have learned to improve crops to meet their changing needs. Since the Green Revolution, traditional breeding and the use of chemicals have dramatically increased crop yield, enabling modern society to achieve food security. However, the reduction of agricultural biodiversity results in imbalance of nutrients, thereby increasing the risk of micronutrient and phytonutrient malnutrition among consumers. The Food and Agriculture Organization of the United Nations estimates that approximately 792 million people worldwide are malnourished, out of whom 780 million live in developing countries (McGuire, 2015). Also, about two billion people around the world suffer from “hidden hunger,” resulting from insufficient intake of micronutrients (vitamins and minerals) in the daily diet (Muthayya et al., 2013; Hodge, 2016). Since the beginning of the twenty-first century, biofortification has been used as an additional and complementary strategy to produce “healthier foods.” Biofortification is the process by which nutritionally enhanced food crops are developed and grown using modern biotechnology, traditional plant breeding, and agronomic practices (Saltzman et al., 2017). Biofortification differs from conventional fortifications, which increases the nutrient levels in crops by breeding and cultivation, rather than by artificial means during food processing.

Long breeding cycles and limited nutrient content variability limit the application of traditional breeding for crop nutritional quality improvement. Modern biotechnological methods (metabolic engineering and synthetic biology) provide alternative and effective approaches for the development of biofortified crops (Garg et al., 2018). Metabolic engineering is the process to improve or implement the production of target compounds in vivo through modulating one or more genes or gene networks (Farré et al., 2014; Fu et al., 2018). Synthetic biology is defined as design and creation of new biological pathways to biosynthesize new compounds in organisms (Liu and Stewart, 2015; Hanson and Jez, 2018). There is overlap between the two fields. At present, most plant-based approaches for developing and producing new bioactive components require a combination of synthetic biology and metabolic engineering (Figure 1). This combined method can
be called “synthetic metabolic engineering” (Nielsen and Keasling, 2011; Ye et al., 2012; Prouvera et al., 2018). Synthetic metabolic engineering involves three major steps (Figure 2): to learn about the metabolic pathways or genetic pathways that exist in natural organisms; to reconstruct (design, assemble, and transform) the artificial metabolic pathways in the target host organisms; and to test the practical effects of the reconstructed pathways in the transformed target organisms (Prouvera et al., 2018; Mortimer, 2019). After the first cycle, the “learn” step analyses the results of the cycle to enhance system-level knowledge and drive subsequent learn/reconstruction/test (L-R-T) cycles. The iterative L-R-T cycles promote the development of better synthetic metabolic engineering approaches. A good example of the use of this cyclic approach is the development of Astaxanthin Rice using the knowledge gained from the development of Golden Rice. The first cycle involved learning the \( \beta \)-carotene biosynthesis in plants, yeast, and bacteria; reconstructing the pathway using a plant \( PSY \) gene encoding phytoene synthase and a bacterial \( CrtI \) gene encoding \( \beta \)-carotene desaturase in rice; and then evaluating the \( \beta \)-carotene product in the transgenic rice (Ye et al., 2000; Paine et al., 2005). The first cycle enhanced our understanding of the carotenoid synthetic pathway. On the basis of this \( \beta \)-carotene engineering, we continued to learn about astaxanthin biosynthesis in algae and yeast, reconstructed the astaxanthin pathway in rice using \( PSY \), \( CrtI \), \( BHY \) (encoding \( \beta \)-carotene hydroxylase), and \( BKT \) (encoding \( \beta \)-carotene ketolase) genes, and finally successfully produced astaxanthin in rice endosperm (Zhu et al., 2018) (Figure 2).

With the evolution of molecular biotechnology, a large number of new research tools and strategies have been exploited to analyze and reconstruct metabolic pathways or genetic circuits in target organisms, and this has accelerated the development of synthetic metabolic biology (García-Granados et al., 2019). In this review, we discuss the latest advances in crop biofortification using new strategies and technologies for synthetic metabolic engineering.

**STRAATEGIES FOR METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY IN PLANTS**

Plant metabolic engineering and synthetic biology involve manipulation of one or more key genes or rate-limiting enzyme genes in metabolic or synthetic pathways, and even all the genes that make up the entire pathways. Therefore, the selections of different key genes and the methods to deliver and express multiple genes in host plants will affect the development of plant synthetic metabolic engineering, which is discussed in the following two sections.

**Strategies for Synthetic Metabolic Engineering**

A metabolic pathway is a chemical chain reaction in which a series of enzymes catalyzes the conversion of substrates to target products and possible by-products in a certain order. Therefore, to increase the synthesis of target compounds, several strategies are usually used: (1) increasing the expression of upstream genes encoding rate-limiting enzymes or multiple key enzymes in the target pathway to ensure adequate supply of precursors and increase metabolic flux through the target pathway; (2) inhibiting the expression of, or knocking out, the key enzyme genes involved in the competitive pathway of the branch point or the catabolic pathway of the target product, to avoid intermediates being diverted and prevent the decomposition of target metabolites; (3) overexpressing transcription factors (or together with certain key enzyme genes) to simultaneously activate multiple pathway-related endogenous key genes to enhance the synthesis of the metabolites; and (4) combining the above methods to maximize the synthesis of the target compounds. In addition, CRISPR/dCas9-based activation/repression systems can also be used in metabolic engineering manipulation (Zalatan et al., 2015; Li et al., 2017). These strategies are shown in Figure 3.

**Strategies for Multigene Transformation**

Genetic engineering of complex metabolic pathways in plants often requires simultaneous expression of multiple target genes, or even entire genes in a pathway to ensure unrestricted metabolic flux. For this purpose, different strategies for multigene stacking have been developed (Figure 4), including the early time-consuming and labor-intensive iterative strategies (sexual crossing and re-transformation) and co-transformation (using particle bombardment and mixed \( Agrobacterium \)-transformation), and the current multigene vector transformation (multiple gene expression cassettes being linked in a single T-DNA region), polycistronic transgenes (using self-cleave peptide 2A to link different protein sequences) and plastid transformation (Dafny-Yelin and Tzfira,
Among these strategies, the multigene vector transformation has a profound advantage over the other methods (Dafny-Yelin et al., 2007), which makes the multiple transgenes being integrated and inherited as a single unit. The multigene vector approach requires the assembly of multiple target genes in single T-DNA regions of binary vectors for *Agrobacterium*-mediated transformation, or in single plasmid vectors for other transformation methods (see section below). Plastid transformation is another effective method for certain purposes, but it requires transferring multiple genes into the plastid genomes. The differences, advantages, and disadvantages of the multigene vectors and plastid transformation have been described elsewhere (Boehm and Bock, 2019). Because of the obvious advantages of the multigene vector transformation, this approach is more widely used in many current crop improvement projects.

**PLANT MULTIGENE TRANSFORMATION VECTOR SYSTEMS**

*Agrobacterium* binary vectors are the crucial tool for plant genetic transformation. Delivering multiple genes using a single binary vector has a significant advantage over the application of the other multigene transformation strategies as discussed above. By only a single transformation, all genes assembled into the T-DNA region of a multigene vector can be simultaneously integrated into a single chromosomal site in the host plant genome, and these genes are inherited together. Therefore, only a small number of transgenic plants are needed to select ideal transgenic events. Although the assembly and transformation of a large transformation construct carrying multiple genes have been a challenge, various multigene vector systems using a variety of approaches have been developed to facilitate the stacking and delivery of different numbers of genes into plants (as exemplified below, Figure 5). Currently, these approaches have employed the use of rare-cutting homing endonucleases and zinc-finger nucleases (Zeevi et al., 2012), type IIS restriction enzymes (Golden Gate cloning) (Engler et al., 2008), Cre/loxP recombination (Lin et al., 2003; Zhu et al., 2017), Gateway recombination (Chen et al., 2006; Wakasa et al., 2006), Gibson Assembly (Gibson et al., 2009), and homologous recombination in yeast (Shih et al., 2016). The multigene vector systems with easy manipulation and large DNA-carrying capacity (e.g., using artificial chromosomes as vector backbone) have more advantages. An example is the recently developed TransGene Stacking II (TGS II) system (Zhu et al., 2017; Zhu and Qian, 2017; Shen et al., 2019).

**Using Restriction Enzyme-Based Assembly**

Various gene expression cassettes can be cloned into a multigene binary plasmid by using rare-cutting restriction enzymes. Examples include the pAUX/pRCS and pSAT vector systems that use rare-cutting homing endonucleases (Goderis et al., 2002; Tzfira et al., 2005; Dafny-Yelin et al., 2007), and their upgrade pRCS11.1 vector system (Figure 5) using homing endonucleases and zinc-finger nucleases (Zeevi et al., 2012). Although these vectors can assemble a small number of genes, the overall efficiency is low, and it is time-consuming due to the low specificity and low cutting efficiency of the rare-cutting endonucleases. In addition, the BioBrick method using isocaudarners (e.g., Xba I and Spe I) (Knight, 2003) and Golden Gate-related systems using the type IIS restriction enzymes (e.g., Bsa I, Esp3 I, and Bbs I, which recognize 6-bp sites and can generate 4-nt non-palindromic sticky ends) (Engler et al., 2008; Emami et al., 2013; Sarrion-Perdigones et al., 2013) also can assemble small DNA fragments or several genes; these methods are more commonly used in microbial metabolic engineering. However, the high frequent presence of these restriction-cutting sites in plant genomic DNA sequences limits the use of these methods in plants (Liu et al., 2013; Ghareeb et al., 2016).

**Using Cre Site-Specific Recombination for Multigene Stacking**

The strategy using a site-specific recombinase for multigene stacking, mainly including Cre/loxP system and Gateway
recombination, can overcome the limitations of the restriction-ligation methods. In a previous report, Lin et al. (2003) developed the first transgene-stacking system that uses a combination of the Cre/loxP-mediated recombination system and two homing endonucleases (I-Sce I and PI-Sce I). This system contains a transformation-competent artificial chromosome (TAC)-based acceptor vector and two donor delivery vectors; the donor vectors with target genes are used alternately to recombine with the acceptor vector to sequentially stack multiple genes into the acceptor vector. Because of the operational difficulty in using homing endonuclease digestion and linker ligation for the plasmid circularization, the utilization of this early vector system to assemble multigenes (more than five) remained a challenge.

To solve these problems, on the basis of this early system, Zhu et al. (2017) recently developed a simpler and high-efficient multigene assembly and transformation vector system, TGS II. TGS II used the Cre-mediated recombination system with the wild-type loxP sites and mutant loxP sites (for irreversible recombination), which can automatically remove the donor vector backbones during the multigene assembly cycles in a special Escherichia coli strain (that expresses the Cre enzyme) (Figure 5). In addition, a selection marker gene-deletion construct can be introduced into the acceptor vector (with assembled target genes) by a Gateway reaction to obtain marker-free transgenic plants. There are some other Cre/loxP-mediated multigene systems, for examples, the MISSA system based on conjugational transfer using the Cre/loxP recombination for multigene assembly and Gateway recombination for deletion of the donor vector backbone in vivo (Chen et al., 2010), and the recombinase-mediated in planta transgene-stacking strategy that requires multirounds of plant transformation (Ow, 2011). Among these systems, TGS II is the most simple one in operation and high efficient for multiple gene assembly, thus is suitable to engineer complex metabolic pathways (Zhu and Qian, 2017; Fang et al., 2018). This system has been successfully used to synthesize anthocyanins, canthaxanthin, and astaxanthin in rice endosperm by stacking and transferring 10 foreign genes (C24 31 kb) involved in anthocyanin biosynthesis, and 4 and 6 ketocarotenoid biosynthetic foreign genes (C24 15–19 kb), respectively, into rice (Zhu et al., 2017, 2018). Using this system to stack multiple genes of photorespiration, Shen et al. (2019) developed a new rice germplasm with high light efficiency and productivity. Furthermore, the TGS II system currently is the only reported multigene vector system that is marker gene-free competent.

Figure 3. Strategies for Synthesizing Target Products in Plants.
Ⓐ, Ⓑ, and Ⓒ are the precursors of a target product Ⓓ. ⒴ is a branch or by-product. The key enzymes of the pathway are indicated as E1–E6.
(A) Enhancing the activity of the rate-limiting enzyme (E5) by overexpression (OX) or improving upstream precursor content by overexpression of one or more key enzymes (such as E4-OX).
(B) Increasing upstream precursors by overexpressing one or more rate-limiting enzymes (E1), and simultaneously blocking the branch point (E6) by RNA interference or CRISPR/Cas-mediated knockout (KO), to enhance the metabolic flux in the main pathway.
(C) Improving levels of the precursors by overexpressing transcription factors (TFs) to activate multiple key enzyme genes of the pathway, and simultaneously knockdown/knockout of the branch point (E6).
(D) Comprehensively increasing contents of the precursors by simultaneously overexpressing all key enzyme genes and the transcription factor genes, along with blocking the branch point.
(E) Enhanced accumulation of the target metabolite by overexpressing a possible multifunctional or fused enzyme (Ex) that can catalyze Ⓓ into ⒴, and blocking the original reaction steps, so as the consumption of the intermediates is reduced, together with improving rate-limiting enzymes activity by overexpression and blocking the by-product production.
Using Gateway Recombination Methods

The Gateway recombination system is another commonly used method to prepare multigene vectors using commercial recombinase LR clonase for attLa and attRs site reactions and BP clonase for attBa and attPs site reactions (Walhout et al., 2000). The multisite Gateway system consists of a set of destination binary vectors with several different modified attR sites and a set of three entry plasmids (each carrying a different attL sites); this system uses LR clonase reaction to assemble several genes in a single recombination step (Wakasa et al., 2006). However, this system cannot stack more genes due to the limited number of available att sites (Dafny-Yelin et al., 2007). Another Gateway-based system is MultiRound Gateway strategy that alternates use of two different Gateway entry vectors to sequentially assemble multiple genes into one Gateway destination vector by multiple rounds recombination reactions (Chen et al., 2006). Recently, Collier et al. (2018) reported an Agrobacterium-based GAANTRY system (gene assembly in Agrobacterium by nucleic acid transfer using recombinase technology) for assembling multigene into the T-DNA of an Agrobacterium rhizogenes pRi virulence plasmid by in vivo MultiRound Gateway BP recombination. The assembly strategy of MultiRound Gateway and GAANTRY system is similar to Cre/loxP-based multigene vector system (Lin et al., 2003), except for the use of different recombinases.

Using Homologous Recombination in Yeast

Yeast homologous recombination in vivo is a robust technique to assemble large linearized DNA fragments (Gibson et al., 2008; Shao et al., 2009). For example, Gibson et al. (2008) reported the complete synthesis of the Mycoplasma genitalium genome (580 kb) by one-step assembly of 25 overlapping large DNA fragments in yeast. For utilizing this method, a jStack vector system was developed for plant multigene assembly, which uses yeast-compatible plant binary pYB vectors modified from the pCAMBIA2301 binary vector by adding yeast replication and selection systems (Shih et al., 2016). By co-transferring linearized multiple DNA fragments and pYB vector backbone with overlapping homologous sequences, multiple DNA molecules can be assembled into pYB vector in yeast in a single step (Figure 5). This approach allows efficient assembly of multiple genes or DNA fragments, but the size of the assembled DNA molecules hardly exceeds 20 kb, limited by the carrying capability of the pBR322 backbone of pYB. Therefore, it is possible to improve this system for carrying more genes by adopting the TAC-based vector backbone.

Using Overlapping DNA Sequence Assembly

The overlapping DNA assemblies use the “chew-back-and-anneal” strategy, which requires digesting one strand of overlapping DNA ends to produce short complementary end sequences for annealing without ligation. These methods include a uracil-specific excision reagent (Nour-Eldin et al., 2010), InFusion (Sleight et al., 2010), sequence- and ligase-independent cloning (Li and Elledge, 2007), and Gibson Assembly (Gibson et al., 2009). They are suitable for the assembly of small DNA fragments or gene expression cassettes. Among these...
(A) The TransGene Stacking II system for multigene assembly based on Cre/loxP-mediated reversible and irreversible recombination in an E. coli strain (NS3529) expressing Cre (Zhu et al., 2017). The first round assembly (round I) includes the processes: (i) Cre/loxP recombination of the pYL322d1-A/B plasmid (containing target genes A and B) into the pYLTAC380GW acceptor binary plasmid, and (ii) automatic removing of the pYL322d1 backbone by irreversible recombination between the mutant loxP1L(1L) and loxP1R(1R) sites. These processes form the first recombinant plasmid pYLTAC380GW-A/B. Round II: Cre/loxP recombination of the pYL322d2-C/D plasmid into pYLTAC380GW-A/B and deletion of the pYL322d2 backbone by irreversible recombination between the mutant loxP1L(2L) and loxP1R(2R) sites to form plasmid pYLTAC380GW-A/B/C/D. Round III is similar with round I. After all target genes are assembled by repeated assembly cycles, the selectable marker/marker-excision cassette on the marker-free donor plasmid is recombined into the acceptor vector by Gateway BP reaction to generate the final binary construct. In transgenic plants, the Cre expression driven by the pollen-specific promoter Pv4 (or another inducible promoter) deletes the marker/Cre cassettes.

(B) The pRCS11.1 vector system using homing endonucleases and zinc-finger nucleases (Zeevi et al., 2012). Assembly of a multigene binary plasmid is achieved by successive cloning of various gene expression cassettes into pRCS11.1 vector using cutting and ligation.

(C) Multigene assembly using homologous recombination in yeast. Various gene expression cassettes with homologous end sequences and the linearized pYB vector are co-transferred into yeast cells, and the target genes can be recombined into the pYB vector in vivo.

(D) Gibson cloning for multiple genes (Gibson et al., 2009). Multiple genes or fragments with overlapping end sequences (20–30 bp) are mixed with a linearized vector, then the collaborative reactions of a 5’ exonuclease, a DNA polymerase, and a DNA ligase produce single-strand ends of the overlapping sequences, annealing between the ends, filling of the gaps, and finally ligating the nicks to complete the multigene assembly.
Metabolic Engineering for Enhancing Crops

methods, Gibson Assembly is a powerful technique for joining multiple overlapping DNA fragments simultaneously in a single reaction to synthesize large DNA molecules \textit{in vitro}. Gibson Assembly uses a mixed reagent with a 5’ exonuclease for generating single strands of overlapping sequences, a DNA polymerase for filling gaps of annealed products, and a DNA ligase for sealing the nicks (Figure 5). Nevertheless, application of these approaches is limited for the assembly of DNA fragments with repetitive sequences. Also, as the number of DNA fragments to be assembled in one reaction increases, the efficiency and accuracy rate decrease (Liu et al., 2013; Ghareeb et al., 2016).

ENGINEERING PHYTONUTRIENTS AND MICRONUTRIENTS IN CROPS

Phytonutrients (nutraceuticals) and micronutrients play important roles in human nutrition and health, but they are often deficient to various degrees in major staple crops (Mattoo et al., 2010). Moreover, some health-promoting nutraceuticals (e.g., astaxanthin) are lacking in food crops. Therefore, biofortification is of great significance for enhancing nutritional and health-promoting qualities of food crops. The use of synthetic metabolic engineering is a suitable strategy for such endeavor (de Steur et al., 2017). At present, a variety of biofortified crops have been developed through these approaches, such as the most well-known β-carotene-enriched “Golden Rice” (Ye et al., 2000; Paine et al., 2005), anthocyanin-enriched “Purple Tomatoes” (Butelli et al., 2008), anthocyanin-enriched “Purple Endosperm Rice” (Zhu et al., 2017), and astaxanthin-enriched “aSTARice” (Zhu et al., 2016).

Flavonoid and Anthocyanin Biofortification in Crops

Anthocyanins are a class of flavonoid compounds widely distributed in fruits and vegetables, which have strong antioxidant properties for promoting human health (Deng et al., 2013). In cereal grains, anthocyanins are only present in the pericarp of particular species or varieties (such as black rice, black corn, and purple wheat), and are completely absent in cereal endosperms. In addition, the health-promoting properties of the grains are further reduced due to the habit of eating refined grains without the pericarp in East Asian people. Anthocyanin biosynthesis is one of the well-understood metabolic pathways (Figure 6). It involves multiple structural genes (encoding enzymes for forming a series of anthocyanin metabolites), decorating genes and transporters, as well as the crucial transcription factors that form an MYB-bHLH-WD40 (MBW) complex to control anthocyanin structural gene expression (Hichri et al., 2011; Dixon et al., 2013; Zhang et al., 2014; Yuan and Grotewold, 2015). By using the key structural genes (Muir et al., 2001; Ogo et al., 2013), the transcription factor genes (Bovy et al., 2002; Butelli et al., 2008; Zhang et al., 2015; Jian et al., 2019), or the key structural genes plus the transcription factor genes (Zhu et al., 2017; Liu et al., 2018a, 2018b), biofortification with flavonoids and anthocyanins have been achieved in some crops (e.g., rice, maize, and tomato) (Table 1).

Anthocyanins naturally exist in many fruits and vegetables, but not in fruit of most tomato cultivars. Overexpression of a single key enzyme gene or a transcription factor gene (such as the petunia chalcone isomerase or the regulator AtMYB12) (Muir et al., 2001; Zhang et al., 2015), or transcription factor genes of the regulatory complex (such as maize anthocyanin transcription factors Lc and C1) (Bovy et al., 2003), all could increase the content of flavonols in tomato, but did not produce anthocyanins. However, co-expression of the snapdragon anthocyanin regulator complex genes AmDel and AmRos1 (Butelli et al., 2008), or expression of the tomato regulatory gene SiMYB75 (Jian et al., 2019), could achieve a large accumulation of anthocyanins in tomato fruit (Figure 6B). These results suggest that the ability of the transcription factors from different sources is different in activating the structural genes. Although the genetic manipulation is simple, the strategy of only using the transcription factor genes or their combination may not be successful.

Compared with anthocyanin-engineering of tomato, biofortification of cereal crops with anthocyanins is more complicated and difficult. For example, in rice endosperm, some of the structural and transcription factor genes involved in the anthocyanin biosynthetic pathway are functionally defective. As a result, expression of structural genes or co-expression of the bHLH- and MYB-type regulatory genes (such as maize anthocyanin regulators ZmR-S and ZmC1) could not complete the target anthocyanin biosynthesis, but only produced the intermediate flavonoid products (the anthocyanin upstream precursors) in the rice endosperm (Shin et al., 2006; Ogo et al., 2013).

Recently, we explored a new strategy involving transformation of the maize regulatory genes (ZmLC and ZmPl) and a complete set of the six structural genes from the coleus anthocyanin biosynthesis pathway, all driven by endosperm-specific promoters (Zhu et al., 2017). By using the multigene stacking system TGS II, the eight genes were assembled and transformed into rice to generate a novel biofortified germplasm Purple Endosperm Rice with high anthocyanin content and antioxidant activity in the endosperm (Figure 6C and 6D). Using a similar strategy, the embryo and endosperm anthocyanin-enriched “Purple Maize” (Figure 6E) was developed by using seed-specific bidirectional promoters to drive the target gene coding sequences linked by the self-cleavage peptide 2A linker (Liu et al., 2018a, 2018b). These results suggest that the strategy of using transcription factor genes plus multiple structural genes has a wider adaptability in anthocyanin metabolic engineering. With this strategy, it is also possible to obtain other purple endosperm cereals.

Carotenoid Biofortification in Crops

Carotenoids are a large class of important lipid-soluble phytonutrients that play important roles in promoting human nutrition and health. For example, β-carotene is the precursor for vitamin A synthesis and astaxanthin is the most powerful antioxidant. Carotenoids are rich in vegetables and fruits but low in cereal grains (Saltzman et al., 2017). Therefore, biofortification of carotenoids in crop grains is crucial and urgent. Carotenoid biosynthesis is another well-studied pathway (Figure 7A). The use of metabolic engineering strategies for carotenoid enrichment is an effective method, such as the famous β-carotene-enriched Golden Rice (Ye et al., 2000; Paine et al., 2005).
expanding the plastid metabolic sink strength (Lu et al., 2006; Zhou et al., 2015; Yazdani et al., 2019). Thus, the main
transcription factors (Stanley and Yuan, 2019), except for the
regulators genes, little is known about the regulation of the key enzymes in the core carotenoid biosyn-
thesis are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanone 3’-hydroxylase (F3’H), and flavanone 3’-hydroxylase (F3’H). Anthocyanins are synthesized by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). Decorating genes encoding enzymes such as GT (glucosyltransferase) and MT (methyltransferase) are involved in the formation of stable anthocyanins from anthocyanidins. GST, glutathione S-transferase. The flavonoid intermediate products, flavonols, are synthesized by flavonol synthase (FLS) from dihydroflavonol (marked by gray background) and flavones are synthesized by flavone synthase (FNS). The transcription regulators form an MBW complex (MYB-bHLH-WD40) to control the expression of the anthocyanin biosynthesis pathway genes.

Although the key enzymes genes in the core carotenoid biosynthesis pathway have been cloned, little is known about the regulator genes (Stanley and Yuan, 2019), except for the ORANGE (OR) gene that encodes a key regulator for carotenoid accumulation by interacting with the plastid metabolic sink strength (Lu et al., 2006; Zhou et al., 2015; Yazdani et al., 2019). Thus, the main engineering strategy for carotenoid biofortification is to use a combination of multiple key enzyme genes from plants, algae, and microbe, to directly synthesize target products in crops, by Agrobacterium-mediated multigene vector transformation (Huang et al., 2013; Pierce et al., 2015; Zhu et al., 2018; Ha et al., 2019; Tian et al., 2019) or particle bombardment (e.g., maize and wheat) (Zhu et al., 2008; Wang et al., 2014). One notable example is the *de novo* synthesis of astaxanthin in rice endosperm (Zhu et al., 2018; Ha et al., 2019).

Astaxanthin is a red-colored ketocarotenoid synthesized from β-carotene in astaxanthin-producing organisms, and has very high antioxidant activity. However, astaxanthin is not synthesized in most higher plants due to lacking the *BKT* gene (encoding β-carotene ketolase) (Zhu et al., 2009). Recently, Zhu et al. (2018) reported the first real *de novo* synthesis of astaxanthin in rice endosperm to produce a rice variety *aSTARice*, by using the TGS II vector system by assembling and transferring four synthetic gene expression cassettes (PSY1, *CrtI*, *BHY*, and *BKT*), driven by the rice endosperm-specific promoters, into transgenic rice. In addition, Ha et al. (2019) used the polycistronic transgene strategy, which used the fused genes linking with self-cleavage peptide 2A, to produce lower content of astaxanthin in rice endosperm.

In the past 20 years since the generation of Golden Rice, a number of biofortified crops have been developed for production of various nutritive carotenoids (Table 2). However, the contents of the synthesized carotenoids in the biofortified crops, especially in rice, are still not high enough for better human nutrition and health. Therefore, introduction of more genes for increasing contents of the precursors (e.g., the upstream genes *DXS* and *HGRM*) (Bai et al., 2016; Tian et al., 2019), for improving the carotenoid stability (e.g., the *HGGT* gene) Che et al. (2016), and for effective regulators (e.g., OR) (Kim et al., 2019; Yazdani et al., 2019), may produce higher levels and more stable carotenoids in biofortified crops. Further research and

---

**Figure 6. Anthocyanins Biofortification in Crops.**

(A) The anthocyanin biosynthesis pathway in plants. The key enzymes of general upstream phenylpropanoid pathway include phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaryl CoA ligase (4CL). Structural genes encoding enzymes involved in anthocyanin biosynthesis are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanone 3’-hydroxylase (F3’H), and flavanone 3’-hydroxylase (F3’H). Anthocyanins are synthesized by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). Decorating genes encoding enzymes such as GT (glucosyltransferase) and MT (methyltransferase) are involved in the formation of stable anthocyanins from anthocyanidins. GST, glutathione S-transferase. The flavonoid intermediate products, flavonols, are synthesized by flavonol synthase (FLS) from dihydroflavonol (marked by gray background) and flavones are synthesized by flavone synthase (FNS). The transcription regulators form an MBW complex (MYB-bHLH-WD40) to control the expression of the anthocyanin biosynthesis pathway genes.

(B) The anthocyanin biofortified purple tomato by fruit-specific co-expression of the anthocyanin regulators *AmRos1* and *AmDel* (Butelli et al., 2008). (C and D) Purple anthocyanins accumulated in the transgenic developing rice seeds (C), and polished and cross-sections of the anthocyanin biofortified purple rice endosperm (D), by introducing two maize regulator genes (*ZmPI* and *ZmLC*), and six anthocyanin structural genes (*SsCHS*, *SsCHI*, *SsF3H*, *SsF3’H*, *SsDFR*, and *SsANS* from coleus), all driven by endosperm-specific promoters (Zhu et al., 2017). (E) Grains and cross-sections of purple embryo and endosperm of maize by bidirectional promoter driving co-expression of four maize anthocyanin biosynthetic genes (*ZmR2*, *ZmC1*, *ZmBz1*, and *ZmBz2*) linked by a 2A self-cleavage peptide (Liu et al., 2018a, 2018b). (B–E) Reproduced with permission from Butelli et al. (2008), Zhu et al. (2017), and Liu et al. (2018a, 2018b), respectively.
| Species | Transgene<sup>a</sup> | Promoters | Transgene assembly and transformation | Target tissue | Major products | Total content (μg/g DW) | Reference |
|---------|----------------|-----------|---------------------------------------|---------------|---------------|------------------------|-----------|
| Rice    | ZmR-S, ZmC1 | Endosperm-specific promoter npr33 | Binary vector and Agrobacterium-mediated transformation | Pericarp (dark brown) and the outer layer endosperm | Flavonoids (dihydroquercetin, 3'-O-methyl dihydroquercetin, and 3'-O-methylquercetin) | 12 800<sup>*</sup> | Shin et al. (2006) |
|         | OsPAL, OsCHS | Endosperm-specific promoter GluB-1 | Multigene vectors (Multisite Gateway) and Agrobacterium-mediated transformation | Endosperm | Flavonoids (Naringenin) | 1–12 | Ogo et al. (2013) |
|         | OsPAL, OsCHS, AtF3H, AtFLS | Endosperm-specific promoter GluB-1 | Seed-specific promoter Ole | Endosperm | Flavonol (Kaempferol) | 10–60 | |
|         | OsPAL, OsCHS, GmIFS | Endosperm-specific promoter GluB-1 | Seed-specific promoter Ole | Endosperm | Isoflavone (genistein) | 10–40 | |
|         | OsPAL, OsCHS, PcFNSI, GmFNSII | Endosperm-specific promoter GluB-1 | Seed-specific promoter Ole | Endosperm | Flavone (Apigenin) | 40–120 | |
|         | OsPAL, OsCHS, PcFNSI, GmFNSII, OsOMT, ViolaF3’5’H | Endosperm-specific promoter GluB-1 | Seed-specific promoter Ole | Endosperm | Flavone (Tricin) | 110 | |
| Maize   | ZmC1, ZmR2, ZmANS, ZmGST | Embryo-specific bidirectional promoter P<sub>2R5SGPA</sub> | Multigene vectors (Cre/loxP-based TGS II system) and Agrobacterium-mediated transformation | Endosperm | Anthocyanin (cyanidin 3-O-glucoside and peonidin 3-O-glucoside) | ~1000 | Zhu et al. (2017) |

Table 1. Flavonoid and Anthocyanin Biofortification in Major Transgenic Crops.

(Continued on next page)
understanding of the mechanism underlying the regulation of carotenoid metabolism, combined with the use of metabolic engineering strategies and new research tools (e.g., genome editing), will dramatically improve our capacity to manipulate plant carotenoid biosynthesis for crop biofortification. Some carotenoid-enriched biofortified crops (e.g., rice, maize, wheat, tomato, and soybean) are shown in Figure 7B–7F, and summarized in Table 2.

Vitamin Biofortification in Crops

Vitamins (e.g., vitamin B9, vitamin B6, vitamin C, and vitamin E) are a group of essential micronutrients for the growth and development of animals. Human cannot synthesize these compounds, thus must obtain them from the diet, especially from plant foods (Garcia-Casal et al., 2017). However, as the main dietary components for human population, the most consumed stable crops and many edible plants deliver inadequate amounts of these micronutrients (Strobbe et al., 2018). Biofortification via genetic engineering provides a crucial tool to enrich crop vitamins in the fight against micronutrient malnutrition.

At present, the contents of some vitamins in certain crops have been improved by increasing the biosynthetic efficiency and promoting the storage stability though metabolic engineering methods (Strobbe and Van Der Straeten, 2018). Expressing one or multiple key enzyme genes involved in folate (vitamin B9) biosynthesis and stability, i.e., GTPCHI (GTP cyclohydrolase I), ADCS (aminodeoxychorismate synthase), FPGS (folypolyglutamate synthetase), or FBP (folate binding protein), significantly increases the amount of folates in rice, maize, potato, and tomato (Diaz de la Garza et al., 2004; Storozhenko et al., 2007; Naqvi et al., 2009; Blancquaert et al., 2015; De Lepeleire et al., 2018). For example, in rice, expression of the transgene GTPCHI resulted in only about 10-fold increase of the folate content, but co-expression of GTPCHI and ADCS led to 100-fold folate enhancement (Storozhenko et al., 2007). When simultaneously expressing three genes (GTPCHI, ADCS, and FPGS) or four genes (GTPCHI, ADCS, FPGS, and FBP), the folate levels were increased by 100- and 150-fold, respectively, and the product was more stable during post-harvest storage (Blancquaert et al., 2015). Thus, the multigene stacking strategy is a high-efficiency method for folate biofortification in crops.

Similarly, overexpressing the PDX gene (encoding a pyridoxal phosphate synthase, a key enzyme of vitamin B6 biosynthesis) also could significantly increase the content of vitamin B6 in casava (Chen and Xiong, 2009; Li et al., 2015). In addition, using a similar strategy expressing one or more key enzyme genes involved in vitamin C or E biosynthesis, vitamin C biofortification was achieved in tomato, potato, and maize (Naqvi et al., 2009; Qin et al., 2011; Bulley et al., 2012), and vitamin E content was enhanced in rice, maize, and barley (Babura et al., 2017; Chen et al., 2017; Strobbe et al., 2018).

Omega-3 Fish Oil in Crops

Omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFAs), eicosapentaenoic acid (EPA) (20:5n-3), and docosahexaenoic acid (DHA) (22:6n-3), play a vital role in human health and development, and the lack of these fatty acids increases the risk
or severity of cardiovascular and inflammatory diseases (Ruxton et al., 2007). However, these two unsaturated fatty acids are not present in higher plants (Napier et al., 2015), and their intake depends only on marine fish oil. The research on engineering plants with accumulation of LC-PUFAs began in the late 1990s (Broun et al., 1999). Biosynthesis of EPA and DHA involves many enzymes that do not exist in higher plants. Recently, several research groups have successfully synthesized EPA and DHA in *Arabidopsis*, *Brassica juncea*, *Camelina sativa*, and canola (Ruiz-Lopez et al., 2012; Ruiz-Lopez et al., 2013; Amjad Khan et al., 2017). Two different strategies are employed: one is the introduction of seven desaturase and elongase genes from algae or fungal (Wu et al., 2005; Petrie et al., 2012, 2014; Ruiz-Lopez et al., 2014); another is transferring three unsaturated fatty acid synthase subunit genes and one phosphopantetheinyl transferase gene from microalgae (Walsh et al., 2016). Currently, several research groups in the U.K., the USA, and Australia have performed animal feeding experiments and confirmed that the plant-derived sources of EPA and DHA can completely replace marine fish oil (Betancor et al., 2015; Tejera et al., 2016).

### Mineral Iron and Zinc Biofortification in Crops

Iron and zinc are essential elements for many metabolic processes in human (Underwood, 1977; Prasad, 1978). Among the top 10 global risk factors for disease burden, zinc and iron
| Species | Transgene | Transgene assembly and transformation | Target tissue | Target products, µg/g DW (fold change relative WT) | Total carotenoid, µg/g DW (fold change relative WT) | Reference |
|---------|-----------|--------------------------------------|---------------|---------------------------------------------------|-----------------------------------------------|-----------|
| Rice    | ZmPSY1, PaCrtI | Multigene vectors (restriction- ligation) and Agrobacterium-mediated transformation | Endosperm | \( \beta \)-Carotene, 30.9 | 36.7 | Paine et al. (2005) |
|         | sCaPSY, sPaCrtI | Multigene vectors (linked by 2A) and Agrobacterium-mediated transformation | | \( \beta \)-Carotene, 2.35 | 4.18 | |
|         | ZmPSY1, PaCrtI, tHMGR1 | Multigene vectors (restriction- ligation) and Agrobacterium-mediated transformation | | \( \beta \)-Carotene, 10.5 | 14.5 | Tian et al. (2019) |
|         | ZmPSY1, PaCrtI, sCrBKT | Co-transformation and particle bombardment | | Canthaxanthin, 4.0 | 8.8 | |
|         | sZmPSY1, sPaCrtI, sCrBKT | Multigene vectors (Cre/loxP-based TGS II system), and Agrobacterium-mediated transformation | | Canthaxanthin, 25.8 | 31.4 | Zhu et al. (2018) |
|         | sZmPSY1, sPaCrtI, sCrBKT, sHpBHY | Multigene vectors (polyacistronic transgene with 2A) and Agrobacterium-mediated transformation | | Astaxanthin, 16.2 | 21.9 | |
|         | CaPSY, PaCrtI, sCaBch, stHpBHY | Multigene vectors (polyacistronic transgene with 2A) and Agrobacterium-mediated transformation | | Zeaxanthin, 0.8 | 1.9 | Ha et al. (2019) |
|         | CaPSY, PaCrtI, stCaBch, stHpBHY | Intercrossing a CaPSY, PaCrtI, stCaBch transgenic rice line with a CaCcs transgenic rice line | | Astaxanthin, 1.1 | 1.8 | |
|         | CaPSY, PaCrtI, CaBch, CaCcs | Intercrossing a CaPSY, PaCrtI, stCaBch transgenic rice line with a CaCcs transgenic rice line | | Capsanthin, 0.33 | 2.2 | |
| Maize   | ZmPSY1, PaCrtI | Co-transformation and particle bombardment | Endosperm | \( \beta \)-Carotene, 57.35 (410) | 156.1 (142) | Zhu et al. (2008) |
|         | ZmPSY1, PaCrtI, GiLycB | Co-transformation and particle bombardment | | \( \beta \)-Carotene, 48.87 (349) | 148.8 (135) | |
|         | ZmPSY1, PaCrtI, GiLycB, CrtW | Co-transformation and particle bombardment | | Astaxanthin, 4.5 | 146.8 (134) | |
|         | ZmPSY1, CrtZ, BKT, RNAi-ZmLycE | Co-transformation and particle bombardment | Endosperm and kernels | Astaxanthin, 16.8 | 35.1 (2.6) | |
|         | ZmPSY1, PaCrtI | Co-transformation and particle bombardment | Endosperm | \( \beta \)-Carotene, 59.3 (148) | 163.2 (109) | Naqvi et al. (2009) |
| Wheat   | CrtB, CrtI | Co-transformation and particle bombardment | Endosperm | \( \beta \)-Carotene, 3.2 (64) | 4.1 (7) | Wang et al. (2014) |
|         | CrtB | Particle bombardment | | \( \beta \)-Carotene, 2.7 (16) | 7.4 (6) | |
|         | RNAi-BHY | Particle bombardment | | \( \beta \)-Carotene, 2.3 (14) | 3.7 (3) | |
|         | CrtB, RNAi-BHY | Co-transformation and particle bombardment | | \( \beta \)-Carotene, 5.6 (35) | 9.3 (8) | |

Table 2. Carotenoid Biofortification in Major Transgenic Crops.
| Species   | Transgene | Transgene assembly and transformation | Target tissue | Target products, µg/g DW (fold change relative WT) | Total carotenoid, µg/g DW (fold change relative WT) | Reference |
|-----------|-----------|--------------------------------------|---------------|---------------------------------------------------|-----------------------------------------------------|-----------|
| Sorghum   | ZmPSY1, PaCrtI | Multigene vectors (multiple Gateway) and Agrobacterium-mediated transformation | Endosperm     | β-Carotene, 9.1 (8)                                | 26.3 (4)                                           | Che et al. (2016) |
|           | ZmPSY1, PaCrtI, AtDXS |                                       |               | β-Carotene, 5.2 (11)                               | 26.41 (3)                                          |           |
|           | ZmPSY1, PaCrtI, AtDXS, HGGT |                                       |               | β-Carotene, 9.3 (19)                               | 31.7 (6)                                           |           |
| Soybean   | CrtB, CrtW | Co-transformation and particle bombardment | Seed          | Canthaxanthin, 52, β-Carotene, 666                | 915 (61)                                            | Pierce et al. (2015) |
|           | CrtB, BKT |                                       |               | Canthaxanthin, 45, β-Carotene, 195                | 324 (22)                                            |           |
| Tomato    | CrtI      | Binary vectors and Agrobacterium-mediated transformation | Fruit         | β-Carotene, 520 (2)                               | 1372 (0.5)                                         |           |
|           | CrtB      |                                       |               | β-Carotene, 825 (3)                               | 5912 (2)                                           |           |
|           | SiLycB    |                                       |               | β-Carotene, 205 (47)                              | 215 (2)                                            |           |
|           | CrtBKT    |                                       |               | Canthaxanthin, 2249.7, Astaxanthin, 926.1        | 5813.1 (5)                                         | Huang et al. (2013) |
|           | CrBKT, HpBHY |                                       |               | Canthaxanthin, 338.4, Astaxanthin, 16 104.7    | 19 054.4 (17)                                      |           |
|           | AtORWT    | Agrobacterium-mediated transformation  |               | β-Carotene, 22.6                                 | 90.8 (1)                                           | Yazdani et al. (2019) |
|           | AtORHis   |                                       |               | β-Carotene, 25.6                                 | 160.7 (2)                                          |           |
| Potato    | OR        | Binary vectors (restriction- ligation) and Agrobacterium-mediated transformation | Tuber          | Zeaxanthine, 67.6 (1.6)                           | 88.3 (1.6)                                         |           |
|           | CrtZ, CrtW |                                       |               | Astaxanthin, 28                                  | 57.7 (1.1)                                         |           |
|           | CrtZ, CrtW, OR |                                       |               | Astaxanthin, 48.6                                | 93.5 (1.7)                                         |           |

Table 2. Continued

AtORWT, Arabidopsis thaliana wild-type OR; AtORHis, Arabidopsis thaliana OR with mutant at R90H; AtDXS, Arabidopsis thaliana 1-deoxy-D-xylulose 5-phosphate synthase gene; CaPSY, Capsicum annum phytoene synthase gene; CaBch, Capsicum annum β-carotene hydroxylase gene; CaCcs, Capsicum annum capsanthin-capsorubin synthase gene; CrtB, bacterial phytoene synthase gene; CrtI, bacterial phytoene desaturase gene; CrtW, bacterial β-carotene ketolase gene; CrtZ, bacterial β-carotene hydroxylase gene; CrBKT, Chlamydomonas reinhardtii β-carotene ketolase gene; HGGT, homogentisate geranylgeranyl transferase gene; HpBHY, Haematococcus pluvialis β-carotene hydroxylase gene; OR, cauliflower ORANGE gene; PaCrtI, Pantoea ananatis phytoene desaturase gene; SiLycB, Solanum lycopersicum lycopene β-cyclase gene; sCrBKT, a rice codon-optimized synthetic Chlamydomonas reinhardtii β-carotene ketolase gene; sCaBch, a rice codon-optimized synthetic Capsicum annum β-carotene hydroxylase gene; stHpBKT, a rice codon-optimized synthetic Haematococcus pluvialis β-carotene ketolase gene; stCaBch, a rice codon-optimized synthetic Haematococcus pluvialis β-carotene hydroxylase gene; sPaCrtI, a rice codon-optimized synthetic Pantoea ananatis phytoene desaturase gene; sZmPSY1, a rice codon-optimized synthetic Zea may phytoene synthase gene; tHMGR, truncated 3-hydroxy-3-methylglutaryl coenzyme A reductase from Saccharomyces cerevisiae; ZmPSY1, synthetic Zea may phytoene synthase gene; ZmLycE, Zea may lycopene ε-cyclase gene.

*Rice endosperm does not contain carotenoids.

*In wild-type soybean (cv. Jack), lutein is only detected.
deficiencies rank fifth and sixth, respectively. Plants absorb these minerals from the surrounding environment. The transgenic strategy to increase the contents of iron and zinc in crops is mainly to improve the absorption and utilization efficiency of iron and zinc by enhancing the expression of related transporter genes (Kerkeb et al., 2008; Blancquaert et al., 2017), and reducing the contents of anti-nutrient factors (e.g., phytic acid) (Aluru et al., 2011). In addition, the co-expression of Lactoferrin (iron-chelating glycoprotein) and FERRITIN also increases the iron contents in crops (Goto et al., 1999; Drakakaki et al., 2000; Lucca et al., 2001; Borg et al., 2012). Simultaneous expression of FERRITIN and NAS (nicotianamine synthase) increases not only zinc but also iron content in crops (Lee et al., 2009; Wirth et al., 2009; Zheng et al., 2010). The combined expressions of four genes, FERRITIN, NAS, PSY, and CrtI, significantly increased iron, zinc, and β-carotene contents in transgenic rice, producing a multinutrient-enriched biofortified crop (Singh et al., 2017). Using these methods, a number of biofortified crops (such as rice, maize, and wheat) with enriched iron and zinc have been developed (Kumar et al., 2019). The multiple nutrient-biofortified crops have great potential for combating global human mineral deficiencies.

CURRENT CHALLENGES AND OPPORTUNITIES

Although significant progress has been made in using synthetic metabolic engineering to biofortify crops, there are still some challenges (Garcia-Granados et al., 2019). The primary problem is the lack of understanding of metabolic pathways and key regulators in an organism. Although more and more plant genomes have been sequenced, the lack of effective gene functional annotations makes it difficult to determine the composition of genes encoding key enzymes in complete metabolic pathways. The combined analysis of genomics, transcriptomics, proteomics, and metabolomics will accelerate the understanding of metabolic pathways and their key components (Farre et al., 2014). The constitutive synthesis of metabolites is likely to cause abnormal cell development and growth in plants. At the same time, metabolic pathways are susceptible to influence by feedback regulation and other factors. The development and application of transgenes expressed in specific tissues can accomplish the synthesis and accumulation of metabolites in specific tissues and avoid their negative effects on normal plant growth (Peremarti et al., 2010). Furthermore, the development of synthetic metabolic engineering is also limited by molecular techniques. Most metabolic pathways involve multiple regulatory factors and enzymes. Thus, the use of the high-efficiency multigene expression vector systems (e.g., the TGS II system) and the CRISPR gene editing tool (e.g., CRISPR/dCas9-based knockout or transcriptional activation or inhibition) can simultaneously enable the expression and regulation of the upstream and downstream genes of entire metabolic pathways in more flexible and precise ways (Zalatan et al., 2015; Li et al., 2017; Zhu et al., 2017).

With deep understanding of biosynthetic pathways and increased advances in metabolic engineering technology, synthetic metabolic engineering will more accurately achieve the reconstruction and regulation of multistep complex metabolic networks, and produce more novel varieties of biofortified crops with multiple nutrients (such as phytoneutrients, vitamins, minerals, and functional nutraceuticals), which will meet the needs for better human nutrition and health.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (31971915) and the Major Program of Guangdong Basic and Applied Research (2019B030302006).

ACKNOWLEDGMENTS

No conflict of interest declared.

Received: November 16, 2019
Revised: December 5, 2019
Accepted: December 17, 2019
Published: January 13, 2020

REFERENCES

Aluru, M.R., Rodermel, S.R., and Reddy, M.B. (2011). Genetic modification of low phytic acid 1-1 maize to enhance iron content and bioavailability. J. Agric. Food Chem. 59:12954–12962.

Amjad Khan, W., Chun-Mei, H., Khan, N., Iqbal, A., Lyu, S.W., and Shah, F. (2017). Bioengineered plants can be a useful source of omega-3 fatty acids. Biomed. Res. Int. 2017:7348919.

Babura, S.R., Abdullah, S.N.A., and Khaza Ai, H. (2017). Advances in genetic improvement for tocotrienol production: a review. J. Nutr. Sci. Vitaminol. (Tokyo) 63:215–221.

Bai, C., Capell, T., Berman, J., Medina, V., Sandmann, G., Christou, P., and Zhu, C. (2016). Bottlenecks in carotenoid biosynthesis and accumulation in rice endosperm are influenced by the precursor-product balance. Plant Biotechnol. J. 14:195–205.

Betancor, M.B., Sprague, M., Usher, S., Sayanova, O., Campbell, P.J., Napier, J.A., and Tocher, D.R. (2015). A nutritionally-enhanced oil from transgenic Camelina sativa effectively replaces fish oil as a source of eicosapentaenoic acid for fish. Sci. Rep. 5:8104.

Blancquaert, D., Van Dalee, J., Strobbe, S., Kieken, F., Storozhenko, S., De Steur, H., Gellynck, X., Lambert, W., Stove, C., and Van Der Straeten, D. (2015). Improving folate (vitamin B-9) stability in biofortified rice through metabolic engineering. Nat. Biotechnol. 33:1076–1078.

Blancquaert, D., De Steur, H., Gellynck, X., and Van Der Straeten, D. (2017). Metabolic engineering of micronutrients in crop plants. Ann. N. Y. Acad. Sci. 1390:59–73.

Bock, R. (2013). Strategies for metabolic pathway engineering with multiple transgenes. Plant Mol. Biol. 83:21–31.

Boehm, C.R., and Bock, R. (2019). Recent advances and current challenges in synthetic biology of the plastid genetic system and metabolism. Plant Physiol. 179:794–802.

Borg, S., Brinch-Pedersen, H., Tauris, B., Madsen, L.H., Darbani, B., Noeparvar, S., and Holm, P.B. (2012). Wheat ferritins, improving the iron content of the wheat grain. J. Cereal Sci. 56:204–213.

Bovy, A., de Vos, R., Kemper, M., Schijlen, E., Almenar Pertejo, M., Muir, S., Collins, G., Robinson, S., Verhoeven, M., Hughes, S., et al. (2002). High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Physiol. 129:2509–2526.

Broun, P., Gettner, S., and Somerville, C. (1999). Genetic engineering of plant lipids. Annu. Rev. Nutr. 19:197–216.

Bulley, S., Wright, M., Rommens, C., Yan, H., Rassam, M., Lin-Wang, K., Andre, C., Brewster, D., Karunairetnam, S., Allan, A.C., et al. (2012). Enhancing ascorbate in fruits and tubers through
Metabolic Engineering for Enhancing Crops

overexpression of the L-galactose pathway gene GDP-L-galactosephosphorylase. Plant Biotechnol. J. 10:390–397.

Butelli, E., Titta, L., Giorgio, M., Mock, H.P., Matros, A., Peterek, S., Schijlen, E.G., Hall, R.D., Bovy, A.G., Luo, J., et al. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat. Biotechnol. 26:1301–1308.

Che, P., Zhao, Z.Y., Glassman, K., Dolde, D., Hu, T.X., Jones, T.J., Gruis, D.F., Obukosia, S., Wambuugu, F., and Albertsen, M.C. (2016). Elevated vitamin E content improves all-trans β-carotene accumulation and stability in biofortified sorghum. Proc. Natl. Acad. Sci. U S A 113:11040–11045.

Chen, H., and Xiong, L. (2009). Enhancement of vitamin B6 levels in seeds through metabolic engineering. Plant Biotechnol. J. 7:673–681.

Chen, Q.J., Zhou, H.M., Chen, J., and Wang, X.C. (2006). A Gateway-based platform for multigene plant transformation. Plant Mol. Biol. 62:927–936.

Chen, Q.J., Xie, M., Ma, X.X., Dong, L., Chen, J., and Wang, X.C. (2010). MISSA is a highly efficient in vivo DNA assembly method for plant multiple-gene transformation. Plant Physiol. 153:41–51.

Chen, J., Liu, C., Shi, B., Chai, Y., Han, N., Zhu, M., and Bian, H. (2017). Overexpression of HvHGT enhances tocotrienol levels and antioxidant activity in barley. J. Agric. Food Chem. 65:5181–5187.

Collier, R., Thomson, J.G., and Thilmony, R. (2018). A versatile and robust Agrobacterium-based gene stacking system generates high-quality transgenic Arabidopsis plants. Plant J. 95:573–583.

Dafny-Yelin, M., and Tzfira, T. (2007). Delivery of multiple transgenes to plant cells. Plant Physiol. 145:1118–1128.

Dafny-Yelin, M., Chung, S.M., Frankman, E.L., and Tzfira, T. (2007). pSAT RNA interference vectors: a modular series for multiple gene down-regulation in plants. Plant Physiol. 145:1272–1281.

Diaz de la Garza, R.D., Quinlivan, E.P., Klaus, S.M.J., Basset, G.J.C., Deng, G.F., Xu, X.R., Zhang, Y., Li, D., Gan, R.Y., and Li, H.B. (2017). Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. Plant Physiol. 173:573–583.

Engler, C., Kandzia, R., and Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. PLoS One 3:e3647.

Fang, X., Ma, Y., and Chen, X. (2018). Engineering purple rice for human health. Sci. China Life Sci. 61:365–367.

Farré, G., Blancquaert, D., Capell, T., Van Der Straeten, D., Christou, P., and Zhu, C. (2014). Engineering complex metabolic pathways in plants. Annu. Rev. Plant Biol. 65:187–223.

Fu, R., Martin, C., and Zhang, Y. (2018). Next-generation plant metabolic engineering, inspired by an Ancient Chinese irrigation system. Mol. Plant 11:47–57.

Garcia-Casal, M.N., Peña-Rosas, J.P., and Giyose, B.; consultation working groups (2017). Staple crops biofortified with increased vitamins and minerals: considerations for a public health strategy. Ann. N. Y. Acad. Sci. 1390:3–13.

Garcia-Granados, R., Lerma-Escalera, J.A., and Morones-Ramirez, J.R. (2019). Metabolic engineering and synthetic biology: synergies, future, and challenges. Front. Bioeng. Biotechnol. 7:36.

Garg, M., Sharma, N., Sharma, S., Kapoor, P., Kumar, A., Chunduri, V., and Arora, P. (2018). Biofortified crops generated by breeding, agronomy, and transgenic approaches are improving lives of millions of people around the world. Front. Nutr. 5:12.

Ghareeb, H., Laukamm, S., and Lipka, V. (2016). COLORFUL-circuit: a platform for rapid multigene assembly, delivery, and expression in plants. Front. Plant Sci. 7:246.

Gibson, D.G., Benders, G.A., Axelrod, K.C., Zaveri, J., Algire, M.A., Moodie, M., Montague, M.G., Venter, J.C., Smith, H.O., and Hutchinson, C.A. (2008). One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic Mycoplasma genitalium genome. Proc. Natl. Acad. Sci. U S A 105:20404–20409.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchinson, C.A., and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6:343–345.

Goderis, I.J., de Bolle, M.F., Francois, I.E., Wouters, P.F., Broekaert, W.F., and Cammue, B.P. (2009). A versatile and modular plant transformation vectors for plant synthetic biology. Transgenic Res. 18:9

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., and Lauvergeat, V. (2011). Recent advances in the transcriptional regulation of the zeaxanthin, astaxanthin and capsanthin in rice endosperm. Metab. Eng. 52:178–189.

Hanson, A.D., and Jez, J.M. (2018). Synthetic biology meets plant metabolism. Plant Sci. 273:1–2.

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., and Lauvergeat, V. (2011). Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J. Exp. Bot. 62:2465–2483.

Hodge, J. (2016). Hidden hunger: approaches to tackling micronutrient deficiencies. In Nourishing Millions: Stories of Change in Nutrition, S. Golden Gate Agrobacterium vectors for plant synthetic biology. Front. Plant Sci. 4:339.

Engler, C., Kandzia, R., and Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. PLoS One 3:e3647.

Fang, X., Ma, Y., and Chen, X. (2018). Engineering purple rice for human health. Sci. China Life Sci. 61:365–367.
Plant Communications

Kim, S.E., Kim, H.S., Wang, Z., Ke, Q., Lee, C.J., Park, S.U., Lim, Y.H., Park, W.S., Ahn, M.J., and Kwak, S.S. (2019). A single amino acid change at position 96 (Arg to His) of the sweetpotato Orange protein leads to carotenoid overaccumulation. Plant Cell Rep. 38:1393–1402.

Knight, T. (2003). Idempotent vector design for standard assembly of Biobricks, DSpace@MIT [online]. http://dspace.mit.edu/handle/1721.1/21168.

Kumar, S., Palve, A., Joshi, C., Srivastava, R.K., and Rukhsar. (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods 4:251–256.

Lee, S., Jeon, U.S., Lee, S.J., Kim, Y.K., Persson, D.P., Husted, S., Schjørring, J.K., Kakei, Y., Masuda, H., Nishizawa, N.K., et al. (2009). Iron fortification of rice seeds through activation of the nicotianamine synthase gene. Proc. Natl. Acad. Sci. U S A 106:22014–22019.

Li, M.Z., and Eldledge, S.J. (2007). Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods 4:251–256.

Li, K.T., Moulin, M., Mangel, N., Albersen, M., Verhoeven-Duif, N.M., Ma, Q., Zhang, P., Fitzpatrick, T.B., Gruissem, W., and Vanderschuren, H. (2015). Increased bioavailable vitamin B6 in field-grown transgenic cassava for dietary sufficiency. Nat. Biotechnol. 33:1029–1032.

Li, Z., Zhang, D., Xiong, X., Yan, B., Xie, W., Sheen, J., and Li, J.F. (2017). A potent Cas9-derived gene activator for plant and mammalian cells. Nat. Plants 3:930–936.

Lin, L., Liu, Y.-G., Xu, X., and Li, B. (2003). Efficient linking and transfer of multiple genes by a multigene assembly and transformation vector system. Proc. Natl. Acad. Sci. U S A 100:5962–5967.

Liu, W., Yuan, J.S., and Stewart, C.N., Jr. (2013). Advanced genetic tools for plant biotechnology. Nat. Rev. Genet. 14:781–793.

Liu, W., and Stewart, C.N., Jr. (2015). Plant synthetic biology. Trends Plant Sci. 20:309–317.

Liu, X., Li, S., Yang, W., Mu, B., Jiao, Y., Zhou, X., Zhang, C., Fan, Y., and Chen, R. (2018a). Synthesis of seed-specific bidirectional promoters for metabolic engineering of anthocyanin-rich maize. Plant Cell Physiol. 59:1942–1955.

Liu, X., Yang, W., Mu, B., Li, S., Li, Y., Zhou, X., Zhang, C., Fan, Y., and Chen, R. (2018b). Engineering of ‘Purple Embryo Maize’ with a multigene expression system derived from a bidirectional promoter and self-cleaving 2A peptides. Plant Biotechnol. J. 16:1107–1109.

Lu, S., Van Eck, J., Zhou, X., Lopez, A.B., O’Halloran, D.M., Cosman, K.M., Conlin, B.J., Paolillo, D.J., Garvin, D.F., Vrebalov, J., et al. (2006). The cauliﬂower or green encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of beta-carotene accumulation. Plant Cell 18:3594–3605.

Lucca, P., Hurrell, R., and Potrykus, I. (2001). Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theor. Appl. Genet. 102:392–397.

Mattoo, A.K., Shukla, V., Fatima, T., Handa, A.K., and Yachha, S.K. (2010). Genetic engineering to enhance crop-based phytosnutrients (nutraceuticals) to alleviate diet-related diseases. Adv. Exp. Med. Biol. 698:122–143.

McGuire, S. (2015). FAO, IFAD, and WFP. The state of food insecurity in the world 2015: meeting the 2015 international hunger targets: taking stock of uneven progress. Rome: FAO. Adv. Nutr. 6:623–624.

Mortimer, J.C. (2019). Plant synthetic biology could drive a revolution in biofuels and medicine. Exp. Biol. Med. (Maywood) 244:323–331.

Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., Ric De Vos, C.H., van Tunen, A.J., and Verhoeven, M.E. (2001). Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonoids. Nat. Biotechnol. 19:470–474.

Muthaya, A., Rah, J.H., Sugimoto, J.D., Roos, F.F., Kraemer, K., and Black, R.E. (2013). The global hidden hunger indices and maps: an advocacy tool for action. PLoS One 8:e67860.

Napier, J.A., Usher, S., Haslam, R.P., Ruiz-Lopez, N., and Sayanava, O. (2015). Transgenic plants as a sustainable, terrestrial source of fish oils. Eur. J. Lipid Sci. Technol. 117:1317–1324.

Naqui, S., Zhu, C.F., Farre, G., Ramessar, K., Bassie, L., Breitenbach, J., Conesa, D.P., Ros, G., Sandmann, G., Capell, T., et al. (2009). Transgenic multivitamin corn through biofortiﬁcation of endosperm with three vitamins representing three distinct metabolic pathways. Proc. Natl. Acad. Sci. U S A 106:7762–7767.

Nielsen, J., and Keasling, J.D. (2011). Synergies between synthetic biology and metabolic engineering. Nat. Biotechnol. 29:693–695.

Nour-Eldin, H.H., Geu-Flores, F., and Halkier, B.A. (2010). USER cloning and USER fusion: the ideal cloning techniques for small and big laboratories. Methods Mol. Biol. 643:185–200.

Oyo, G., Ozawa, K., Ishimaru, T., Murayama, T., and Takaiwa, F. (2013). Transgenic rice seed synthesizing diverse flavonoids at high levels: a new platform for flavonoid production with associated health beneﬁts. Plant Biotechnol. J. 11:734–746.

Ow, D.W. (2011). Recombinase-mediated gene stacking as a transformation operating system. J. Integr. Plant Biol. 53:512–519.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L., et al. (2005). Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat. Biotechnol. 23:482–487.

Peremarti, A., Twyman, R.M., Gómez-Galera, S., Naqvi, S., Farré, G., Sabalza, M., Miralpeix, B., Dasevskaya, S., Yuan, D., Ramessar, K., et al. (2010). Promoter diversity in multigene transformation. Plant Mol. Biol. 73:363–378.

Petrie, J.R., Shrestha, P., Zhou, X.R., Mansour, M.P., Liu, Q., Belide, S., Nichols, P.D., and Singh, S.P. (2012). Metabolic engineering plant seeds with fish oil-like levels of DHA. PLoS One 7:e49165.

Petrie, J.R., Shrestha, P., Belide, S., Kennedy, Y., Lester, G., Liu, Q., Divi, U.K., Mulder, R.J., Mansour, M.P., Nichols, P.D., et al. (2014). Metabolic engineering Camelina sativa with fish oil-like levels of DHA. PLoS One 9:e85061.

Pierce, E.C., Lafayette, P.R., Ortega, M.A., Joyce, B.L., Kopsell, D.A., and Parrott, W.A. (2015). Ketocarotenoid production in soybean seeds through metabolic engineering. PLoS One 10:e0138196.

Pouvreau, B., Vanhercke, T., and Singh, S. (2018). From plant metabolic engineering to plant synthetic biology: the evolution of the design/build/test/learn cycle. Plant Sci. 273:3–12.

Prasad, A.S. (1978). Trace Elements and Iron in Human Metabolism (New York/Chichester: Wiley).

Qin, A., Shi, Q., and Yu, X. (2011). Ascorbic acid contents in transgenic potato plants overexpressing two dehydroascorbate reductase genes. Mol. Biol. Rep. 38:1557–1566.

Ruiz-Lopez, N., Haslam, R.P., Napier, J.A., and Sayanava, 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.

Ruiz-Lopez, N., Haslam, R.P., Usher, S.L., Napier, J.A., and Sayanava, O. (2013). Reconstitution of EPA and DHA biosynthesis in Arabidopsis: iterative metabolic engineering for the synthesis of n-3 LC-PUFAs in transgenic plants. Metab. Eng. 17:30–41.

Ruiz-Lopez, N., Sayanava, O., Napier, J.A., and Haslam, R.P. (2012). Metabolic engineering of the omega-3 long chain polyunsaturated
Metabolic Engineering for Enhancing Crops

fatty acid biosynthetic pathway into transgenic plants. J. Exp. Bot. 63 (7):2397–2410.

Ruxton, C.H.S., Reed, S.C., Simpson, M.J.A., and Millington, K.J. (2007). The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. J. Hum. Nutr. Diet 20:275–285.

Saltzman, A., Birol, E., Oparinde, A., Andersson, M.S., Asare-Marfo, D., Diressie, M.T., Gonzalez, C., Lividini, K., Moursi, M., and Zeller, M. (2017). Availability, production, and consumption of crops biofortified by plant breeding: current evidence and future potential. Ann. N. Y. Acad. Sci. 1390:104–114.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palaci, J., Casteljins, B., Forment, J., Ziaroso, P., Blanca, J., Granell, A., and Orzaez, D. (2013). GoldenBraid 2.0: a comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol. 162:1618–1631.

Shao, Z., Zhao, H., and Zhao, H. (2009). DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res. 37:e16.

Shen, B.R., Wang, L.M., Lin, X.L., Yao, Z., Xu, H.W., Zhu, C.H., Teng, H.Y., Cui, L.L., Liu, E.E., Zhang, J.J., et al. (2019). Engineering a new chloroplastic photosynthetic bypass to increase photosynthetic efficiency and productivity in rice. Mol. Plant 12:199–214.

Shih, P.M., Vuu, K., Mansoori, N., Ayad, L., Louie, K.B., Bowen, B.P., Northen, T.R., and Loqué, D. (2016). A robust gene-stacking method utilizing yeast assembly for plant synthetic biology. Nat. Commun. 7:13215.

Shin, Y.M., Park, H.J., Yim, S.D., Baek, N.I., Lee, C.H., An, G., and Woo, Y.M. (2006). Transgenic rice lines expressing maize C1 and R-S regulatory genes produce various flavonoids in the endosperm. Plant Biotechnol. J. 4:303–315.

Singh, S.P., Gruissem, W., and Bhullar, N.K. (2017). Single genetic locus improvement of iron, zinc and β-carotene content in rice grains. Sci. Rep. 7:6883.

Sleigh, S.C., Bartley, B.A., Lieviant, J.A., and Sauro, H.M. (2010). In-Fusion BioBrick assembly and re-engineering. Nucleic Acids Res. 38:2624–2636.

Stanley, L., and Yuan, Y.W. (2019). Transcriptional regulation of carotenoid biosynthesis in plants: so many regulators, so little consensus. Front. Plant Sci. 10:1017.

Strobbe, S., De Lepeleire, J., and Van Der Straeten, D. (2018). From in planta function to vitamin-rich food crops: the ACE of biofortification. Front. Plant Sci. 9:1862.

Strobbe, S., and Van Der Straeten, D. (2018). Toward eradication of B-vitamin deficiencies: considerations for crop biofortification. Front. Plant Sci. 9:443.

Storozhenko, S., De Brouwer, V., Volckaert, M., Navarrete, O., Blancoquinta, D., Zhang, G.F., Lambert, W., and Van Der Straeten, D. (2007). Folate fortification of rice by metabolic engineering. Nat. Biotechnol. 25:1277–1279.

Tejera, N., Vauzour, D., Betancor, M.B., Sayanova, O., Ushe, S., Cochart, M., Rigby, N., Ruiz-Lopez, N., Menoyo, D., Tocher, D.R., et al. (2016). A Transgenic Camelina sativa seed oil effectively replaces fish oil as a dietary source of eicosapentaenoic acid in mice. J. Nutr. 146:227–235.

Tian, Y.S., Wang, B., Peng, R.H., Xu, J., Li, T., Fu, X.Y., Xiong, A.S., Gao, J.J., and Yao, Q.H. (2019). Engineering carotenoid biosynthesis in rice endosperm by metabolic engineering. Plant Biotechnol. J. 17:849–851.

Tzfira, T., Tian, G.W., Lacroix, B., Vyas, S., Li, J., Leitner-Dagan, Y., Krichevsky, A., Taylor, T., Vainstein, A., and Oltovsky, V. (2005). pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol. Biol. 57:503–516.

Underwood, E.J. (1977). Trace Elements in Human Nutrition. 4th edn (New York: Academic), p. 545.

Wakasa, Y., Yasuda, H., and Takaiwa, F. (2006). High accumulation of bioactive peptide in transgenic rice seeds by expression of introduced multiple genes. Plant Biotechnol. J. 4:499–510.

Walthouw, A.J., Temple, G.F., Brasch, M.A., Hartley, J.L., Lorson, M.A., van den Heuvel, S., and Vidal, M. (2000). GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. Methods Enzymol. 328:575–592.

Walsh, T.A., Bevan, S.A., Gachotte, D.J., Larsen, C.M., Moskal, W.A., Merlo, P.A., Sidorenko, L.V., Hampton, R.E., Stoltz, V., Preedy, D., et al. (2016). Canola engineered with a microalgal polyketide synthase-like system produces oil enriched in docosahexaenoic acid. Nat. Biotechnol. 34:881–887.

Wang, C., Zeng, J., Li, Y., Hu, W., Chen, L., Miao, Y., Deng, P., Yuan, C., Ma, C., Chen, X., et al. (2014). Enrichment of provitamin A content in wheat (Triticum aestivum L.) by introduction of the bacterial carotenoid biosynthetic genes CrtB and CrtL. J. Exp. Bot. 65:2545–2556.

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., Tohge, T., Fernie, A.R., Günter, D., Gruissem, W., et al. (2009). Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. Plant Biotechnol. J. 7:631–644.

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

Yazdani, M., Sun, Z., Yuan, H., Zheng, S., Thannhauser, T.W., Vrabalov, J., Ma, Q., Xu, Y., Fei, Z., Van Eck, J., et al. (2019). Ecotropic expression of ORANGE promotes carotenoid accumulation and fruit development in tomato. Plant Biotechnol. J. 17:33–49.

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

Ye, X., Honda, K., Sakai, T., Okano, K., Omasa, T., Hirotta, R., Kuroda, A., and Ohtake, H. (2012). Synthetic metabolic engineering—a novel, simple technology for designing a chimeric metabolic pathway. Microb. Cell Fact. 11:120.

Yuan, L., and Grotewold, E. (2015). Metabolic engineering to enhance the value of plants as green factories. Metab. Eng. 27:83–91.

Zalatan, J.G., Lee, M.E., Almeida, R., Gilbert, L.A., Whitehead, E.H., La Russa, M., Tsai, J.C., Weissman, J.S., Dueber, J.E., Qi, L.S., et al. (2015). Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. Cell 160:339–350.

Zeevi, V., Liang, Z., Arieli, U., and Tzfira, T. (2012). Zinc finger nucleases and homing endonuclease-mediated assembly of multigene plant transformation vectors. Plant Physiol. 158:132–144.

Zhang, Y., Butelli, E., and Martin, C. (2014). Engineering anthocyanin biosynthesis in plants. Curr. Opin. Plant Biol. 19:81–90.

Zhang, Y., Butelli, E., Alseekh, S., Tohge, T., Rallapalli, G., Luo, J., Kawar, P.G., Hill, L., Santino, A., Fernie, A.R., et al. (2015). Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato. Nat. Commun. 6:8635.

Zheng, L., Cheng, Z., Ai, C., Jiang, X., Bei, X., Zheng, Y., Glehn, R.P., Welch, R.M., Miller, D.D., Lei, X.G., et al. (2010). Nicotianamine, a novel enhancer of rice iron bioavailability to humans. PLoS One 5:e10190.
Zhou, X., Welsch, R., Yang, Y., Álvarez, D., Riediger, M., Yuan, H., Fish, T., Liu, J., Thannhauser, T.W., and Li, L. (2015). Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis. Proc. Natl. Acad. Sci. U S A 112:3558–3563.

Zhu, L., and Qian, Q. (2017). Development of “Purple Endosperm Rice” by engineering anthocyanin biosynthesis in endosperm: significant breakthrough in Transgene Stacking System, new progress in rice biofortification (in Chinese). Chin. Bull. Bot. 52:539–542.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., and Capell, T. (2008). Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. Proc. Natl. Acad. Sci. U S A 105:18232–18237.

Zhu, C., Naqvi, S., Capell, T., and Christou, P. (2009). Metabolic engineering of ketocarotenoid biosynthesis in higher plants. Arch. Biochem. Biophys. 483:182–190.

Zhu, Q., Yu, S., Zeng, D., Liu, H., Wang, H., Yang, Z., Xie, X., Shen, R., Tan, J., Li, H., et al. (2017). Development of “purple endosperm rice” by engineering anthocyanin biosynthesis in the endosperm with a high-efficiency transgene stacking system. Mol. Plant 10:918–929.

Zhu, Q., Zeng, D., Yu, S., Cui, C., Li, J., Li, H., Chen, J., Zhang, R., Zhao, X., Chen, L., et al. (2018). From golden rice to aSTARice: bioengineering astaxanthin biosynthesis in rice endosperm. Mol. Plant 11:1440–1448.