



Improving nutritional quality of rice for human health

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Abstract

Key message This review surveys rice nutritional value, mainly focusing on breeding achievements via adoption of both genetic engineering and non-transgenic strategies to improve key nutrients associated with human health.

Abstract Rice (*Oryza sativa*) is an essential component of the diets and livelihoods of over 3.5 billion people. Polished rice is mostly consumed as staple food, fulfilling daily energy demands and part of the protein requirement. Brown rice is comparatively more nutritious, containing more lipids, minerals, vitamins, dietary fiber, micronutrients, and bioactive compounds. In this article, we review the nutritional facts about rice including the level of γ -aminobutyric acid, resistant starch, lysine, iron, zinc, β -carotene, folate, anthocyanin, various carotenoids, and flavonoids, focusing on their synthesis and metabolism and the advances in their biofortification via adoption of both conventional and genetic engineering strategies. We conclude that besides representing a staple food, rice has the potential to become a source of various essential nutrients or bioactive compounds through appropriate genetic improvements to benefit human health and prevent certain chronic diseases. Finally, we discuss the available, non-genetically engineering strategies for the nutritional improvement of rice, including their main strengths and constraints.

Introduction

Rice (*Oryza sativa*) is an important crop that feeds approximately half of the human population, providing over 21% of the human caloric needs and up to 76% of the caloric intake of Southeast Asian inhabitants (Fitzgerald et al. 2009). It was estimated that the world rice production reached 741 million tons in 2016 (FAO 2018). Rice is widely cultivated in South Asia, China, Thailand, Japan, and Korea. According to the caryopsis color, rice can be mainly classified into white rice, black rice, red rice, and green rice. Among them, white rice is the most common variety. In addition to steamed rice, rice can also be consumed as fried rice or parched rice, and several rice-based preparations such as rice cake, noodle, dumpling, and glutinous rice flour. The annual per-capita consumption of rice ranges between 62 and 190 kg in rice-consuming countries (Graham et al. 1999).

People generally consume polished (or milled) rice, which leaves only endosperm with the removal of embryo and bran layers through processing. The nutritional value of polished rice is therefore determined by rice endosperm, which mainly comprises starch (70–80%), with proteins and lipids in small proportions, about 7–10% and less than 1%, respectively (Yang et al. 2019, Table 1). It is generally known that rice endosperm lacks many micronutrients or phytonutrients. Hidden hunger is pervasive in low- and middle-income countries and holds significant individual, social, and economic consequences (Ruel-Bergeron et al. 2015). Biofortification is a feasible and cost-effective approach to increase micronutrients or phytonutrients in crops via conventional breeding or genetic engineering, which is proven to greatly improve nutritional quality of crop and benefit human health (Bouis and Saltzman 2017). Since 2003, HarvestPlus and its partners have demonstrated that breeding efforts have resulted in the development of several staple food crops with significant levels of the three micronutrients that are most limiting in diets: zinc, iron, and vitamin A (Bouis and Saltzman 2017; HarvestPlus 2019). Provitamin A-enriched maize, high-iron bean, high-zinc rice, and high-iron pearl millet have been developed and released to farmers successfully (Saltzman et al. 2013) and more than 38 million people were growing and eating biofortified

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Table 1 Nutritional composition of rice. Modified from OECD (2016)

| Nutrient | Brown rice | Milled rice |
|---|------------|-------------|
| Proximate, carbohydrate components (% of dry matter) and energy content | | |
| Water (% of fresh weight) | 11.37–16.4 | 12.31–15.5 |
| Crude protein | 6.5–10.0 | 7.3–8.3 |
| Crude fat | 1.9–3.9 | 0.3–0.65 |
| Crude ash | 1.2–1.7 | 0.3–0.9 |
| Carbohydrates | 85.2–88.9 | 91.07 |
| Digestible carbohydrates | 84.8–88.2 | 89.1–91.2 |
| Starch | 77.2 | 90.2 |
| Free sugars | 0.8–1.5 | 0.3–0.5 |
| Neutral detergent fiber | 4.5 | 0.8–2.7 |
| Dietary fiber/insoluble | | 0.5 |
| Total dietary fiber | 3.9 | 0.5–2.8 |
| Crude fiber | 0.7–1.2 | 0.2–0.6 |
| Hemicelluloses | | 0.1 |
| Pentosans | 1.4–2.4 | 0.6–1.6 |
| Lignin | | 0.1 |
| Energy (kJ/g) | 17.2–18.7 | 17.0–18.1 |
| Protein fraction (% of total protein) | | |
| Albumin (soluble in water) | | 2–5 |
| Globulin (soluble in salt water) | | 2–10 |
| Prolamin (soluble in alcohol) | | 20–25 |
| Glutelin (soluble in aqueous alkaline solution) | | 60–65 |
| Amino acid composition (% of dry matter) | | |
| Alanine | 0.37–0.59 | |
| Arginine | 0.52–0.88 | |
| Aspartic acid | 0.59–0.96 | |
| Cystine | 0.15–0.28 | |
| Glutamic acid | 1.06–1.88 | |
| Glycine | 0.32–0.48 | |
| Histidine | 0.16–0.27 | |
| Isoleucine | 0.22–0.40 | |
| Leucine | 0.51–0.85 | |
| Lysine | 0.26–0.40 | |
| Methionine | 0.14–0.34 | |
| Phenylalanine | 0.32–0.55 | |
| Proline | 0.25–0.46 | |
| Serine | 0.30–0.53 | |
| Threonine | 0.23–0.38 | |
| Tryptophan | 0.05–0.13 | |
| Tyrosine | 0.21–0.51 | |
| Valine | 0.37–0.59 | |
| Fatty acid component (% of total fatty acids) | | |
| Myristic (14:0) | 0.5–1.1 | |
| Pentadecanoic (15:0) | 0.1–0.3 | |
| Palmitic (16:0) | 18.2–31.2 | |
| Palmitoleic (16:1) | 0.1–0.2 | |
| Heptadecanoic (17:0) | 0.1–0.6 | |

Table 1 (continued)

| Nutrient | Brown rice | Milled rice |
|--|------------|-------------|
| Stearic (18:0) | 1.5–2.8 | |
| Oleic (18:1) | 30.9–40.2 | |
| Linoleic (18:2) | 26.1–39.0 | |
| Linolenic (18:3) | 0.9–1.6 | |
| Arachidic (20:0) | 0.4–0.7 | |
| Eicosenoic (20:1) | 0.4–0.6 | |
| Behenic (22:0) | 0.2–0.6 | |
| Docosenoic/erucic (22:1) | 0.1–0.2 | |
| Lignoceric (24:0) | 0.4–0.9 | |
| Tetracosenoic (24:1) | 0.1–0.3 | |
| Others | 4 | |
| Macro-minerals (mg/g dry matter) | | |
| Calcium | 0.1–0.6 | 0.1–0.3 |
| Magnesium | 0.2–1.7 | 0.2–0.6 |
| Phosphorus | 2.0–5.0 | 0.9–1.7 |
| Potassium | 0.7–3.2 | 0.8–1.5 |
| Silicon | 0.7–1.6 | 0.1–0.5 |
| Sulfur | 0.3–2.2 | 0.9 |
| Micro-minerals ($\mu\text{g/g}$ dry matter) | | |
| Copper | 1–7 | 2–3 |
| Iron | 2–60 | 2–33 |
| Manganese | 2–42.24 | 7–20 |
| Sodium | 20–395 | 6–100 |
| Zinc | 7–33 | 7–27 |
| Vitamin ($\mu\text{g/g}$ dry matter) | | |
| Retinol (A) | 0–0.13 | 0-trace |
| Thiamine (B ₁) | 3.4–8.1 | 0.2–1.3 |
| Riboflavin (B ₂) | 0.2–1.6 | 0.2–0.7 |
| Niacin (B ₃) | 41–134.7 | 15–28 |
| Pantothenic acid (B ₅) | 11–17 | 4.8 |
| Pyridoxine (B ₆) | 1.8–11 | 0.5–1.4 |
| Biotin (B ₇) | 0.05–0.12 | 0.01–0.07 |
| Choline, total | 1100 | 450–1020 |
| Folic acid (B ₉) | 0.1–0.6 | 0.03–0.16 |
| Cyanocobalamin (B ₁₂) | 0–0.005 | 0–0.0016 |
| Alpha-tocopherol (E) | 8.9–29 | Trace-3 |
| Beta-tocopherol | 0.5–1.4 | |
| Gamma-tocopherol | 2.2–4.8 | |
| Delta-tocopherol | 0.1–0.6 | |

crops in 2018. Moreover, with the rapid development of biotechnology, several biofortified rice germplasms have been developed, such as β -carotene-enriched “Golden Rice” (GR) (Paine et al. 2005; Ye et al. 2000), folate-enriched rice (Blancquaert et al. 2015), anthocyanin-enriched “Purple Endosperm Rice” (Zhu et al. 2017), astaxanthin-enriched rice (Zhu et al. 2018). In addition, several colored rice germplasms have been engineered, displaying yellow, pinkish red, and orange-red colors due to the accumulation of

zeaxanthin, astaxanthin, capsanthin and other carotenoids (Ha et al. 2019). As a staple food, the nutritional quality of rice strongly influences consumers' health, especially in the case of poor populations, because their diet mostly consists of rice, given that other foodstuffs such as fruits, fish, and meat are usually unaffordable. Therefore, strengthening the nutrition of rice is a matter of great benefit to human health.

In this article, we describe the structure of rice grain and examine its nutritional quality, focusing on nutrient composition and distribution. We also reviewed the metabolic pathways involved in their synthesis. The main efforts for improving rice nutritional value through both conventional and genetic engineering strategies were also discussed. Finally, a summary and perspective concerning rice nutritional quality and its impact on human health are presented.

Rice grain structure and nutrients distribution

Brown rice, obtained by direct dehulling of rice seeds without polishing, consists of endosperm (accounting for ~90% of grain weight), the embryo (~2–3%), and the bran layers (~6–7%). It is known that the polishing process improves rice cooking and eating quality, and extends its shelf life; however, it also decreases the nutrient contents because the embryo and the bran layers are rich in lipids, proteins, dietary fiber, and other components (Cicero and Gaddi 2001; Wu et al. 2013). Significant reductions in almost all main nutritional components (except for protein) including fat, dietary fiber, vitamin B, iron, calcium, and bioactive compounds such as γ -aminobutyric acid (GABA) have been documented after rice polishing. Although eating brown rice has been advocated by a fraction of health-conscious people, consumer's acceptance of this rice is hampered by a comparatively inferior texture.

Germination treatment seems to be a solution to this dilemma between nutrition and sensory qualities. During germination, the hydrolytic enzymes are activated to hydrolyze the starches, non-starch polysaccharides, and proteins, which may result in an improved eating quality while keeping nutrients; this type of rice is called germinated brown rice (GBR) (Cho and Lim 2016; Lee et al. 2007). Germination or water-soaking has positive effects on amino acid composition and protein availability, and also increases the contents of total sugars and bioactive components, while antinutrients concentrations diminish (Caceres et al. 2014; Liang et al. 2008; Xia et al. 2017). Researches have confirmed that GBR intake has many beneficial effects on human health including antihyperlipidemic, antihypertensive, and antidepressant-like actions, and reduces the risk of some chronic diseases, such as diabetes and cancer (Cho and Lim 2016; Mamiya et al. 2007; Roohinejad et al. 2010; Wu et al. 2013).

Because nutrient proportions vary markedly among different rice grain fractions, the structural modification of rice grain is expected to alter rice nutritional quality significantly. Giant embryo rice (GER) is a type of rice mutants with large embryos, in which the embryo size is two to three (but sometimes even more, up to five) times that of common rice. As a result of increased proportion of embryo, which is more nutritious than the endosperm, GER has higher contents of protein, calcium, sodium, vitamin E, vitamin B₆, dietary fiber, and other nutritional components. The *GE* gene, responsible for the large embryo mutation, has been determined by map-based cloning, and proved to be a cytochrome P450 gene (*CYP78A13*). *GE* is predominantly expressed in embryo/endosperm-interface tissues and regulates the embryo/endosperm size balance (Nagasawa et al. 2013; Xu et al. 2015a).

The most noteworthy nutrient in GER is GABA, which is a non-protein amino acid with a peculiar biological effect on alleviating hypertension as an inhibitory neurotransmitter in the central nervous system for animals (Hayakawa et al. 2002, 2005). Because GABA mainly exists in rice embryo, GER is rich in GABA (Kim et al. 2013). Moreover, germination or water-soaking under proper conditions can further increase GABA contents in both wild type brown rice and GER (Komatsuzaki et al. 2007; Lu et al. 2010; Oh 2003).

Zhao et al. (2017) examined the molecular mechanism underlying high GABA contents in the *GE* mutant. They concluded that up-regulation of gene transcripts and intermediate contents of GABA in the polyamine pathway, along with down-regulation of GABA catabolic genes, would explain the enhanced GABA levels in the mutant as compared with those in the original rice variety. Furthermore, they showed that higher GABA accumulation in germinated brown rice resulted from both glutamate-derived and polyamine-derived pathways, as Glu, Arg, and putrescine levels were all significantly increased in germinated brown rice as compared to those before germination for both *GE* and common rice varieties (Zhao et al. 2017).

The endosperm of brown rice consists of the outer aleurone layer and the inner starchy endosperm. The aleurone is mostly a single-cell layer (except for a small region near the dorsal vascular bundle, with three to four cell layers), which contains proteins, vitamins, and minerals, and is more nutritious than the inner starchy endosperm (which mainly contains starch) (Becraft and Yi 2011; Wu et al. 2016). Therefore, increasing the number of aleurone cell layers may improve the nutritional values of rice. The study by Liu et al. (2018) demonstrated that a weak allele mutation (*thick aleurone 2-1*, *ta2-1*) of *OsROS1*, a DNA demethylase gene, caused a thickened aleurone (4.8 ± 2.2 cell layers on average). As expected, and except for starch, all nutritional features measured including lipids, proteins, vitamins, minerals, and dietary fibers significantly increased in brown rice

of *ta2-1* mutants compared with the wild type control (Liu et al. 2018).

GABA-enriched rice

In addition to structural modification such as GE mutant, GABA enrichment can also be acquired by genetic manipulation. In plants, GABA is mainly generated from the decarboxylation of glutamate by glutamate decarboxylase (GAD). Because the autoinhibitory C-terminal domain in rice *OsGAD2* lowers the activity itself, the constitutive overexpression of a C-terminal domain truncated *OsGAD2ΔC* in rice increased the GABA content in the callus up to 100-fold compared with wild type ones. However, the regenerated plants showed aberrant phenotypes (pale green leaves and dwarfism), probably caused by the constitutive overexpression of *OsGAD2ΔC* (Akama and Takaiwa 2007). Then, the endosperm-specific overexpression of *OsGAD2ΔC* was conducted in rice, and the resultant rice plants did not exhibit obvious difference in agronomic traits compared with the wild type ones, except the significant increase of GABA content (up to 30-fold) in brown rice (Akama et al. 2009). Oral administration of this GABA-enriched rice for 6 weeks brought about a 20 mm Hg decrease in blood pressure in spontaneously hypertensive rats (Akama et al. 2009).

GABA transaminase (GABA-T) is a key enzyme for GABA catabolism; this enzyme catalyzes GABA breakdown into succinate semialdehyde in the mitochondria. To produce a higher accumulation of GABA in rice, a combined strategy involving the regulation of both GABA synthesis and catabolism was conducted by Shimajiri et al. (2013). Overexpression of the truncated *OsGAD2ΔC* and knockdown of *GABA-T* gene mediated by RNA interference (RNAi) led to sustained high levels of GABA in polished rice (75–350 mg/100 g); contrarily, there was only 0.1 mg/100 g in the non-transgenic control (Shimajiri et al. 2013). In the study of Shimajiri et al. (2013), the target genes involved in either overexpression of *OsGAD2ΔC* or knockdown of *GABA-T* are rice endogenous ones, therefore exploring the alleles with natural variations in rice germplasm resource or creating allelic mutants of the target genes through targeting inducing mutagenesis methods, such as Targeting Induced Local Lesions in Genomes (TILLING) based on conventional chemical mutagenesis (Chen et al. 2014a), may provide non-transgenic strategies for breeding GABA-enriched rice.

High resistant starch rice

Resistant starch (RS) is defined as a portion of starch products able to resist the enzymatic hydrolysis during digestion, as they pass through the small intestine and reach the colon, thus becoming available for fermentation by

the microbiota (Chen et al. 2017). Dietary RS can reduce calorie intake and improve gut health and insulin resistance, thus reducing body fat and decreasing cardiovascular disease risk factors and so on (Keenan et al. 2015).

Unfortunately, RS content in rice is generally low, and too low RS values cannot confer health benefits. In cooked rice of wild type cultivars, the estimated RS content ranged from 0.6 to 1.21% (Yang et al. 2016b; Butardo et al. 2012). In rice, both the ratio of amylose to amylopectin and the ratio of long to short branches within the amylopectin influence RS content. In this sense, it has been reported that RS level in steamed rice is positively correlated with the apparent amylose content when RS values are relatively low (less than 4%), whereas a higher correlation with the number of long chains in amylopectin is found when RS values in steamed rice exceed 15% (Tsuiki et al. 2016).

Starch biosynthesis in plants involves multiple enzymes whose activation is regulated through phosphorylation. Initially, cytosolic ADP-glucose pyrophosphorylase (AGPase) decomposes sucrose from photosynthesis to generate ADP-glucose (ADPGlc), which is then transported into the amyloplast, as the direct substrate. The granule-bound starch synthase (GBSS) completes the amylose synthesis independently, whereas starch synthase (SS), starch-branching enzyme (SBE), and debranching enzyme (DBE) are responsible for the amylopectin synthesis. Some rice mutants with high RS content, such as Goami2, RS111, and Jiangtangdao1, have been obtained through mutagenesis (Yang et al. 2012). Linkage analyses indicated that *OsSBE3*, which encodes a starch-branching enzyme, was the putative gene responsible for the high RS rice mutant Jiangtangdao 1 (Yang et al. 2012).

High RS rice can be developed through genetic engineering. The simultaneous inhibition of expression of *SBEI* and *SBEIIb*—two isoforms of SBE enzyme—by RNAi increased amylose content from 27.2 to 64.8%, RS from 0 to 14.6%, and total dietary fiber from 6.8 to 15.2% in grains of transgenic rice when compared with the wild type control (Zhu et al. 2012). Rat feeding tests indicated that consuming this transgenic high-amylose rice may reduce blood glucose, inhibit weight gain, and improve gut health (Zhu et al. 2012). Targeted mutagenesis of rice *SBEI* and *SBEIIb* mediated by clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) genome editing system was also conducted to increase rice RS content (Sun et al. 2017). According to the findings of these authors, *sbeI* knockout mutation by CRISPR/Cas9 did not affect starch synthesis relative to non-transgenic controls (Sun et al. 2017). This finding was consistent with those reported by Zhu et al. (2012). The *sbeIIb* mutants showed significant increases in the amylose content, RS, and proportion of long chains in amylopectin. Thus, both studies

proved that *sbeIIb* instead of *sbeI* is the effective target gene for developing high RS rice.

Improving grain protein and essential amino acids in rice

Rice grain protein is mainly stored in proteasomes of cells, 80% of which are in the endosperm and 20% in the embryo and aleurone layer. Polished rice usually contains 5–17% of protein on dry weight basis, making it an important source of protein for people consuming rice as a staple food. Grain protein content (GPC) varies among rice varieties, ranging in *indica* rice from 4.9 to 19.3% and in *japonica* rice from 5.9 to 16.5% (Yang et al. 2015). Several genetic studies have been conducted to dissect the molecular mechanism that determining GPC (Peng et al. 2014; Yang et al. 2019). By map-based cloning, Peng et al. (2014) identified a putative amino acid transporter gene *OsAAP6*, which functions as a positive regulator of rice GPC by enhancing root absorption of amino acids and affecting their distribution. Moreover, two common variations in the *OsAAP6* 5'-untranslated region seem to be associated with GPC differences, mainly in *indica* cultivars (Peng et al. 2014). Recently, another positive GPC regulator *OsGluA2*, encoding a glutelin type-A2 precursor, was isolated by map-based cloning as well. A single nucleotide polymorphism (SNP) located in the promoter region of *OsGluA2* is associated with its expression level and GPC fluctuations (Yang et al. 2019). These two studies demonstrated that the functional research of key genes determining rice nutritional traits in combination with the corresponding identification of naturally allelic variations in rice germplasm resource may facilitate the conventional breeding for rice varieties with higher nutrients and provide potential non-transgenic strategies for rice biofortification (Peng et al. 2014; Yang et al. 2019).

Besides GPC, the types of amino acids and the proportions of essential amino acids determine rice nutritional quality. Lysine (Lys) is considered the first limiting essential amino acid in rice and its content in polished rice is particularly low. Due to a deficiency in lysine and other essential amino acids, rice protein is considered to be nutritionally incomplete. In plants, the Lys metabolic pathway has been well elucidated. Lys is synthesized from aspartate through a branched biosynthetic pathway, characterized by a complex metabolic regulation. The accumulation of Lys is not only inhibited by feedback itself, but also related to degradation, as well as many other metabolic pathways (Galili 1995, 2002). Aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) are two key enzymes for Lys synthesis, while Lys ketoglutaric acid reductase/saccharopine dehydrogenase (LKR/SDH) is a bifunctional enzyme playing a central role in Lys degradation (Long et al. 2013). Boosting Lys synthesis through the constitutive expression

of a maize Lys feedback-insensitive DHPS was proved to be an inefficient way, as this only led to a slight increase (up to 2.5-fold) of free Lys levels in mature seeds of transgenic rice compared with that of the wild type (Lee et al. 2001).

Long et al. (2013) demonstrated that depressing Lys catabolism was more effective than boosting its synthesis for improving free Lys levels in rice grains. By combining the constitutive expression of Lys feedback-insensitive AK and DHPS and endosperm-specific inhibition of LKR/SDH, free Lys levels in rice grains increased by up to ~60-fold (Long et al. 2013). The further field trial showed that these rice varieties with high levels of free Lys did not exhibit any significant difference in the main agronomic traits including yield, except for plant height and grain color, indicating that this strategy might be useful for developing high Lys rice (Yang et al. 2016a). It should be noted that high free Lys levels in rice grains result in a color change of endosperm (which acquires a dark-brown appearance). It was proved that high accumulation of free Lys in rice endosperm induced the jasmonate signaling pathway, which resulted in enhanced serotonin biosynthesis and led to the dark-brown endosperm appearance reported (Yang et al. 2018). Animal feeding experiments confirmed that this Lys biofortified rice contributed to improving growth performance, food efficiency conversion, and Lys availability in growing rats (Yang et al. 2017).

Besides modifying the metabolic pathway of Lys, direct overexpression of Lys-rich proteins in rice grains is also a practical way to develop high Lys rice varieties (Wong et al. 2015; Liu et al. 2016). Moreover, Wu et al. (2003) attempted a different strategy to improve the Lys content of proteins in rice seeds, in which the recombinant tRNA^{lys} species with modified anticodons were introduced into rice, causing the replacement of Gln, Asn, Glu or stop codon (UAG) with Lys in proteins. Apparently, rice was tolerant to these substitutions because the derived transgenic rice plants did not show any significant heritable abnormality (Wu et al. 2003).

Iron and zinc biofortified rice

Iron (Fe) and zinc (Zn) are two essential minerals that the human body needs. Fe and Zn deficiencies are among the most prevalent micronutrient deficiencies worldwide, affecting 2 billion people and causing more than 0.8 million deaths annually (WHO 2011). Polished rice of common varieties contains approximately 2 µg/g Fe and 16 µg/g Zn on average. The goal is to achieve Fe- and Zn-enriched rice by increasing these values by about six and two times, respectively, to reach the target concentrations (Fe: 13 µg/g; Zn: 28 µg/g) in biofortified rice, which will allow people to get on average about 30% of the estimated average daily requirements (Trijatmiko et al. 2016).

Several quantitative trait loci (QTLs) studies directed at obtaining high Fe and Zn rice using biparental populations have been conducted, and three meta-QTLs for grain Fe and two for grain Zn were identified based on multiple studies (Dixit et al. 2019). The identification of these QTLs has facilitated the breeding of high grain Fe and Zn rice varieties using marker-assisted selection (MAS). Thus, Dixit et al. (2019) selected five rice backcross inbred lines of RP Bio-226/Sampada showing higher Fe (more than 13 µg/g) and Zn (more than 21 µg/g) grain contents, with comparatively better yields, indicating the feasibility of developing Fe- and Zn-enriched rice varieties by non-transgenic strategies.

To date, the development of transgenic rice with elevated Fe and Zn levels in the grains has mainly involved two types of genes that function in either Fe uptake and translocation or Fe storage (Connorton and Balk 2019; Ludwig and Slamet-Loedin 2019). Most strategies for Fe biofortification have been shown to lead to the elevation of Zn content as well, because most components associated with Fe uptake, translocation, and storage are also associated with Zn biotransformation except some Fe specific transporters such as barley Yellow Stripe 1 (HvYS1) (Banakar et al. 2017). Goto et al. (1999) reported the first iron biofortified rice through overexpression of a soybean ferritin gene *SoyferHI* and achieved an approximate doubling of Fe content in brown rice when compared with the non-transgenic control. Thereafter, many studies about Fe and Zn biofortification in rice have been reported, using single-gene approaches such as the Fe-storage protein *ferritin* (Goto et al. 1999; Oliva et al. 2014; Vasconcelos et al. 2003), nicotianamine (a metal ion chelating agent) synthase *NAS* (Lee et al. 2009; Masuda et al. 2009; Moreno-Moyano et al. 2016), iron transporter genes *OsIRT1* (Lee and An 2009; Tan et al. 2015) and HvYS1 (Banakar et al. 2017) or using gene combination approaches. There are two gene combination strategies including combining Fe storage-associated genes with Fe uptake and translocation-associated genes (Boonyaves et al. 2016; Masuda et al. 2012; Trijatmiko et al. 2016; Wu et al. 2019) or combining Fe biofortification-associated genes with bioavailability associated genes such as a *phytase* (Lucca et al. 2001; Wirth et al. 2009). In general, the gene combination approaches are more effective than single-gene ones because an Fe storage protein and iron uptake and translocation-associated proteins usually have synergistic effects.

To date the most promising example is the rice event with high Fe and Zn grain concentrations, NASFer-274. This event involved two transgenes: rice *NAS* gene (*OsNAS2*) and soybean *ferritin* gene (*Sfer-HI*). Field evaluation in two countries showed that NASFer-274 polished rice contained as much as 15 µg/g of Fe and 45.7 µg/g of Zn, both of which are over the target levels of Fe and Zn set by Harvestplus. In addition, no yield changes or harmful effects related to heavy metals accumulation in NASFer-274 grains were observed

(Trijatmiko et al. 2016). These results demonstrated the great potential of the NASFer-274 variety in developing Fe- and Zn-enriched rice varieties to alleviate Fe and Zn malnutrition (Trijatmiko et al. 2016). Recently, Fe and Zn biofortification in rice involving an Fe intracellular transportation gene was reported for the first time (Wu et al. 2019). In addition to the Arabidopsis *NAS* gene (*AtNAS1*) and *Phaseolus vulgaris* FERRITIN gene (*PvFER*), the Arabidopsis NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (*AtNRAMP*) encoding a transporter that exports Fe from vacuoles to the cytosol were transformed into rice. The best resultant lines achieved 12.67 µg/g Fe and 45.60 µg/g Zn in polished grains with the recipient variety Nipponbare and 13.65 µg/g Fe and 48.18 µg/g Zn in polished grains with the recipient variety IR64 (Wu et al. 2019), both of which reached the recommended Fe and Zn contents by Harvestplus.

Vitamin biofortified rice

Both polished rice and brown rice lack provitamin A (mainly β-carotene), the precursor of vitamin A that can be conveniently converted to vitamin A in human body. Preformed vitamin A only exists in animal products, while β-carotene is mainly contained in dark green, yellow, and orange parts of plants, as well as in animal products (Dawe et al. 2002). Diet diversification is an efficient way to prevent vitamin A deficiency (VAD). However, VAD is a worldwide health problem that prevails in the poor populations especially in the developing countries of South and Southeast Asia, where rice is the staple food, and poverty severely restricts the access to a balanced diet. VAD causes visual problems such as night blindness, and severe VAD can even lead to blindness. In addition, VAD results in various disorders and increased susceptibility for infection like measles and diarrhea, and further lead to increased child mortality (WHO 2009; Wirth et al. 2017).

The complete β-carotene biosynthetic pathway is absent in rice endosperm. Wild type rice has only the biosynthetic capacity to produce the precursor of carotenoids geranylgeranyl diphosphate (GGPP) in the endosperm. In plants, five enzymes including phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), carotene *cis-trans*-isomerase (CRTISO), lycopene-β cyclase (LCYB) function successively to produce β-carotene from GGPP (Fig. 1).

The β-carotene biofortified rice was first reported in 2000. In that rice, the *Psy* gene from daffodil (*Narcissus pseudonarcissus*) and the bacterial phytoene desaturase (*CrtI*) from *Pantoea ananatis* (formerly, *Erwinia uredovora*), which can replace the functions of four plant enzymes (PDS, ZISO, ZDS, and CRTISO), were introduced into rice under the control of an endosperm-specific promoter (Ye et al. 2000).

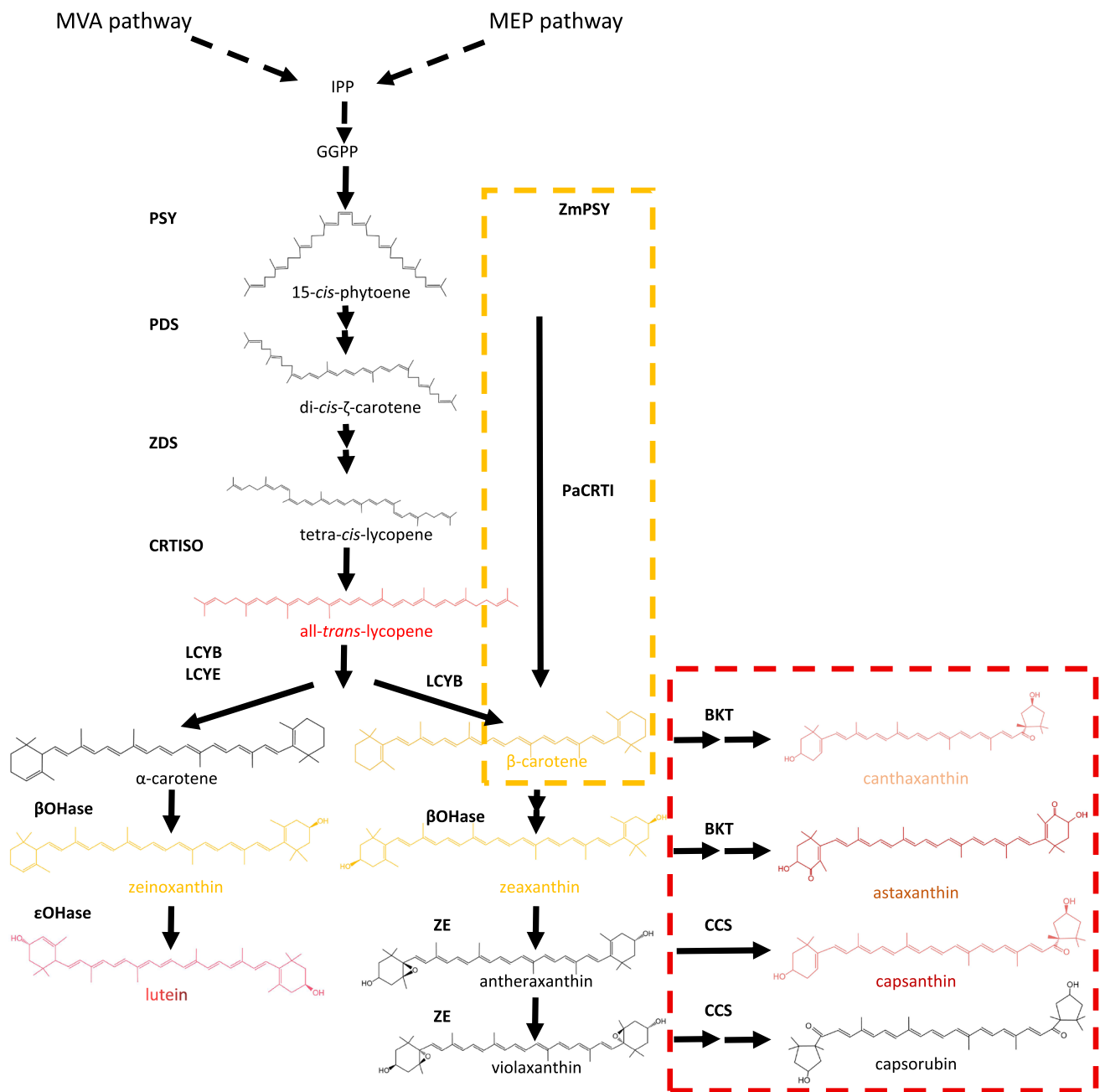


Fig. 1 Carotenoid synthesis in rice. The yellow dotted box represents the engineered biosynthetic pathway of GR2; the red dotted box represents engineered biosynthetic pathway other carotenoid derivatives of zeaxanthin, astaxanthin, capsanthin and other carotenoids. *IPP* isopentenyl diphosphate, *LYCE* lycopene ϵ -cyclase, β *OHase* β -carotene

hydroxylase, ϵ *OHase* ϵ -carotene hydroxylase, *ZE* zeaxanthin epoxidase, *ZmPSY* phytoene synthase from maize, *CRTI* bacterial carotene desaturase, *BKT* β -carotene ketolase, *CCS* capsanthin-capsorubin synthase (Paine et al. 2005; Zhu et al. 2017; Ha et al. 2019) (color figure online)

This transgenic rice harboring daffodil *Psy* and bacterial *CrtI* accumulated up to 1.6 $\mu\text{g/g}$ of carotenoids in the endosperm and was named “Golden Rice” (GR) for its distinctive yellow or orange hue (Ye et al. 2000). Later, an improved version of GR, GR2, was developed by Syngenta scientists (Paine et al. 2005). The β -carotene content in GR2 grains was approximately 23 times higher than in GR lines, with up

to 37 $\mu\text{g/g}$ of carotenoids and β -carotene as the predominant component. In GR2, the accumulation of β -carotene in the endosperm was significantly boosted when the daffodil *Psy* gene was replaced by a maize-derived ortholog (the *Pantoea CrtI* remained unchanged), indicating that the type of *Psy* is the main rate-limiting step for β -carotene synthesis in GR (Paine et al. 2005). Thereafter, several attempts to generate

new versions of GR were reported (Bai et al. 2016; Ha et al. 2010; Jeong et al. 2017; Tian et al. 2019).

Ha et al. (2010) simplified the GR expressing constructs by combining Korea pepper (*Capsicum annuum*) *Psy* and *Pantoea CrtI* in a single bicistronic gene, using the 2A sequence from the foot-and-mouth disease virus or the internal ribosome entry site (IRES) sequence from the crucifer-infecting tobamovirus as the linker. The resulting two bicistronic genes, *PAC* (*Psy-2A-CrtI*) and *PIC* (*Psy-IRES-CrtI*), were introduced into rice under the control of endosperm-specific promoters. Although both *PAC* and *PIC* rice accumulated carotenoids in their endosperms, the concentrations achieved were limited. *PAC* rice accumulated an average of 1.3 µg/g of total carotenoids in the endosperm, a higher value than that recorded for *PIC* rice (Ha et al. 2010). Jeong et al. (2017) optimized the codon in the *PAC* gene for rice plants and synthesized a new gene version: *stPAC*. The seeds of *stPAC* rice exhibited an enhanced level of total carotenoids (2.9-fold, 3.50 µg/g) and β-carotene (fourfold, 2.37 µg/g) as compared with that of *PAC* rice (Jeong et al. 2017), but their concentrations were still much lower than those of GR2 (Paine et al. 2005).

Strategies for the enhancement of β-carotene content in GR grains by increasing supply of upstream isoprenoid precursors or promoting downstream metabolic sinks were also assessed. In the study of Bai et al. (2016), the maize *Psy* gene (*ZmPsy1*) and the *Pantoea CrtI* (*PaCrtI*) gene were introduced into rice in combination with *Arabidopsis thaliana* genes *AtDXS* or *AtOR*. *AtDXS* encodes the enzyme 1-deoxy-D-xylulose 5-phosphate synthase, involved in isoprenoid precursors supply upstream of 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, whereas *AtOR* encodes the *ORANGE* gene, which promotes the formation of a metabolic sink by switching the differentiation of non-colored plastids into chromoplasts. The accumulation of β-carotene in the endosperms of rice plants expressing either *ZmPSY1* + *PaCRTI* + *AtDXS* or *ZmPSY1* + *PaCRTI* + *AtOR* proteins (7.5–16.6 µg/g and 5.9–10.5 µg/g, respectively) was higher than that of control plants expressing only *ZmPSY1* + *PaCRTI* (1.2–2.2 µg/g) (Bai et al. 2016).

Besides the plastidial MEP pathway, plants also have another pathway to supply isoprenoids: the cytoplasmic mevalonate (MVA) pathway. The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is considered to be the major rate-limiting enzyme in the MVA pathway. Tian et al. (2019) attempted a new way to produce GR by combining *ZmPSY1* + *PaCRTI* expression with an improved MVA pathway. They introduced into rice *ZmPSY1* + *PaCRTI* along with *tHMGI*, which encodes a truncated HMGR from *Saccharomyces cerevisiae*. The resulting rice accumulated more β-carotene and total carotenoids than *ZmPSY1* + *PaCRTI* rice (Tian et al. 2019), indicating that higher β-carotene

synthesis may be achieved by boosting the upstream flux from either MVA or MEP pathways.

The introduction of a plant-derived *Psy* gene and a bacterial *CrtI* gene along with other synthetic carotenogenic genes allowed the synthesis of other carotenoid derivatives beyond β-carotene. Astaxanthin, a red-colored ketocarotenoid, is a significant target product for plant metabolic engineering because it can benefit human health due to its strong antioxidant activity. Two enzymes, β-carotene ketolase (BKT) and β-carotene hydroxylase (BHY), are needed to convert β-carotene into astaxanthin through the 4-ketolation of both ionone end groups and the 3-hydroxylation, respectively (Zhu et al. 2018, Fig. 1). *ZmPsy1*, *PaCrtI*, and a synthetic *Chlamydomonas reinhardtii* BKT (*sCrBkt*) gene were introduced into rice plants under the control of endosperm-specific promoters. In the resulting rice plants, total carotenoid levels in the endosperms ranged between 7.1 and 8.8 µg/g, with a predominant accumulation of ketocarotenoids such as canthaxanthin (3.0–4.0 µg/g) and adonirubin (1.1–1.4 µg/g). However, very low levels of astaxanthin (0.1–0.2 µg/g) were detected in these transgenic lines. These results implied that the limitation in endogenous BHY activity in rice endosperm was the bottleneck of astaxanthin synthesis (Bai et al. 2017). In order to produce astaxanthin-rich rice, four synthetic carotenogenic genes, *sZmPSY1*, *sPaCrtI*, *sCrBkt*, and *sHpBhy*, were introduced into an *indica* variety Huaguang 1. High-performance liquid chromatography (HPLC) analysis showed that the transgenic rice containing these four genes accumulated up to 21.9 µg/g of total carotenoids, and the predominant carotenoid was astaxanthin (16.2 µg/g), as expected (Zhu et al. 2018). Recently, *Capsicum* β-carotene hydroxylase gene (*CaBch*), a marine algal *Haematococcus pluvialis* Bkt (*HpBkt*), and *Capsicum* capsanthin-capsorubin synthase gene (*CaCcs*) were introduced into rice in combination with endosperm-expressing *stPSY* (a synthetic *CaPsy*) and *stCrtI* (a synthetic *PaCrtI*). These biofortified rice plants may accumulate diverse carotenoid derivatives such as zeaxanthin (0.8 µg/g), astaxanthin (1.1 µg/g), capsanthin (0.3 µg/g), and others, depending on the portfolios of different carotenogenic genes introduced (Ha et al. 2019; Fig. 1).

Folate-enriched rice is another successful example of vitamin biofortified rice. Folate (vitamin B₉) is a water-soluble vitamin; its deficiency is associated with several health disorders including anemia, depression, and cardiovascular disease, and also with pregnancy disorders such as neural tube defects (Iyer and Tomar 2009). In plants, folate is generated by the condensation of pterin, para-aminobenzoic acid and glutamic acid in mitochondria. The synthesis of pterin takes place in the cytoplasm, and the first step is catalyzed by GTP cyclohydrolase I (GTPCHI), whereas the synthesis of para-aminobenzoic acid is accomplished in the

plastid, and the first step is catalyzed by aminodeoxychorismate synthase (ADCS).

To achieve folate biofortified rice, *GTPCHI* and *ADCS* genes from *A. thaliana* were endosperm-specifically over-expressed in rice, resulting in transgenic rice which accumulated 15–100 times more folate in the seeds compared with the wild type control. These rice grains reached up to 17.23 µg folate/g of fresh weight (Storozhenko et al. 2007). However, these folate-biofortified rice lines showed poor folate stability during storage, such that folate concentration decreased to 50% of the original level after 4 months of storage at various temperature conditions (21 °C or 28 °C), and to 40% after 8 months (Storozhenko et al. 2007). To address this drawback, second-generation of folate-biofortified rice was developed by introducing folate-binding proteins, which complex folate, together with *GTPCHI* and *ADCS*. Higher folate concentrations and improved folate storage stability were thus achieved (Blancquaert et al. 2015).

Colored rice with proanthocyanidins and anthocyanins accumulation

Red rice and black (or purple) rice are two main types of colored rice that contain rich proanthocyanidins (PAs) and anthocyanins deposit in the pericarp, respectively (Abdel-Aal et al. 2006; Bordiga et al. 2014; Kim et al. 2010; Pereira-Caro et al. 2013). Both PAs and anthocyanins belong to flavonoids that are a type of secondary metabolites widely distributed in plants with multiple biological functions. PAs and anthocyanins are all well-recognized antioxidants, and their health-beneficial effects such as the reduction in the risk of obesity, diabetes, cardiovascular disease (CVD), and certain cancers have been reported (Tsuda 2012; Vinayagam and Xu 2015; Wallace et al. 2016; Wang and Stoner 2008).

PAs and anthocyanins biosynthesis pathways share most synthetic enzymes and a similar regulation pattern. Like other flavonoids, PAs and anthocyanins biosynthesis originates from the general phenylpropanoid pathway and involves various biosynthetic and regulatory genes (Winkel-Shirley 2001; Xu et al. 2015b). As shown in Fig. 2, the biosynthetic genes encode catalytic enzymes, including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3' hydroxylase (F3'H), flavonoid 3'5' hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) or leucoanthocyanidin dioxygenase (LDOX), UDP-flavonoid glucosyl transferase (UFGT), leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR). Among them, LAR and ANR are specifically in PAs biosynthesis, while UFGT is an anthocyanin specific biosynthetic enzyme. MYB, basic helix-loop-helix (bHLH), and WD40 repeat protein are main families of transcription factors involved in the regulation of flavonoid synthesis (Akhter et al. 2019; Pang et al. 2009;

Ramsay et al. 2003). Generally, MYB alone or the ternary protein complex MBW (MYB-bHLH-WD40) participate in the regulation of the biosynthetic genes by activating a subset of structural genes, such as the regulation pathway in rice leaves (Zheng et al. 2019).

The major anthocyanins in the pericarp of black rice are cyanidin-3O-glucoside (C3G) and peonidin-3O-glucoside (P3G), whereas red rice is rich in PAs and flavan-3-ols oligomers in which catechin is the main extension unit (Chen et al. 2019). Red rice and black rice are relatively rare among cultivated rice varieties, and they are evolutionarily distinct. The red pericarp is a wild trait typical of wild rice, the progenitor of cultivated rice. A loss-of-function mutation in *Rc*, a bHLH transcription factor gene regulating PA synthesis in rice, and the subsequent artificial selection of non-functional *rc* caused the loss of the red pericarp in most cultivated rice varieties (Sweeney et al. 2006, 2007). Black rice, instead, originated from a gain-of-function mutation in *OsB2*, also a bHLH gene which regulates anthocyanin synthesis in rice. A rearrangement in the promoter region of *OsB2* caused a substantial increase in its expression level both in seeds and leaves (Oikawa et al. 2015; Zheng et al. 2019). Black rice is even rarer than red rice among rice cultivars.

Although the mechanisms underlying rice pericarp color have been elucidated, breeding rice varieties with high PAs and anthocyanins contents in the pericarp using conventional programs is hampered by the limited knowledge about the regulatory networks involved in these pathways. Moreover, both PAs and anthocyanins exist exclusively in the pericarp. Therefore, people have to eat brown rice to obtain PAs and anthocyanins from colored rice; however, most people used to consume polished rice. Recently, the “purple endosperm rice” exhibiting anthocyanin accumulation in the endosperm was developed by the simultaneous introduction of eight anthocyanin synthesis-related genes, including two regulatory genes (*ZmLc* and *ZmPl*) from maize and six structural genes (*SsCHS*, *SsCHI*, *SsF3H*, *SsF3'H*, *SsDFR*, and *SsANS*) from *Coleus* (*Solenostemon scutellarioides*) into a common white rice variety. The purple endosperm rice thus predominantly produced two anthocyanins in rice endosperm, P3G and C3G in relatively high amounts (ca. 1 mg/g of dry weight). It should be noted that the grain weight in the purple endosperm rice was significantly reduced (25%) as compared with that of the wild type control (Zhu et al. 2017), suggesting that an excessive anthocyanins accumulation in the endosperm might adversely affect endosperm development.

Ogo et al. (2013) demonstrated that rice endosperm is an ideal platform to synthesize different classes of flavonoids. When these researchers introduced *phenylalanine ammonia lyase* (*PAL*) and *CHS* genes into rice, naringenin, a flavanone and central precursor for most flavonoids, is accumulated in rice grains with large quantity. Other types

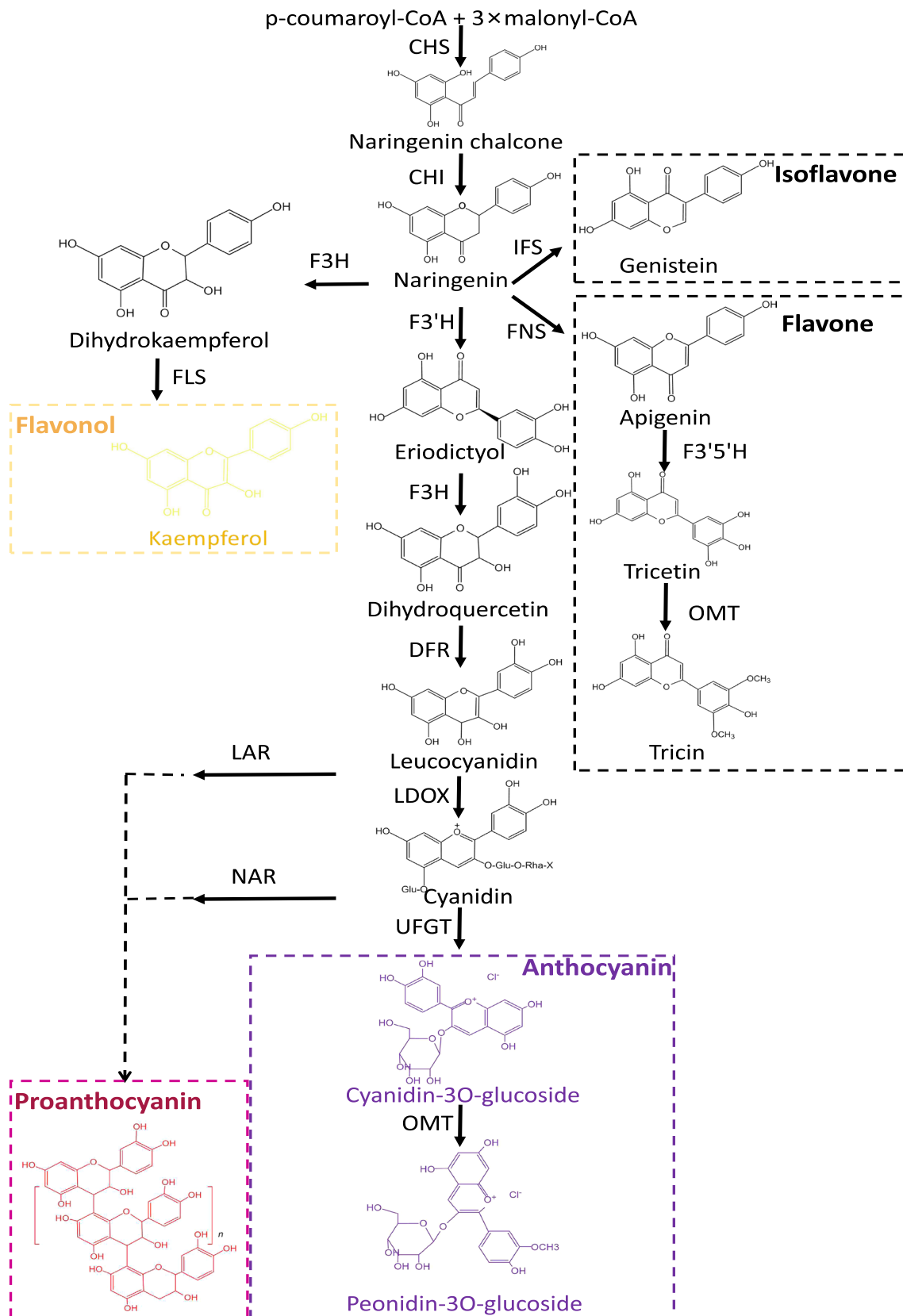


Fig. 2 Flavonoid synthesis in rice. *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *F3'H* flavonoid 3' hydroxylase, *F3'5'H* flavonoid 3'5' hydroxylase, *FLS* flavonol synthase, *IFS* isoflavone synthase, *FNS* flavone synthases, *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanidin synthase, *LDOX* leucoanthocyanidin dioxygenase, *LAR* leucoanthocyanidin reductase, *ANR* anthocyanidin reductase, *UFGT* UDP-flavonoid glucosyl transferase (Ogo et al. 2013; Zheng et al. 2019)

of flavonoids were then produced in rice endosperm on the basis of naringenin synthesis. Together with *PAL* and *CHS*, the target flavonoids kaempferol, genistein, and apigenin was generated by introducing genes encoding flavonol synthase (*FLS*) + flavanone3-hydroxylase (*F3H*), isoflavone synthase (*IFS*), and flavone synthases (*FNS*), respectively (Fig. 2). Furthermore, when introducing hydroxylase and methyltransferase *tricin* was accumulated as expected (Fig. 2). Ogo et al. (2013) concluded that rice endosperm could be conveniently manipulated for flavonoid metabolic synthesis based on the introduction of exogenous enzymes due to the presence of appropriate organelles, in addition to the less interference on flavonoid production caused by endogenous enzymes.

Multi-nutrient biofortification in rice

In general, each biofortified nutrient only addresses an individual deficiency. However, there are also a few examples of multi-nutrient biofortification in rice. Singh et al. (2017) reported the development of the biofortified rice enriched with Fe, Zn and β -carotene simultaneously, as a means to address these micronutrient deficiencies that are prevalent in similar populations worldwide. This multi-nutrient biofortified rice was developed using a combination of four transgenes *AtNAS1*, *PvFERRITIN*, *PaCRT1* and *ZmPSY*. As expected, simultaneous biofortification with improved Fe, Zn, and β -carotene was achieved. However, only up to 3.4 $\mu\text{g/g}$ β -carotene was detected in the endosperm among all transgenics, which was significantly lower than the level recorded in GR2 (Paine et al. 2005). It seems that the increased accumulation of Fe and Zn impaired the biosynthesis of β -carotene, but the authors did not discuss the potential reasons behind this observation (Singh et al. 2017).

Another representative example of multiple biofortification in rice is the research on black rice with giant embryo (BGR), which was derived from the hybridization of black rice and giant embryo (GE) mutant rice (Kim et al. 2013). BGR contains high levels of anthocyanins, as evident from its black pericarp, and enhanced GABA and other nutrients when compared with common rice because of the traits of GE. Animal tests with mouse models demonstrated that diet with BGR significantly alleviated obesity-associated metabolic disorders (Lee et al. 2016) and anxiety-related behavior (Jung et al. 2017).

Conclusion and perspective

As a staple food, rice can provide enough energy to maintain daily human activities. Rice is also a valuable source of protein, and even when its total protein content is comparatively low, the amino acid profile in rice proteins is better balanced when compared with other cereals such as wheat, corn, millet, and sorghum (Hegsted 1969). People commonly consume polished rice, which is nutritionally incomplete, considering daily human nutrient requirements. Polished rice contains only low levels of lipids, minerals, vitamins, and other micronutrients, whereas brown rice has higher levels of most macro- and micronutrients. In addition, some peculiar health-promoting metabolites such as PAs and anthocyanins (provided by colored rice) exist exclusively in the pericarp of brown rice grains. Brown rice consumption is crucial for full dietary nutrition and substantially improves the nutritional value of this staple food (Table 1). A higher intake of brown rice should be advocated in all rice-consuming countries. Interventional and epidemiological studies have demonstrated that brown rice consumption reduces the risk of developing chronic disorders such as cardiovascular diseases, obesity, type II diabetes, and certain cancers (Mattei et al. 2015; Shao and Bao 2015). However, compared with polished rice, brown rice has some disadvantages, especially the inferior texture. Pre-treatment such as germination or water-soaking may improve both sensory qualities and nutritional values of brown rice. In China, brown rice of black rice varieties is generally consumed as rice soup (“congee”) instead of steamed rice. Sticky rice with extremely low amylose content (< 2%) is more suitable when cooking rice soup. Therefore, breeding colored rice to obtain sticky varieties would improve rice soup taste and promote its consumption.

Genetic engineering is a demonstrably powerful tool to elevate micronutrients in rice grains (Table 2). For instance, Fe and Zn, β -carotene, folate, GABA, anthocyanin, and astaxanthin fortified rice germplasm resources have been developed successfully. Moreover, it has also been shown to be a feasible approach for producing health-promoting metabolites such as carotenoid or flavonoid derivatives using rice endosperm (Ha et al. 2019; Ogo et al. 2013). Nevertheless, given the regulatory complexity, public acceptance issues and continuing debate around of genetically engineered crops, non-transgenic strategies are always preferable. Conventional technologies including mutagenesis and MAS have been applied successfully to generate biofortified rice resources as well, which may be used as functional food, such as giant embryo rice for hypertensive patients, high RS for patients with diabetes, and Fe- and Zn-enriched rice for the impoverished populace.

Table 2 Summary of main achievements in rice biofortification

| Nutritional component | Main health benefit | Content in the wild type control | Maximum achievement | Target gene | References |
|-----------------------|--|----------------------------------|---|--|--------------------------|
| GABA | Antihypertensive | 1 µg/g in endosperm | 3.5 mg/g in endosperm | Endosperm-specific expression of the truncated <i>GAD</i> and knockdown of <i>GABA-T</i> gene | Shimajiri et al. (2013) |
| Resistant starch | Improving gut health, adiposity and insulin resistance; decreasing cardiovascular disease risk factors | 0% | 14.6% | Endosperm-specific inhibition of expression of <i>SBEI</i> and <i>SBEIIb</i> | Zhu et al. (2012) |
| Free lys | The first limiting essential amino acid | – | ~60-fold increase | Constitutive expression of <i>Lys</i> feedback-insensitive <i>AK</i> and <i>DHPS</i> , and endosperm-specific inhibition of <i>LKR/SDH</i> | Long et al. (2013) |
| Fe | Fe deficiency causes anemia | ~2 µg/g | 15 µg/g | Constitutive expression of <i>OxNAS2</i> and endosperm-specific expression of <i>SferH-1</i> | Trijatmiko et al. (2016) |
| Zn | Zn deficiency causes child stunting | ~16 µg/g | 45.7 µg/g | Constitutive expression of <i>OxNAS2</i> and endosperm-specific expression of <i>SferH-1</i> | Trijatmiko et al. (2016) |
| β-carotene | Vitamin A deficiency causes night blindness, blindness, and increased child mortality | 0 µg/g | ~37 µg/g total carotenoid with 84% β-carotene | Endosperm-specific expression of <i>ZmPSY</i> and <i>PaCRTI</i> | Paine et al. (2005) |
| Astaxanthin | Strong antioxidant | 0 µg/g | 16.23 µg/g | Endosperm-specific expression of <i>ZmPSY1</i> , <i>PaChl</i> , <i>C7BKT</i> , and <i>HpBHY</i> | Zhu et al. (2017) |
| Folate | Preventing neural tube defects and megaloblastic anemia | <0.5 µg/g | 25.3 µg/g | Endosperm-specific expression of <i>GTPCHI</i> , <i>ADCS</i> , and <i>CAFBP</i> | Blancaert et al. (2015) |
| Anthocyanin | Decreasing the risk of certain cancers, cardiovascular disease, diabetes, and other chronic disorders | 0 µg/g in rice endosperm | ~1 mg/g in rice endosperm | Endosperm-specific overexpression of <i>SsCHS</i> , <i>SsCHI</i> , <i>SsFSH</i> , <i>SsF3H</i> , <i>SsDFR</i> , <i>SsANS</i> , <i>ZmPl</i> , and <i>ZmLc</i> | Zhu et al. (2017) |

Emerging genomic technologies may provide new breeding approaches and greatly facilitate the nutritional improvement of rice. The functional gene research of nutrition-associated traits in rice especially the identification of various alleles with natural variations is crucial to providing necessary germplasm resources for nutritional improvement. Conventional breeding, MAS or genomic breeding can all transfer agronomically important alleles into the desired varieties through one cross and subsequently recurrent backcrosses. However, genomic breeding that comprises of two major components—genomic design and whole-genome selection for the genetic background in the late generations of backcrosses are much more high-throughput, precise, and timesaving when compared with conventional breeding or MAS. Several types of rice high-density SNP chips have been developed that facilitates the deployment of the whole-genome selection (Chen et al. 2014b, Singh et al. 2015; Yu et al. 2014). The whole procedure commonly comprises one cross, four backcrosses and one final selfing (Wing et al. 2018).

Though genomic breeding has shown great efficacy for the fast and precise introgression of existing variations into the desired varieties, many genes or traits may lack or have insufficient natural variations, which is a vital constraint for the utilization of genomic breeding. However, target induced mutagenesis technologies such as genome editing especially that based on CRISPR/Cas systems are available to create site-targeted mutations (Manghwar et al. 2019). Initially, genome editing is mainly utilized to introduce gene-targeted knockouts based on a mechanism of DNA double-stranded breaks and non-homologous end joining. Therefore, nutritional improvement using gene editing is generally involved in the knockout of those negative genes, thus impairing the synthesis or accumulation of target nutrients in desired organs. For instance, the inhibition of *SBEIIb* by CRISPR/Cas9 caused high RS content in rice (Sun et al. 2017).

Continuous innovation in this area has allowed genome editing to become much more powerful. Many CRISPR/Cas-based novel technologies have arisen including base editing, gene replacement, and gene knock-in among others, which may be potentially applied to tune protein activation or activate target genes in addition to gene-targeted knockout. However, political divergence in different countries is one of the most important risk factors, which may constrain the commercial application of genome editing in some countries and potentially cause trade frictions of genome edited products (Schulman et al. 2019). For instance, genome editing is a feasible method for creating various mutations of target genes in the United States of America, where the resultant germplasm are regarded as non-genetically engineered ones (Waltz 2016). Whereas the European Union decided to regulate gene-edited crops as stringently as the conventional genetically engineered ones (Callaway 2018), and in

most countries including China, the regulation policy for gene-edited crops remains ambiguous. For those countries in which genome editing is not feasible, TILLING based on conventional chemical mutagenesis may be an alternative to genome editing. TILLING has been shown to be an effective method for generating loss-of-function mutants with various degrees of impairment and even gain-of-function mutants for crop breeding (Chen et al. 2014a). Once the proper mutant alleles (either natural or target induced) are determined, the whole genomic selection technology based on DNA microarray can be used for precise and highly effective incorporation of target alleles into desired recipient varieties (Wing et al. 2018).

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Author Contribution statement MCZ performed most of the references collection and drafted the manuscript. YJL and HC provided revisions to the manuscript and improved the manuscript. All authors have read the revised manuscript and HC approved the final version.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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