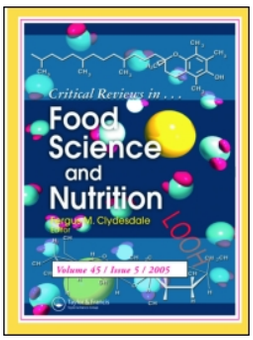

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Progress and challenges in improving the nutritional quality of rice (*Oryza sativa* L.)

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ABSTRACT

Rice is a staple food for more than 3 billion people in more than 100 countries of the world but ironically it is deficient in many bioavailable vitamins, minerals, essential amino- and fatty-acids and phytochemicals that prevent chronic diseases like type 2 diabetes, heart disease, cancers, and obesity. To enhance the nutritional and other quality aspects of rice, a better understanding of the regulation of the processes involved in the synthesis, uptake, transport, and metabolism of macro-(starch, seed storage protein and lipid) and micronutrients (vitamins, minerals and phytochemicals) is required. With the publication of high quality genomic sequence of rice, significant progress has been made in identification, isolation, and characterization of novel genes and their regulation for the nutritional and quality enhancement of rice. During the last decade, numerous efforts have been made to refine the nutritional and other quality traits either by using the traditional breeding with high through put technologies such as marker assisted selection and breeding, or by adopting the transgenic approach. A significant improvement in vitamins (A, folate, and E), mineral (iron), essential amino acid (lysine), and flavonoids levels has been achieved in the edible part of rice, i.e., endosperm (biofortification) to meet the daily dietary allowance. However, studies on bioavailability and allergenicity on biofortified rice are still required. Despite the numerous efforts, the commercialization of biofortified rice has not yet been achieved. The present review summarizes the progress and challenges of genetic engineering and/or metabolic engineering technologies to improve rice grain quality, and presents the future prospects in developing nutrient dense rice to save the everincreasing population, that depends solely on rice as the staple food, from widespread nutritional deficiencies.

KEYWORDS

Biofortification; metabolic engineering; grain and nutritional quality; rice; human health

Introduction

Rice is the predominant staple food meeting over 25% of the calorific needs of half of the world's population (Kusano et al., 2015). However, it provides up to 76% of the daily calories for most of the people in South East Asia (Fitzgerald et al., 2009; Miura et al., 2011). One-fifth of the world's inhabitants depend upon rice cultivation for livelihoods. According to FAO statistics, China is the largest producer of rice with about 204.23 MT, followed by India and Indonesia (FAO, 2012). World rice production has witnessed significant increase during the last half-century due to: (1) increase in harvest index (proportion of plant biomass in the harvested grains) by the use of semi-dwarf varieties (with short stiff stems to prevent lodging) that requires high inputs of fertilizers, pesticides, and water. However, the application of chemical pesticides and fertilizers is costly and causes environmental problems, outbreak of diseases and resistant insect pests and affects human health, (2) by exploiting heterosis through production of hybrids using the three-line or cytoplasmic male sterile system in 1970s (Yuan, 1987) but it suffers from some drawbacks such as expensive seeds, and farmers' dependency on the seeds as they need to buy new seeds in every season (as the seeds deliver expected yields in the first generation). However, since mid 1980, rice yield levels are reaching a plateau and no significant increase in rice yield is observed. Further the everincreasing population along with the adverse effects of the ongoing global climate change, scarcity of water, depletion of ozone, and an increase in frequency and severity of extreme weather conditions (Stocker et al., 2013) have potentially affected not only the rice plant growth and yield, but also the chemical and physical characteristics of the grains (Chen et al., 2012; Zhao and Fitzgerald, 2013; Goufo et al., 2014; Halford et al., 2014). To feed the growing population, rice production has to be increased by about 40% before 2030 (Khush et al., 2005; Macovei et al., 2012). This elevated demand will have to be met with the same amount of land that we have today, probably with lesser water and fewer chemicals. Use of conventional breeding which requires sufficient genetic variation for a given trait in a species has met with limited success in improving rice yield and grain quality because it is cumbersome, time-consuming and sometimes introduces adverse genes along with desirable ones due to linkage drag. The earliest breeding work includes introgression of biotic and abiotic resistance genes from wild relatives to cultivated varieties. But it could lead to narrowing of the gene pool resulting in cultivars prone to biotic and abiotic stresses (Breseghello and Coelho, 2013). Therefore, it is imperative to find novel methods such as molecular markers, genomics and transgenic approaches to complement rice breeding to break the

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yield ceiling and to improve grain quality. However, until recently, rice breeders' efforts have focused mainly on improving production while grain quality traits were largely neglected. Identification of molecular markers and their use for direct genotypic identification/selection of traits irrespective of the developmental stage of the plant (marker-assisted selection, MAS) has accelerated the rice breeding (Rao et al., 2014). The work on molecular breeding, i.e., MAS and identification of QTLs for grain quality traits has been reviewed recently (Brar et al., 2012; Bao, 2014; Rao et al., 2014). Further, with the availability of high quality genomic sequence of rice (Goff et al., 2002; Yu et al., 2002), significant progress has been made in developing functional genomics resources which have greatly accelerated identification, isolation, characterization and cloning of novel genes controlling rice yield and grain quality (Duan and Sun, 2005; Jiang et al., 2012). Advances in genetic engineering have been dominated by the transfer of one or a few well-characterized desirable genes, affecting mainly the output traits such as herbicide and insect resistance, etc., in very precise and faster way to develop the first generation genetically modified (GM) rice plants (Bajaj and Mohanty, 2005; Kathuria et al., 2007; Dunwell, 2014). Metabolic engineering is a genetic engineering approach that has been used to alter the existing metabolic pathways in plants or introduce a novel metabolic pathway in order to raise the content of a desirable substance and/or inhibit the accumulation of an undesirable one (see Jaiwal et al., 2006; Farre et al., 2014). This has been achieved by using different strategies. The most logical strategies involve: (i) the overexpression of a known rate-limiting enzyme of the metabolic pathway using a feedback insensitive enzyme (Zhu et al., 2008). Increase in the expression of enzymes in upstream pathway ensures a sufficient supply of the precursor and increase in the expression of first committed enzyme in the target compound pathway directs the flux to the subsequent downstream steps (Morris et al., 2006; Farre et al., 2014); (ii) enhancement of the activities of all the genes involved in the pathway using a transcription factor; (iii) introduction of a novel pathway to produce new compounds that are not normally produced by the plant such as very long polyunsaturated fatty acids, etc.; (iv) decreasing the flux through competing or catabolic pathways via RNAi (through small RNAs, short interfering RNA, siRNA; microRNA, miRNA, and artificial microRNA, amiRNA) or antisense technologies so as to direct the flux in the required pathway (Diretto et al., 2006; Yu et al., 2008); and (v) creation of sink compartments that store the target metabolite (Farre et al., 2014). Current efforts in developing rice with output traits including nutritional enhancement, the second generation transgenics are on rise and are under advance stage of development. Thus, to overcome the food and nutritional security, there is an urgent need of new high yielding and superior quality rice varieties that are more resilient to stress/climate change and contain higher levels of bioavailable vitamins, essential amino acids, minerals and phytochemicals that provide nutritional and health benefits (Khush, 2005). Moreover, such rice varieties with improved grain quality will be more acceptable to consumers, provide profit to farmers from increased commercial value or higher price of high quality rice, and find multiple uses in food processing industries (Hsu et al., 2014). In the present review, the progress and challenges in developing biofortified rice enriched with primary (macro-) as well as secondary (micro-) metabolites and future prospects to alleviate the widespread nutrient deficiencies in humans are discussed.

Nutrient composition

The rice grain is made up of the hull, the pericarp or the seed coat, the starchy endosperm along with germ or embryo. During the milling process, hull is removed, and whole brown rice left thereafter contains the bran coat and the germ. Further removal of the outer layer (called bran) from brown rice yields white rice. The embryo consists of majority of the mineral matter of the grain, a fourth of the protein, nearly all of the vitamins and about three-fourths of the fat whereas endosperm contains mainly the starch and protein. The bran layer of rice is laden with minerals, phenolic compounds, sterols, various vitamins like niacin, thiamine, tocopherol, tocotrienol, β-carotene and lutein along with other health promoting phytochemicals with antioxidant, anti-inflammatory and anti-hypercholestric properties (Goffman et al., 2004; Lonsdale, 2006; Esa et al., 2013). Despite of all these benefits, brown rice is not as popular as its white counterpart with consumers owing to their short shelf life and variable sensory properties (Fitzgerald et al., 2008). Differences in nutrients concentration of husked and milled rice are shown in Table 1. The short shelf life and nutritional quality deterioration of brown rice during storage is due to lipid peroxidation via lipoxygenases (LOXs), LOX1, LOX2, and LOX3 (Shirasawa et al., 2008; Kaewnaree et al., 2011). RNAi- and antisense-mediated down regulation of *LOX1* and *LOX3* genes under the control of *Oleosin-18* (specific to aleurone and embryo only) and rice endosperm specific promoters, respectively, have reduced quality deterioration and enhanced seed longevity during storage (Gayen et al., 2014; Xu et al., 2015). On the other hand, over-expression of Os*LOX2* in transgenic rice lines has resulted in faster germination rate whereas suppression of *OsLOX2* by hpRNAi has caused loss of seed germination capacity, though seed longevity during seed storage was enhanced (Huang et al., 2014). White milled rice is composed of about 90% starch, including both the amylose and amylopectin components, about 5–7% protein and nearly 0.5–1% lipids. Among micronutrients, Fe, Zn, Ca, iodine, and vitamin A are seriously deficient among many people with rice as their staple diet (Bhullar and Gruissem, 2013). Recently, whole rice grain ionome has been evaluated to identify diverse rice

Table 1. Showing nutrient content of husked rice versus milled rice (Mandal et al., 2000).

Constituents (%)		Husked Rice	Milled Rice	RDA values
Carbohydrates		77.2	79.4	130 g/d
Fats		2.0	0.3	20–35 g/d
Proteins		8.9	7.6	56 g/d
Ash		1.9	0.4	—
Vitamins (ppm)	Thiamin (B1)	3–5	0.6–1	1.2 mg/d
	Riboflavin (B2)	0.8–1	0.28	1.3 mg/d
	Nicotinic Acid (Niacin)	55.0	15.20	16 mg/d
	Pantothenic Acid	17.0	6.4	5 mg/d
Minerals (%)	Calcium	0.084	0.009	1000 mg/d
	Phosphorus	0.290	0.096	700 mg/d
	Iron	0.002	0.001	8 mg/d

accession with high elemental composition (Pinson et al., 2015). Further, the potential health benefits of whole rice grain consumption have been correlated with the problems of malnutrition and chronic diseases (Dipti et al., 2012). Rice genotypes with enlarged embryos and reduced endosperms contain more phytochemicals than genotypes with normal embryo/endosperm ratios. Thus manipulation of embryo size is important for nutritional composition of rice grain. Rice *giant embryo* (*ge*) mutants have been derived from wild-type by chemical mutagenesis. Such mutants are used to clone a gene controlling the giant embryo (*GE*) trait (Nagasawa et al., 2013) by a map-based approach (Chen et al., 2015). It encodes cytochrome P450 protein CYP78B5. Loss of function of *OsGE/CYP78B5* produces giant embryo seeds. The large embryo results from enlargement of cell size mediated by a decrease in auxin.

Strategies for enhancing the grain/nutritional quality

There are various methods to enhance the nutritional quality of food including choosing a more nutrient rich food within the same commodity group, combining different components of food to make up for the lack of a nutrient in one type of diet, looking out for nutritionally superior varieties among various cultivars to be used for breeding which is the basis of current biofortification strategies for iron-, zinc-, and vitamin-A (carotenoid)-dense rice (Bouis, 2002). Another strategy includes processing and cooking techniques for minimal loss of nutrients during postharvest. It can play an important role for each of the major micronutrient deficiencies (iron, iodine, zinc, vitamin A, folic acid) (Bouis and Hunt, 1999). Other methods like mineral supplementation and post-harvest food fortification (adding essential nutrients during food processing) are less relevant to rice because it is usually not ground into flour (Impa and Johnson-Beebout, 2012). Moreover, these methods require additional costs and are inaccessible to developing countries (Hotz and Brown, 2004). The breeding approaches used for the biofortification of rice have recently been reviewed (Brar et al., 2012). Genomics can aid in improvement of rice breeding programs with efficient identification of genes for quality traits, analyzing and scanning for available genetic variation with precisely tailored genes; along with faster transfer of genes between *Oryza* species and improved tools for molecular tracking (Varshney et al., 2006).

Besides conventional breeding, mutation breeding played a significant role in developing rice mutant lines with random changes in genes using insertion mutagenesis such as T-DNA insertion and transposon or retrotransposon tagging, and chemical/irradiation mutagenesis to create novel traits for crop improvement and to identify the gene functions (see review Wang et al., 2013). Many mutants derived by chemical mutagens (usually EMS and sodium azide) have been identified by a reverse genetic technique using high throughput genome-wide screening for point mutations in desired genes called TILLING (Chen et al., 2014). Targeting Induced Local Lesions in Genomes (TILLING) resource developed in rice (Wu et al., 2005; Till et al., 2007) may be used to target the genes involved in grain quality such as starch synthesis.

Improving nutrients' accumulation into edible parts of staple food crops (biofortification) via genetic engineering is a fast, sustainable and cost-effective alternative to the above-said methods. The genetic engineering of the rice is a potential option as rice can be easily transformed by *Agrobacterium* or biolistic methods. Genetic engineering also takes less time as compared to conventional breeding besides the added advantage of targeted expression in desirable part(s) of the plant with the use of specific promoters and even multiple genes can be stacked, using successive crosses between different transgenic lines, sequential transformation or co-transformation using same transformation or different transformation plasmids, allowing multiple traits to be transferred (Naqvi et al., 2009, 2010; Farre et al., 2014).

Quality characteristics of milled rice

Rice grain quality is a comprehensive combination of multidimensional traits involving the appearance, cooking, nutritional qualities, and milling (Yu et al., 2008). Grain quality is dependent upon variety, production, and harvesting conditions and postharvest management, milling, and marketing techniques (Fig. 1). Various factors that influence different aspects of grain quality are as follows.

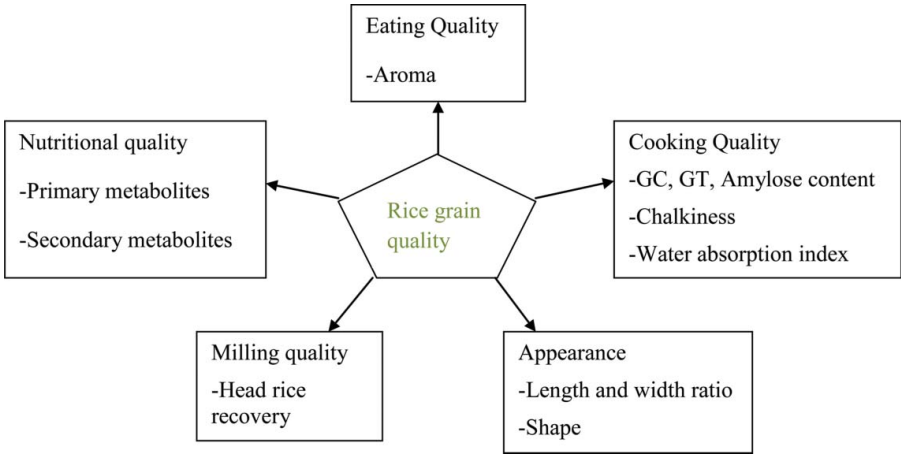

Figure 1. Factors affecting the rice grain quality (GC- Gel consistency, GT- gelatinization temperature).

Physical qualities

These include the length and width ratio, shape, and appearance of grains along with millout percentage. A long, slender, white translucent grain is desired in most of the markets. Head rice recovery, which is a measure of the percentage of unbroken grains after milling, is dependent upon the grain length and shape. The genetic basis underlying the grain size in rice has been extensively studied (Aluko et al., 2004; Li et al., 2004; Wan et al., 2005, 2006). Various QTLs such as GRAIN SIZE 3 (GS3), SEED WIDTH 5 (SW5), GRAIN WEIGHT 2 (GW2), GW8, OsSPL16, GL3 controlling seed weight, size, shape, and length have been cloned and characterized (Fan et al., 2006; Song et al., 2007; Shomura et al., 2008; Wang et al., 2012; Zhang et al., 2012; see review Huang et al., 2013). In rice, *GIF1* (Grain Incomplete Filling 1), a domestication-associated gene, has been shown to regulate grain weight by affecting the rate of grain filling. *GIF1* overexpression driven by its native promoter resulted in increased grain production while ectopic expression of cultivated *GIF1* under the action of 35S or rice *Waxy* promoter led to the production of small grains (Wang et al., 2008). Auxin responsive factor (ARF), have been shown to be linked with seed development in rice. Developing seeds show approx 40-fold higher IAA content as compared to other tissues (Xue et al., 2009) indicating the role of auxin and its signal transduction during seed development. The constitutive expression of heterotrimeric G-protein α subunit gene in *d1* mutant substantially increased the seed length and weight (Oki et al., 2005). Expression of a brassinosteroids biosynthesis in rice plants produced heavier seeds (Wu et al., 2008). Genetic engineering of seed size regulating genes have improved rice yield (Kitagawa et al., 2010). Recently miRNA has been found to control seed size and yield in rice. Rice overexpressing OsmiRNA397 produced more grain bearing branches with larger and more grains per branch than wild type rice plants by downregulation of the gene *OsLAC* whose product results in an enhanced sensitivity of plants to growth promoting hormone brassinosteroids (Zhang et al., 2013).

Chalkiness

Even though all grains become equally translucent after cooking and chalkiness has no effect on taste or texture, rice with a clear endosperm is preferred generally by the consumers over the rice that has opaque endosperm. Temperature is the most important factor which affects chalkiness (Lisle et al., 2000) along with other factors such as soil fertility and water management. Cheng et al. (2005) analyzed how chalky and translucent parts in rice grains have different cooking and eating properties. Several QTLs have been studied which are associated with chalk (Wan et al., 2005).

Milling quality

The ability of rice grains to resist breaking while being mechanically hulled is known as milling quality. Three factors that play a key role in the assessment of milling quality are brown rice ratio, milled rice ratio and head rice ratio. Many QTLs have been reported for the milling quality (Dong et al., 2004; Kepiro et al., 2008).

Chemical, cooking, and eating qualities

The cooking qualities are principally determined by the composition, structure, and interaction of the following components of the milled rice.

Aroma

2-acetyl-1-pyrroline, found in the volatile compounds of cooked rice, is the chemical which is behind the famous aroma of the Indian Basmati and Thai Jasmine rice (Buttery et al., 1983). Aroma is controlled by a single recessive gene (*fgr*, fragrance) present on chromosome 8 which encodes for betaine aldehyde dehydrogenase 2 (*badh2*). Mutations in *OsBADH2* responsible for aromatic phenotype have been confirmed by transgene complementation (Chen et al., 2008) or RNAi-induced suppression (Chen et al., 2012). The dominant *badh2* allele inhibits the synthesis of 2-acetyl-1-pyrroline (2AP) by exhausting 4-aminobutyraldehyde, a presumed 2AP precursor (Chen et al., 2008). Recently, transcription activator-like effector nuclease (TALEN) has been used to create homozygous mutant aromatic rice with significantly high content of 2AP from nonaromatic via targeted knockout of *OsBADH2* gene in a faster way (Shan et al., 2015).

Alkali spreading value

It helps to measure the temperature and the time required for cooking (Unnevehr et al., 1992).

Water absorption index

It is a measure of the amount of water absorbed during cooking of rice and thus, it determines the expandability upon cooking. Higher water absorption index causes rice to be heavier and to expand more upon cooking.

Nutritional aspects

Like other food crops, rice dietary components are**:** (1) macronutrients that are present in grams per 100 g of rice include proteins, carbohydrates, lipids (oils), and fiber, (2) micronutrients that are present in milligram per 100 g of rice include vitamins, minerals, and secondary metabolites, (3) antinutrients that limit bioavailability of nutrients, such as phytate, etc., and (4) allergens like intolerance and toxins. The first two components are to be enhanced while the latter two are to be limited or removed (Uncu et al., 2013). However, macronutrients are much more difficult to alter quantitatively than micronutrients. But the qualitative composition of the former can be easily modified or altered. The capacity to synthesize carbohydrates, proteins, and fats in seeds of staple crops such as rice, wheat, and maize is an important consideration for enhancement of yield as well as quality. Further improvement in macro- and micronutrients in rice has been reported via genetic engineering (Table 2).

Starch

The rice grain contains 80–90% starch on dry weight basis (Duan and Sun, 2005). Important starch properties like AC (amylose content), GT (Gelatinization temperature), and GC (gel consistency) contribute significantly to the appearance of

Table 2. Showing work done in nutrient enhancement of rice via genetic engineering.

Crop with cultivar	Gene transferred	Promoter used	Remark	Reference
Starch content and composition				
japonica rice cv.M202	Modified maize ADP-glucose pyrophosphorylase (AGP) large subunit sequence (*Sh2r6hs*)	Under control of an endosperm-specific promoter	AGP activity increased by 2.7 fold in the presence of Pi.	Smidansky et al., 2003
			No increase in starch content of individual seed, seed weight/ plant and total plant biomass increased by 23%	
japonica rice cv. Kitaake	*E. coli* AGPase with three amino-acid replacements (*E. coli glgC*-triple mutant)which rendered the enzyme independent of allosteric regulation	Expression in cytoplasm	Transgenic showed 3–6 fold increase in AGPase activity, enhanced starch synthesis, 11% increased in grain yield compared to wild type control	Skulsingharoj et al., 2004, Nagai et al., 2009
Rice	Antisense *ISA1*	—	Abundance of short chain DP$\leq$11 in endosperm of anti-ISA1	Nakamura et al., 1997, Kubo et al., 1999
-do-	*OsBEIIb* gene with its promoter introduced in the *be2b* mutant line	CaMV35S	Starch in *BEIIb* overexpressing line lost crystallinity due to its excess branched soluble α-glucans	Tanaka et al., 2004
japonica cv.taipei 309 & Zhong Hua 11	*OsETR2* (subfamily II ethylene receptor)	CaMV 35S	Overexpression of ETR2 leads to reduced ethylene sensitivity & late flowering, starch promoted accumulation in stem, prevent sugar translocation from stem to grains leading to reduced seed setting & reduced 1000 seed weight	Wuriyangham et al., 2009
	OsETR2-RNAi vector	CaMV35S promoter	Enhanced sensitivity to ethylene and early flowering, failed to accumulate starch in stem, sugar translocation enhanced resulting in higher 1000 seed wt	
	T-DNA insertion mutant			
Japonica rice cv. Zhonghua 11	Homozygous T-DNA insertion mutant rsr1	—	rsr1 seeds are larger in size, increased 1000 seed weight, 7.2% yield increase, increased amylose & altered structure of amylopectin, starch granule of rsr1 are round in shape and loosely packet, decreased gelatinization temperature	Fu and Xue, 2010
	Rice starch regulator 1 (*RSR1*), an APETALA 2 /ethylene-responsive element binding protein family transcription factor gene along with its promoter transferred into *rsr1* mutant (complementary expression of RSR1 gene)	*RSR1* promoter	Complementary expression of RSR1 into rsr1 mutant results in the recovered expression of starch synthesis genes,	
	RSR1 gene transferred to wild type	*RSR1* promoter	RSR1 over expression lines suppressed expression of type 1 starch synthesis genes, delayed flowering, reduced seed size and 1000 seed weight, altered amylopectin structure and increased gelatinization temperature.	
Japonica cv Nipponbare	Starch branching IIb (*SBEIIb*)hairpin RNA	under wheat glutelin promoter	Amylose content of hp-BEIIb and ami-BEIIb was double than control, significant alterations in starch granule morphology, crystallinity and digestibility	Butardo et al., 2011
	SBEIIb artificial microRNA			
Japonica cvs. Kinmaze & Nipponbare	Entire wild type *FLO2* gene introduced in *flo2* mutant (complementation) and in wild type (over expression)	*FLO2* gene promoter	flo2 have white &floury endosperm which is filled with loosely packet small and spherical starch granules, grain size, length and weight smaller, amylose content low, over expression lines produced larger & heavier grains compared with wild type	She et al., 2010

(*Continued on next page*)

Table 2. (Continued)

Crop with cultivar	Gene transferred	Promoter used	Remark	Reference
Japonica cv. Nipponbare	Transposon inserted rice mutant isoamylase3 (*isa3*) *ISA3*- RNAi vector Introduction of *ISA3* in the *sugary1* mutant	under rice α-globulin promoter	Study showed that ISA3 facilitates starch metabolism and affects morphological characteristics of plastids in rice	Yun et al., 2011
Indica rice cv.	RNAi and antisense vectors for inhibition of two isoforms of starch branching enzyme SBE (SBE1 &SBEIIb)	endosperm-specific rice glutelin Gt1 promoter	Amylose content increased from 27.2 to 64.8%, resistant starch content from 0 to 14.6% and total dietary fiber from 6.8 to 15.2%, increased gelatinization temperature and resistance to alkali digestion, High amylose rice have positive effect on lowing the blood glucose response in diabetic rats	Zhu et al., 2012
Protein				
Japonica variety Nagdongbyeo	Maize *dihydrodipicolinate synthase*	CaMV 35S and the rice glutelin GluB-1 promoter	Increased free lysine in seeds	Lee et al., 2001
Oryza sativa cv Nipponbare	Rice *OASA1* and *OASA2 (encodes* Anthranilate synthase)	Maize Ubi1 promoter	mutation of *OASA1* resulted in accumulation of Trp	Tozawa et al., 2001
Oryza sativa L. cv. Taipei	Sunflower Seed Albumin	1Dx5 and Bx17 wheat high molecular weight glutenin promoter	Increased cysteine content, grain showed little change in total sulfur amino acid content	Hagan et al., 2003
Oryza sativa L., *japonica* cv. TNG67	Sesame 2S Albumin precursor polypeptide	glutelin promoter	29–76% rise in methionine and 31–75% rise in cysteine levels	Lee et al., 2003
Oryza sativa L. cv. Nipponbare	Rice *OASA1D*	maize ubiquitin promoter	Double the amount of free Trp as compared to wild plants	Wakasa et al., 2006
Mtr1	Rice *Mtr1* mutant gene *(mtr1-D)*	maize ubiquitin promoter	Accumulation of Phenylalanine and Tryptophan	Yamada et al., 2008
Oryza sativa L., *japonica* cv. Wuxiangjing 9	*E. coli* AK, DHPS and RNAi of LKR/SDH	CaMV 35S promoter, rice endosperm-specific glutelin Gt1 promoter	Increased lysine upto ~12-fold in leaves and ~60-fold in seeds	Long et al., 2013
Oryza sativa L. cv. Taipei 309	*E. coli serine acetyltransferase*	Ubquitin promoter	Increased levels of cysteine, glutathione and methionine	Nguyen et al., 2012
Fatty Acids				
Rice	*NtFAD3*	CaMV35S	1.8- and 1.1- fold higher linolenic acid (18:3) in the root and leaf tissues	Shimada et al., 2000
-do-	*Gm FAD3*	maize ubiquitin and CaMV 35S	13 - and 2.5-fold increase in ALA content than wild type	Anai et al., 2003
-do-	*Gm FAD3–1* and *OsFAD3*	endosperm specific *Gluc* promoter	23.8- and 27.8-fold increase in ALA than wild type	Liu et al., 2012
-do-	*GmFAD3–1* and *OsFAD3*	embryo specific promoter, *REG*	25.4-(in embryo) and 27.9-fold (in bran) increase in ALA content	Yin et al., 2014
Flavonoids				
cv. Murasaki R86	soybean isoflavone synthase (*IFS*)	*CaMV 35S*	Production of isoflavone (genistein) in rice tissues, induce *nod* gene expression in different rhizobia	Sreevidya et al., 2006
-do-cv. Kita-ake	*Os PAL* and *OsCHS; OsC4H, Os4CL, OsCHI*	endosperm specific GluB1 promoter or embryo -and aleurone-specific 18-kDa Oleosin promoter	Increased Flavanone (Naringenin); Flavonol (Kaemferol), Isoflavone (Genistein), Flavone (Tricin)	Ogo et al., 2013
Pro-vitamin A (β-carotene)				
Japonica cv.TP 309	Daffodil phytoene synthase (*psy*), Erwinia uredovora phytoene desaturase (*crt 1*) and daffodil lycopene cyclase (*lcy*)	*psy* driven by endosperm-specific glutelin (Gt-1), *crt 1* (contained pea Rubisco small subunit transit peptide) under 35S, lcy (carried a transit peptide allowing plastid import) under rice glutelin	1.6 μg/g carotenoids in the endosperm	Ye et al., (2000)

Indica cvs. IR64 and MTL250, *Japonica* cv. Taipei 309	Daffodil *psy* and bacterial *crt1 fused with SSU*, PMI as selectable marker	*psy* under endosperm glutelin and *crt1* under CaMV 35S, PMI driven by 35S	0.8 μg/g (indica rice) and 1.2 μg/g (japonica rice) carotenoid in seeds, Υ-Oryzanol increased in endosperm, no effect on vitamin E compounds	Hoa et al., 2003
cv. Asanohikari, cv. Kaybonnet	Maize *psy* with SSU *Erwinia uredovora* crt 1	*psy* and *crt1* under rice glutelin	37 μg/g carotenoids	Paine et al., 2005
Vitamin B_9 (Folate)				
Japonica cv. Nippon Bare	*At GTPCHI* AtACDSAtGTPCHI / *At ADCS* genes on a single T-DNA	Rice endosperm-specific globulin 1 (*glb-1*) promoter Rice endosperm-specific glutelin B1 (*gluB-1*) *AtGTPCHI /At ADCS* driven by globulin 1 (glb-1) and glutelin B1 (*gluB-1*) promoters, respectively	No change in folate, up to 25-fold increase in pterin Folate content was six-times lower than in wild type seeds, PABA leves 49 times higher than in control plants Folate accumulated 100 times higher than wild type, 4-fold increase in Pterin and 25-fold increase in PABA than control seeds	Storozhenko et al., 2007
Australian rice cultivar Jarrah	Wheat HPPK/DHPS	Maize Ubiquitin promoter	75% increase in folate in leaf and 40% in seed, Precursors of folates were not analyzed	Gillies et al., 2008
Japonica cv. Nippon Bare	*AtGTPCHI* & *At ADCS* genes on a single T-DNA	*AtGTPCHI* driven by *globulin-1* and *At ADCS* by glutein B1 promoter	Accumulation of pterin, PABA and folate altered the expression of 235 genes without a significant effect on the expression of endogenous folate biosynthesis genes	Blancquaert et al., 2013
Vitamin E				
cv. EYI 105	Arabidopsis PDS1 (encodIng)	maize ubiquitin (Ubi)	No change in tocopherol level but a significant shift from the Υ to the α-isoform	Farre et al., 2012
Rice ssp. *Japonica* cv. Wuyujing	Arabidopsis Υ-*TMT* (*AtTMT)*	maize ubiquitin (Ubi) or endosperm-specific rice *glutelin* (GluA-3)	Increase in the α-tocotrienol after shifting of Υ-isomers to the α-isomers	Zhang et al., 2013
Iron and Zinc				
IR68144, BR29	Soybean Ferritin	maize ubiquitin (Ubi)	iron content was tripled in the transgenic plants (38.1 $\pm$ 4.5 μg/g DW)	Goto et al., 1999
Japonica rice variety Taipei 309	*Phaseolus vulgaris* Ferritin	maize ubiquitin (Ubi)	increased the iron content by 2-fold, increased iron absorption	Lucca et al. 2001
IR68144	Soybean Ferritin	endosperm-specific glutelin promoter	high accumulation of iron and zinc levels not only in brown rice but also in polished transgenic grains	Vasconcelos et al., 2003
Oryza sativa L. cv. Pusa Basmati	*Glycine max* Ferritin	rice endosperm-specific glutelin promoter (GluB-1)	increase of iron content in endosperm cells, iron-specific histochemical staining reaction	Sivaprakash et al., 2006
2D30228	*Rice nicotianamine synthase* (*NAS*) gene (*OsNAS3)*	rice endosperm-specific glutelin promoter, GluB-1	increased levels of Fe up to 2.9 fold, Zn up to 2.2 fold and Cu up to 1.7 fold, NA level up to 9.6 fold	Lee et al., 2009
Japonica rice (*Oryza sativa* L.) cultivar Tsukinohikari	Barley *HvNAS1*	pGluB-1	4-fold higher NA content	Usuda et al., 2009
Oryza sativa 'Dongjin'	Rice *OsYSL15*	OsYSL15 promoter	3-fold increase in Fe content, 20-fold increase in NA content, 2.7 fold increase in Zn content, improved plant tolerance to Zn deficiency	Lee et al., 2009
Oryza sativa ssp. Japonica cv. Taipei 309	Arabidopsis thaliana NAS (AtNAS1), Phaseolus vulgaris (ferritin), Aspergillus fumigates strain Af293 phytase	Pvferritin and Afphytase- rice seed storage globulin promoter, AtNAS1- CaMV35S promoter	more than six-fold higher iron concentration in the endosperm, 1.3–1.5-fold increase in Zn concentration	Wirth et al., 2009
An activation-tagged mutant (OsNAS2-D1) and transgenic plants over-expressing OsNAS2	Rice *OsNAS2*	Ubiquitin promoter	3-fold rise in Fe concentration of mature seeds	Lee et al., 2012

(*Continued on next page*)

Table 2. (Continued)

Crop with cultivar	Gene transferred	Promoter used	Remark	Reference
Japonica rice (*Oryza sativa* L.) cultivar Tsukinohikari	Soybean *SoyferH2* (Ferritin) and Barley, *HvNAS1, HvNAAT-A* and-*B, IDS3*	Endosperm specific OsGlb and 1.3-kb OsGluB1 promoter	Increase in iron concentration in polished T3 seeds by 4 times along with increased zinc concentration	Masuda et al., 2013
Oryza sativa L. subspecies *indica* cv. Pusa Sugandhi II	Rice *PK1*	Oleosin18 promoter	Accumulation of 1.8-fold more iron in the endosperm	Ali et al., 2013
Oryza sativa cv IR64	Rice and Soybean ferritin	glutelin-1, glutelin-4 and globulin-1	3.4-fold increase in Fe content, marker free selection	Oliva et al., 2014
Low phytate				
rice	*RINO1*	*RINO1* promoter or CaMV35S	Increase in Pi, Detrimental to plant development	Feng and Yoshida (2004)
-do-Cv. Guanglinxiangjiu	Phytase gene of *Aspergillus niger*	Ubiquitin promoter	Pi was 57% higher in mature seeds	Liu et al., 2006
-do-cv. Kita-ake	Antisense of *RINO1*	seed storage protein, glutelin *GluB1* promoter	Phytate content decreased (17%), Pi content increased by 5-fold than nontransgenic, No effect on plant height, number of panicals and wt of 100 seeds	Kuwano et al., 2006
-do-cv. Kita-ake	Antisense of (Ins(3) P1)synthase (*RINO1*)	seed specific *Ole 18* promoter	Phytic acid reduced by 68%, Increase in Pi, no negative effect on seed wt., germination and plant growth	Kuwano et al., 2009
-do-Indica cv. Swarna/IR36	RNAi vector containing myo-inositol-3 phosphate synthase (*MIPS*)	*Ole 18* promoter	Phytate reduced by 58%, Pi increased by 44.7%, 1.6 fold increase in iron, seed germination normal	Ali et al., 2013 a
-do-indica cv. Pusa sugandhi	RNAi vector containing Inositol phosphate kinase (*IPK*)	*Ole 18* promoter	Phytate reduced, Pi increased by 51%, 1.8fold more iron, without hampering growth and development of transgenic plants	Ali et al., 2013 b
-do-Japonica cv. Nipponbare	Artificial miRNA to down regulate *OsMRP5*	*Ole18*	Phytate reduced by 35.8–71.9%, increased Pi by 7.5 times Reduction in seed wt, germination and seedling emergence	Li et al., 2014
Coenzyme Q_{10}				
Oryza sativa L. cv. Nipponbare	decaprenyl diphosphate synthase (DdsA) of *Gluconobacter suboxydans*	CaMV35S promoter with (a) mitochondrial targeting (b) Golgi bodies targeting (c) cytoplasm	Seed CoQ_{10} content was 10 times to that of wild rice, the expression of DdsA having Golgi targeting signal had an inhibitory effect on growth in transgenic plants.	Takahashi et al., 2006
Oryza sativa L. cv. Haiibuki and Chukei-toku 70	decaprenyl diphosphate synthase (DdsA) of *Gluconobacter suboxydans*	CaMV35S promoter with mitochondrial targeting signal	Maximum seed CoQ10 content was 22.1 μg/g.	Takahashi et al., 2009
Oryza sativa sugary and *shrunken* mutants	decaprenyl diphosphate synthase (DdsA) of *Gluconobacter suboxydans*	CaMV35S promoter with mitochondrial targeting signal	Seed CoQ10 content per weight increased upto 1.6 (*sugary*-type) and 1.3 (*shrunken*-type) fold than that of giant embryo CoQ10-enriched lines	Takahashi et al., 2010
Recombinant proteins				
rice	7Crp peptide	Rice AGPase large subunit or maize ubiquitin-1 promoters with the glutelin GluB-1 signal peptide and the KDEL signal	The highest accumulation for the AGPase construct was about 10 μg/grain, whereas those under control of the maize ubiqutin-1 promoter contained only about 0.2 μg/grain.	Takaiwa et al., 2007
rice	IL-10	an endosperm-specific GluB-1 promoter	Accumulation of human IL-10 in transgenic rice	Fujiwara et al., 2010
rice	IL-10	a signal peptide and ER-retention signal (KDEL) with GluB-1 promoter and suppression of prolamins via RNAi	increase in the human IL-10 levels by 3-fold.	Yang et al., 2012

the grain, which in turn determines the cooking, eating, and milling quality (Bao et al., 2008).

Starch improvement

The molecular and genetic basis of starch biosynthesis and effect of various abiotic stresses on starch content and composition has been recently reviewed (Chen et al., 2012; Thisisaksakul et al., 2012; Fujita, 2014). Four main enzymes involved in cereal starch synthesis are: ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (James et al., 2003; Jeon et al., 2010 and Pandey et al., 2012). Starch biosynthetic pathway in a cereal endosperm amyloplast is shown in Fig. 2 (Thitisaksakul et al., 2012). AGPase represents the rate-limiting enzyme of the pathway which produces the activated glucosyl donor ADP-glucose, i.e., the first committed step in starch biosynthesis. SS has two isoforms, namely so-called soluble (SSS) and granule-bound soluble (GBSS) which are responsible for the synthesis of amylopectin and amylose respectively. GBSS, in turn, has two isozymes which are differently expressed in different tissues; GBSSI is expressed in storage tissues while GBSSII is predominant in nonstorage tissues. GBSSI is also known as Waxy (Wx) and it plays a key role in amylose biosynthesis. DBEs are the enzymes that hydrolyze α-(1,6)-linkages, which is important for regularization of the branching and maintenance of amylopectin crystallinity (Jeon et al., 2010). Change in the amylopectin structure and content significantly affect the morphology of starch granules which in turn impact the cooking and consumption characteristics.

Manipulation of the enzymes involved in the starch biosynthesis pathway has also been employed for improvement of quality traits in rice, e.g., enzyme AGPase which is the rate limiting enzyme in the pathway (Smidansky et al., 2003; Nagai et al., 2009). An alternate choice for manipulation of starch content involves the down regulation (via antisense or RNAi approach) of the expression of the enzymes involved in amylopectin production direct the flux towards amylose production (Morell and Meyers, 2005; Regina et al., 2006, 2010; Rahman et al., 2007; Butardo et al., 2011; Zhu et al., 2012). A multigene approach involving overexpression of Bt2, Sh2, Sh1, and GbssIIa and RNAi mediated silencing of SbeI and SbeII was employed to develop transgenic maize with increased total starch content (2.8–7.7%) and the proportion of amylose (37.8–43.7%) along with 20.1–34.7% increase in 100-grain weight, a 13.9–19% increase in ear weight and larger kernels with a better appearance, indicating possibility of a modified starch structure (Jiang et al., 2013). This approach can be utilized for rice to not only modulates the quality and quantity of starch but also for the improvement of starch-dependent agronomic properties. Various transcription factors (TFs), such as OsbZIP33 (Cai et al., 2002), OsBP-5 (Zhu et al., 2003), RSR1 (Fu and Xue, 2010), OsbZIP58 (Wang et al., 2013), and *FLOURY ENDOSPERM2* (She et al., 2010) which act as regulators of starch synthesis provides another target to modify starch content and composition in rice. Recently, three novel alleles of *flo2* were identified which conferred dull grains (Wu et al., 2015). However, the starch pathway is complex and much of the intricate details of the pathway regarding its regulation are still poorly understood. Biselli et al. (2014) analyzed available markers for apparent amylose content through GBSSI allele mining and discovered new markers. Unexpected relationship between grain shape characters and polymorphisms associated to the waxy locus was identified and analyzed. A detailed knowledge about all these parameters would provide more insights to developing new varieties of rice with improved texture, appearance, and cooking time.

Amylose content

Because of their promising health benefits and industrial uses, high amylose cereals are attracting attention. Amylose content generally ranges from 6.3 to 28.2%. The highest reported AAC (apparent amylose content) is only 30% for wild rice types

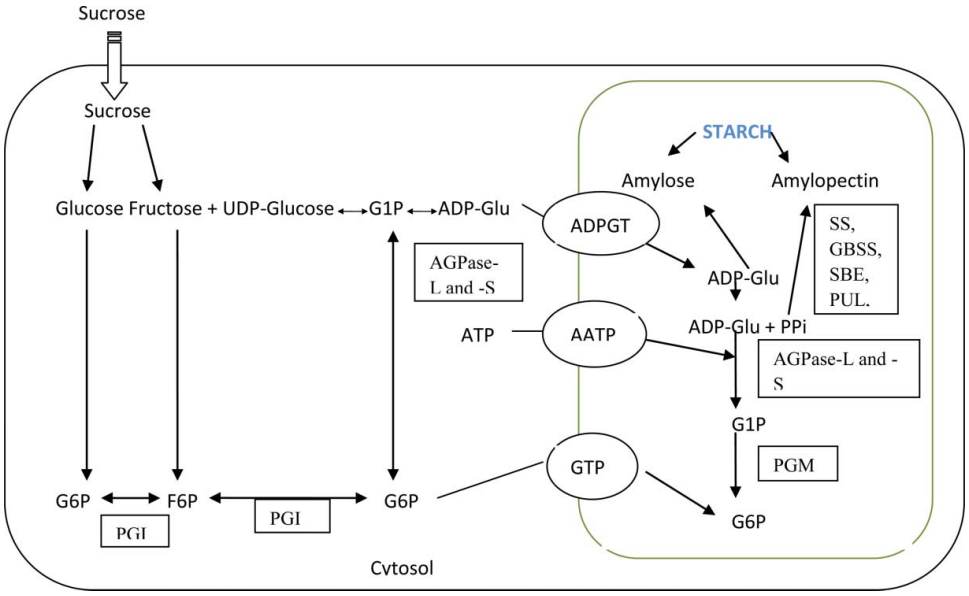

Figure 2. Starch biosynthetic pathway in a cereal endosperm amyloplast (SS, Starch synthase; GBSSI, Granule bound starch synthase; SBE, Starch branching enzyme; PUL, Pullulanase; ISA, Isoamylase; PHO, Starch phosphorylase; AGPase, L and S- Large and small subunit of AGPase respectively; PGM, Phosphoglucomutase; PGI, Phosphoglucoisomerase; modified from Thitisaksakul et al., 2012).

(Juliano, 2003). Varying contents of amylose are conferred by different alleles at the *wx* locus (Chen et al., 2008; Mikami et al., 2008). Various QTLs have been detected for AAC (Yang et al., 2014). Two approaches have been employed to achieve cultivars with high AAC: (1) overexpression of *Wx* alleles (Itoh et al., 2003; Hanashiro et al., 2008) and (2) downexpression of enzymes involved in amylopectin synthesis (Crofts et al., 2012) and SBEs (Butardo et al., 2011; Jiang et al., 2013; Man et al., 2013). However, higher AAC content has relatively inferior eating quality so three strategies have been used to reduce its content: (1) downexpression of *Wx* genes by gene silencing (Terada et al., 2000); (2) use of TFs such as OsBP-5 (Zhu et al., 2003) to reduce *Wx* gene expression; and (3) employing splicing factor genes, such as *Du-1* (Isshiki et al., 2000; Zeng et al., 2007) to lessen the splicing efficiency of *Wx* pre-mRNA (Liu et al., 2014). Starch branching enzyme (SBE) hydrolyze α-(1,4)-linkages and catalyze the synthesis of α-(1,6)-linkages within the polymer. An *indica* rice cultivar with high amylose content (65%) has been generated by transgenic inhibition of *SBE I* and *SBE IIb*, two isoforms of starch branching enzymes. This high amylose rice has also high resistant starch (RS) and total dietary fiber (TDF) content. High amlyose rice was reported to lower blood glucose response in diabetic rats in a rat feeding trial (Zhu et al., 2012).

Protein. The protein content of a polished rice seed is about 5–7% in the most common rice varieties. Glutelins represent the most common protein fraction found in rice, constituting 70–80% of the total seed protein (Katsube et al., 1999). Protein content has been shown to negatively correlate with taste (Ye et al., 2010). Since rice is used by majority of the population as their staple food, particularly in South Asian countries, many attempts have been made to improve its nutrient concentration due to its low nutritional value, mainly with respect to protein. The limiting nutritive value of seed proteins of rice stems mainly from the deficiency in certain amino acids, such as lysine and tryptophan (Lee et al., 2003; Ufaz and Galili, 2008). So far, majority of the approaches to enhance the nutritional quality are limited to maize resulting in development of quality protein maize (QPM) cultivars, which are rich in Lys and Trp, but they have not been successful in other crop species. Reasons that limit the success rate include limited genetic material available for breeding and the side effects associated with biofortification, such as retarded seed germination rate and/or abnormal plant growth as these traits do not function in a seed-specific manner. Genetic engineering approaches seem to be more promising as it allows the specific compartmentalized expression using different promoters such as endosperm-specific promoters (Ufaz and Galili, 2008).

Improvement of lysine content. Cereal grains, the major staple crops worldwide, are limiting in lysine, which is regarded as the most important essential amino acid (Ufaz and Galili, 2008). Lysine belongs to Aspartate family pathway along with three other essential amino acids viz. Methionine, Threonine, and Isoleucine (Fig. 3) (Galili et al., 2005). This pathway is feedback regulated by complex loops operated by the end products leading to reduced accumulation of soluble lysine (Azevedo and Arruda, 2010). Regulation occurs at two levels: during its synthesis when aspartate kinase catalyzes the first step of the pathway, and when dihydrodipicolinate synthase (DHDPS) catalyzes the first step of the dihydropicolinate branch, and during its catabolism, catalyzed by lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) (Galili, 2002).

Efforts have been made to understand the lysine metabolism and how it can be used to increase its content. Three strategies have been used to improve lysine content: (1) by expressing lysine feedback-insensitive forms of aspartate kinase and DHDPS; (2) by modifying seed storage proteins (SSPs), for instance silencing of 13-kDa prolamin raised total lysine content by 56% (Kawakatsu et al., 2010); and (3) overexpression of lysine rich proteins, such as RLRH1 and RLRH2 in seeds (Wong et al., 2014). Expression of feedback insensitive aspartate kinase and DHDPS resulted in higher accumulation of lysine in tobacco (Shaul and Galili, 1992a, b), canola (Falco et al., 1995), soybean (Falco et al., 1995), and Arabidopsis (Zhu and Galili, 2003). However, when the bacterial-feedback insensitive DHDPS was expressed in maize, lysine overproduction occurred only when the expression was confined to the embryo, but not in the endosperm (Frizzi et al., 2008). A constitutive promoter resulted only in the slight increase in the lysine content in the seeds in case of rice (Lee et al., 2001) and other cereals such as barley (Brinch-Pedersen et al., 1996), suggesting different mechanisms of lysine accumulation. RNAi technology has been used to reduce the activity of lysine catabolic enzymes, LKR/SDH which increased the free lysine levels in maize seeds upto 4000 ppm (Houmard et al., 2007; Frizzi et al., 2008). However, when maize lysine feedback-insensitive DHPS was overexpressed in rice, there was only a minimal increase of up to 2.5-fold in lysine content in mature seeds, though the seed germination rate was hampered. Thus developing seeds with higher lysine content without retarding the germination rate seemed to be the technical challenge until Long et al. (2013) genetically engineered rice via RNAi of rice lysine ketoglutaric acid reductase/saccharopine dehydropine dehydrogenase (LKR/SDH) and by expressing bacterial lysine feedback-insensitive AK and DHPS to increase lysine levels upto ~12-fold in leaves and ~60-fold in transgenic seeds, without showing the associated negative changes in plant growth as well as seed germination.

Improvement of cysteine and methionine content. The methionine deficiency has various downsides to it for humans as well as livestock industry. It results in reduction in wool growth in sheep, dairy production in cattle, and a reduction in the quality of meat (Xu et al., 1998). Thus increasing methionine content has been an important goal in breeding and plant biotechnology. Nguyen et al. (2012) elevated methionine (1.4-fold) and cysteine (2.4-fold) levels in rice by ectopic expression of an *Escherichia coli* serine acetyltransferase isoform driven by an ubiquitin promoter. The transgenic lines also showed higher isoleucine, leucine, and valine contents, indicating the conversion of methionine to isoleucine.

Improvement of tryptophan and phenylalanine content. Aromatic amino acids act as precursors for a wide variety of secondary metabolites such as flavonoids, phenylpropanoids, indole alkaloids, and lignin. Tryptophan (Trp) is used to supplement poultry and pig feeds. It is employed in the treatment of depression as a pharmaceutical agent (Massey et al., 1998). Phenylalanine (Phe) is used in the production of aspartame, low-calorie sweetener. Thus, it is desirable to

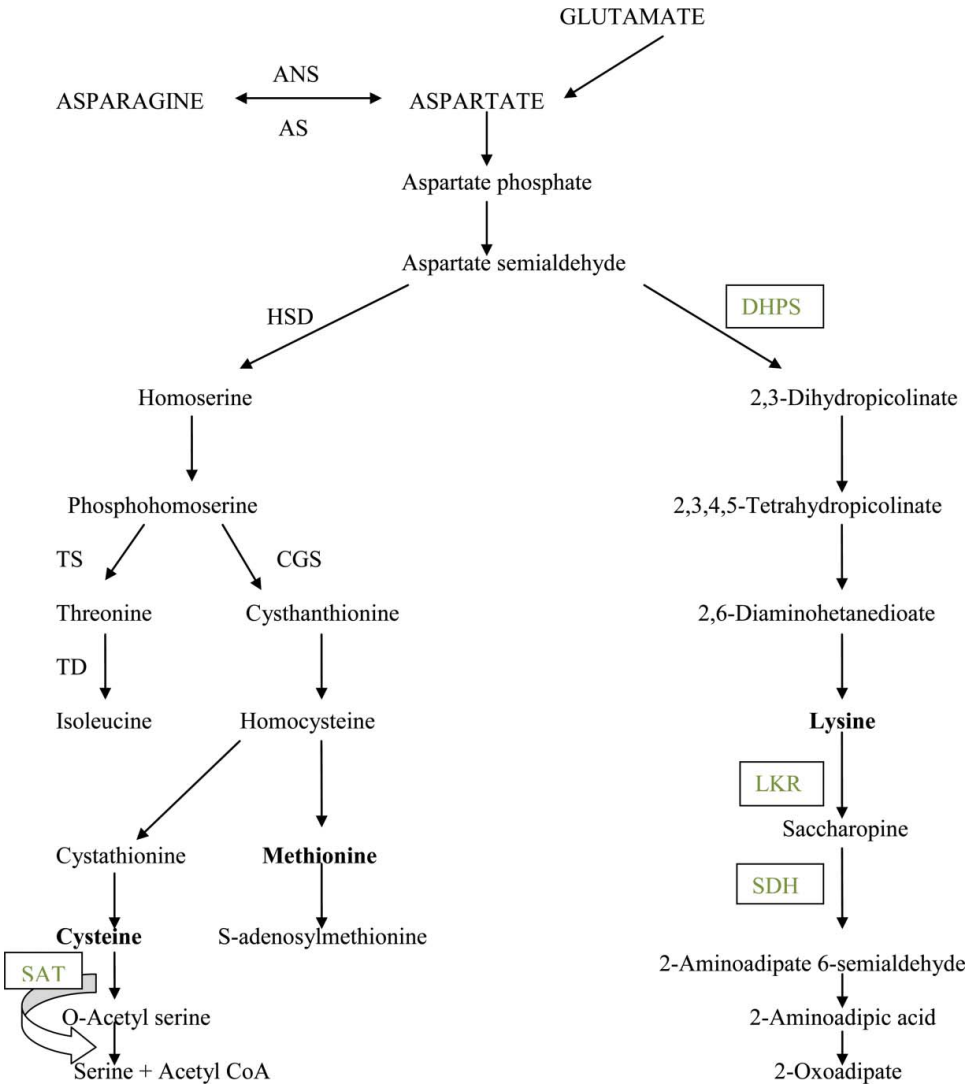

Figure 3. Aspartate family pathway (ANS, asparagine synthetase; AS, anthranilate synthase; HSD, homoserine dehydrogenase; DHPS, dihydrodipicolinate synthase; TS, threonine synthase; TD, threonine dehydratase; CGS, cysthationine γ-synthase; SAT, serine acetyltransferase; LKR, lysine ketoglutaric acid reductase; SDH, saccharopine dehydropine dehydrogenase; Enzymes in boxes signify the enzymes employed for enhancement of the essential amino acids via genetic engineering; modified from Azevedo and Arruda, 2010).

increase Trp and Phe contents in staple foods. Little work has been attempted in increasing Trp content as compared to increasing Lys, (Zhu and Galili, 2003), and Met (Lai and Messing, 2002). In plants, bacteria, and fungi, the aromatic amino acids belong to shikimate pathway and are biosynthesized from a common precursor, chorismate (Fig. 4). Genes containing feedback-insensitive α subunits of anthranilate synthase (AS) has been employed for the accumulation of free Trp in crops. This approach has been used in various crops viz. *Astragalus sinicus* (Cho et al., 2000), tobacco (Zhang et al., 2001), potato, and rice (Tozawa et al., 2001; Yamada et al., 2004; Wakasa et al., 2006). Unlike Trp, there are no mutant plants that accumulate Phe except rice Mtr 1 mutant which has both Phe as well as Trp (Wakasa and Widholm, 1987). Wakasa and Ishihara (2009) over expressed Mtr 1, which catalyzes the final reaction in Phe biosynthesis and encodes for an arogenate dehydratase (ADT)/prehenate dehydratase (PDT), in rice which showed elevated levels of both Phe as well as Trp,

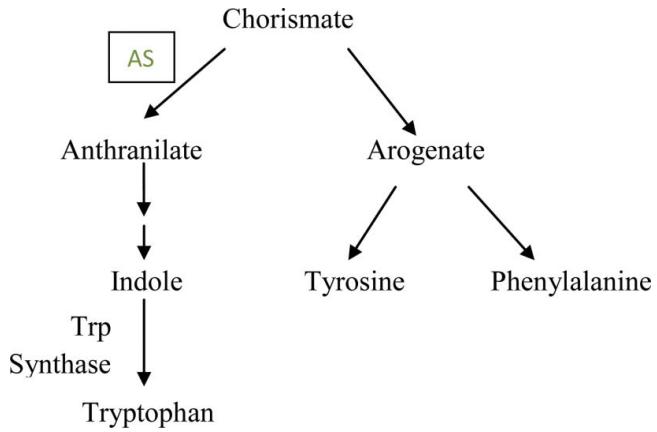

Figure 4. Pathway showing aromatic amino acids biosynthesis (AS, anthranilate synthase; Trp, tryptophan, Enzymes in boxes signify the enzymes employed for enhancement of the above amino acid(s) via genetic engineering; modified from Radwanski and Last, 1995).

indicating that reactions catalyzed by AS and ADT are critical regulatory points in the biosynthesis of Trp and Phe, respectively.

Fatty acids. Very long chain polyunsaturated fatty acids (VLCPUFAs) and long chain polyunsaturated fatty acids (LCPUFAs) are regarded as essential for regulation of cholesterol synthesis and transportation for the maintenance of cellular membrane (Simopoulos, 1991) and eicosanoid synthesis (Kankaanpaa et al., 1999). They form key constituents of neuronal cells in brain and retinal tissues and affect cell function and development and overall human health (Qi et al., 2004) and are known to reduce the incidence of cardiovascular and Alzheimer's diseases (Demaison and Moreau, 2002; Okuyama et al., 2007). The sources of α-linolenic acid (ALA) include deep-sea fish and some oil seed plants like flax, rape, walnut, soybean, and perilla. However, there is a limited supply of deep-sea fish, the oil seed plants are also quite few and high ALA content leads to rancidity and "off" flavors in food products developed using them (Yokoo et al., 2003). Therefore, development of alternate sources of ALA other than oilseed plants is required. Rice seeds, contain very low amounts of ALA (<0.4 mg g/1), therefore developing varieties with higher ALA content would help in overcoming ALA deficiency.

There are different pathways (ω-6 pathway and ω-3 pathway) for the synthesis of VLCPUFAs in nature (Qiu, 2003). Various genes encoding enzymes such as FAD3 (Shimada et al., 2000; Liu et al., 2012), D5-elongase (Chodok et al., 2012), omega-3 fatty acid desaturase, Δ8-desaturase and Δ5-desaturase (Cheah et al., 2013) have been used to elevate levels of various LCPUFAs. FAD3 which catalyzes ALA synthesis in seeds has been used to modulate/enhance ALA content in rice seeds. A tobacco FAD3 under the control of CaMV 35S promoter has been expressed in rice which led to an increase in ALA level up to 2.5-fold (Shimada et al., 2000). ALA content was increased up to a 13-fold when soybean FAD3 driven by the maize ubiquitin-1 promoter was introduced in rice (Anai et al., 2003). CaMV 35S and Ubi-1 are the class of constitutive promoters which are not known to drive the expression of genes strongly enough in rice seeds (Qu and Takaiwa, 2004). Thus there is a need to use strong endosperm-specific promoters to further increase ALA accumulation in rice endosperm. Three FAD3 genes have been cloned and characterized from rice. However, their role in increasing ALA concentration is not clear. ω-3 FAD genes from rice and soybean have been introduced into rice. Different promoters such as an endosperm-specific expression promoter, GluC (Qu et al., 2008), or a constitutive expression promoter, Ubi-1 were used to evaluate their potential to further increase ALA accumulation. ALA content was found to be higher by 23.8- and 27.9-fold when soybean and rice FAD were used, respectively. GluC promoter was found to be better than the Ubi1 promoter (Liu et al., 2012). More than 80% of the daily adult ALA requirement would be met by a meal-size portion of high ALA rice. ALA acts as precursor of important LC-ω3-PUFAs, such as EPA and DHA. Higher plants lack the machinery to convert C18-PUFAs into very long-chain (VLC)-PUFAs. Metabolic engineering have enabled scientists to synthesize EPA and DHA in higher plants. Arabidopsis has been genetically modified to produce EPA (3%) (Qi et al., 2004) along with *Brassica juncea* which led to accumulation of EPA (8%) and DHA (0.2%) (Wu et al., 2005). Thus, rice can also be utilized to produce EPA and DHA by combinational overexpression of D6 and C20 elongases, and D6, D5, and D4 desaturases employing these high ALA rice as hosts.

Oleosin, the most abundant protein present in the oil bodies of plant seeds has also been used to regulate fat content. Overexpression of two soybean oleosin genes under the action of an embryo-specific rice promoter REG-2 in transgenic rice resulted in a rise in lipid content of the seeds up to 36.93 and 46.06%. However, there was no change in the overall fatty acid profiles of the triacylglycerols (Liu et al., 2013). Liu et al (2013)'s review throws a light on the class, distribution, and variation of phospholipids in rice, their effect on rice quality, and human health and the methods of analytical profiling. There is a tremendous interest in the manipulation of rice bran oil which is beneficial for human health. The bran oil also contains antioxidant compounds such as oryzanol (1–2%), lecithin, tocopherol, and tocotrienol (Zullaikah et al., 2005). Introduction of GmFAD3-1 and OsFAD3 genes under the control of an embryo-specific promoter (REG) into rice increased ALA content in embryos and bran. The increased ALA is preferably present at the sn-2 position in triacylglycerols which are digestible and absorbable for humans (Yin et al., 2014). Similarly, rice bran specific expression of *Brassica juncea* FAD3 (BjFad3) significantly increased C18:3 fatty acid content (up to 10-fold) and also improved nutritionally desirable ω6: ω3 ratio (2:1) in one of the transgenic rice lines (Bhattacharya et al., 2014).

Dietary fiber. Whole grains and bran of cereals, such as barley, oat, and wheat are the good source of the dietary fibers whereas rice is not a significant source of dietary fiber. Nonstarch polysaccharides (NSP) are the principal component of dietary fibers which are of two types: soluble and insoluble. Soluble dietary fibers are composed of pectin substances that are composed of arabinoxylans and (1,3;1,4) β-D-glucans. Dietary fibers have beneficial effects on human health, e.g., reduction in constipation, positive effects in certain conditions such as cardiovascular diseases, blood cholesterol, colon cancer, and regulation of glucose absorption and insulin secretion and promotion of the growth of beneficial gut microflora (as a prebiotic). Beside these roles, soluble β-glucans also have immune-stimulatory activity. However, the amount and quantity of these nonstarch polysaccharides which are the principal component of dietary fibers tends to depend upon the type of rice and its cultivar and degree of milling. Brown rice is rich in insoluble and soluble fiber. Since no detailed studies on β-glucans from rice are available, the extent to which these benefits are shared by rice β-glucans is not known. In Arabidopsis plants, the expression of rice cellulose synthase like families (CSLF) genes that encode β-glucan synthases results in detection of β-glucan (not synthesized by Arabidopsis) (Burton et al., 2006). However, downregulation of wheat *β-glucan synthase* gene (CSLF6) using RNAi results in decrease in total β-glucan in endosperm (Nemeth et al., 2010) and similar suppression of glucosyl transferase gene decreases the arabinoxylan content (Lovegrove et al., 2013). These studies indicate that β-glucan amount and properties can be modified to enhance health benefits.

Flavonoids. Flavonoids consists of phenylpropanoid-derived secondary metabolites found in plants that perform an array of functions such as their involvement in UV filtration, pigmentation for flowers and fruits coloration for attracting pollinators and for efficient seed dispersal, their role as messenger molecules in plant-rhizobium and mycorrhizal symbiosis, auxin transport inhibitor, antiherbivores, pollen-viability, and antioxidant as well as antimicrobial compounds (see Jaiwal et al., 2006; Dixon and Pasinetti, 2010; Buer et al., 2010). The various health-promoting effects of these compounds have triggered an intense academic as well as commercial interest in improving their levels in staple food crops (Ogo et al., 2013).

Flavonoid biosynthesis has been studied by various researchers (Fig. 5, Tanaka et al., 2009, 2010; Nishihara and Nakatsuka, 2011). Except for a trace amount of tricin found in bran, rice does not contain significant content of different flavonoids. *IFS* (isoflavone synthase) gene has been used to result in the production of the isoflavone genistein (Sreevidya et al., 2006). Various isoflavones such as genestein and daidzein has been reported to have a variety of health benefits (Fader et al., 2006; Liu et al., 2002). Thus, incorporation of isoflavone synthesis into staple crops may be useful for enhancement of their nutritional value.

Rice transgenic plants expressing well-characterized five flavonoid biosynthetic genes (*OsPAL, OsC4H, Os4CL, OsCHS, and OsCHI*) or their combination under the control of different promoters accumulated high amounts of flavonoids that varied depending on the class of flavonoids (Ogo et al., 2013). It is an excellent example of using a multigene approach to produce and accumulate high levels of various types of flavonoids in the rice endosperm. Improvement in content of sakuranetin, a flavonoid phytoalexin (Shimizu et al., 2012) and resveratrol (Baek et al., 2013) in rice has also been reported. Further, the resveratrol-enriched transgenic rice grain accumulating 1.9 μg/g of resveratrol in addition to fiber and polyphenols has strong anti-obesity effects when fed to animals (Baek et al., 2014).

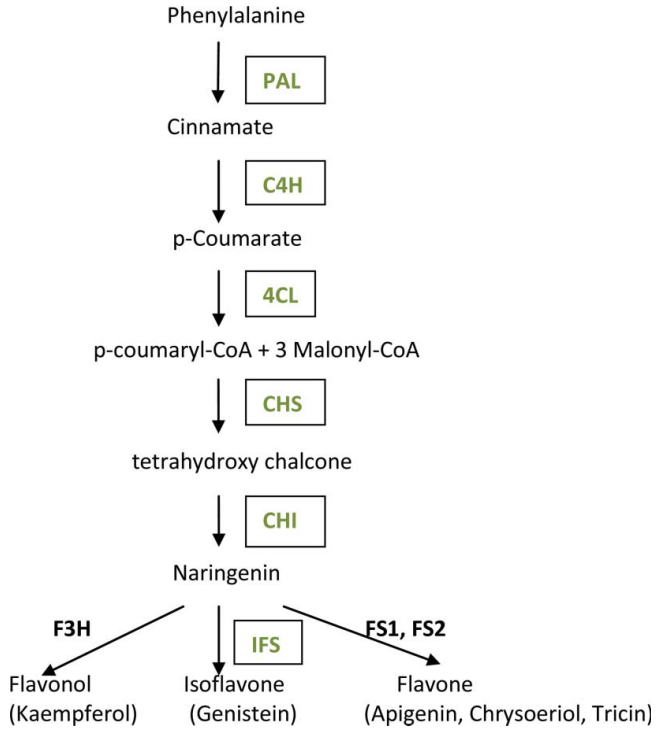

Figure 5. Biosynthetic pathway of flavonoids (PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate : coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; IFS, isoflavone synthase, Enzymes in boxes signify the enzymes employed for enhancement of the various flavonoid(s) via genetic engineering; modified from Nishihara and Nakatsuka, 2011).

Vitamin A. Carotenoids, predominantly β-carotene are cleaved within the body and function as provitamin A (Yeum and Russell, 2002). Vitamin A deficiency is predominant in countries where majority of the population depends on a staple food such as rice which lacks provitamin A in the edible part of the grain, i.e., the endosperm. Vitamin A deficiency affects 250 million people and is associated with permanent blindness and a depressed immune system (Underwood, 2000).

The genes for the enzymes of the biosynthetic pathway of carotenoids (Fig. 6) have been isolated and well characterized from a variety of sources such as of bacteria, fungi and plants (Scolnik and Bartley, 1994; Al-Babili et al., 1996, 1996). Rice plant has the machinery to synthesize carotenoids in the leaves but some of the enzymes of the carotenoid pathway do not express in the endosperm. Rice plant has been genetically altered to produce β-carotene in the endosperm of the grain, giving rise to a characteristic yellow color, hence the name "golden rice" (Ye et al., 2000). The first generation golden rice has 1.6 μg of total carotenoids per g dry weight of rice, amounting to 100 μg retinol equivalents with a daily intake of 300 g of rice per day, which seems to be unrealistic for the children who are at risk for vitamin A deficiency. Paine et al. (2005) developed the second generation of golden rice and reported 23-fold increase in grain carotenoid levels (maximum 37 μg/g) by using maize phytoene synthase (*psy*) instead of daffodil *psy*. Datta et al. (2003) bioengineered β-carotene metabolism in indica rice using the rice seed-specific glutelin promoter leading to the production of the carotenoids in the range of 0.297 mg/g

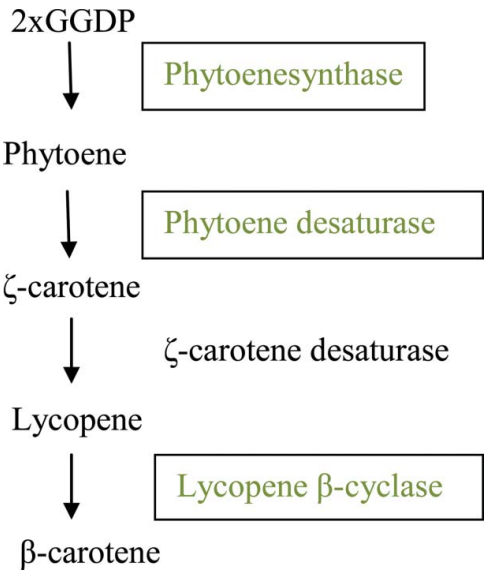

Figure 6. β-carotene biosynthetic pathway (GGDP, geranyl geranyl diphosphate, enzymes in boxes represent the enzymes used to enhance β-carotene levels via genetic engineering; modified from Beyer et al., 2002).

to 1.05 mg/g. Cooking led to a ~10% reduction in total carotenoid content, however, β-carotene levels was not much affected. There have been some other attempts to further increase β-carotene levels in indica as well as japonica rice (Hoa et al., 2003; Al-Babili et al., 2006). Although indica rice grain is bigger than that of japonica rice, the amount of carotenoids was higher in japonica rice.

Thiamine. Rice contains low thiamine or vitamin B1 which is just 18% of the daily dietary allowance (RDA) of vitamin B1 (1.3 mg/day). During polishing of rice, thiamine level further decreases due to the removal of aleurone layer and germ that contain more thiamine than the endosperm. Thus, deficiency of thiamine causes beriberi in humans who are dependent on polished rice (white) as a sole food. This problem can be overcome either by supplementing rice with chemically synthesized thiamine or by parboiling of rice which lead to the retention of thiamine in matrix of the white rice kernels. However, these interventions are expensive and inaccessible. An alternative approach is to enrich rice with thiamine using genetic engineering. In recent years significant improvement has been made in illumination of thiamine biosynthesis pathway in plants (Gerdes et al., 2012). Thiamine synthesis occurs in plastid by the condensation of two separate biosynthesized moieties of pyrimidine (hydroxymethylpyrimidine pyrophosphate, HMP-PP by phosphomethyl pyrimidine synthase, THIC) and thiazole (hydroxyethylthiazole phosphate, HET-P, by thiazole synthase, THI1) by the action of TMP-synthase (TH1) to form thiamine monophosphate (TMP). TMP is then exported from plastid to cytoplasm where it is hydrolyzed to thiamine which is then converted into thiamine pyrophosphate (TPP), a thiamine active form in the cytosol. The pathway is regulated by RNA sequences, riboswitches, where the product TPP binds to the pre mRNA of particular thiamine biosynthetic genes that interferes with gene expression. A TPP riboswitch sequence located in the 3′-UTR of *THIC* gene acts as a negative regulator of thiamine biosynthesis pathway in plants. Introduction of a mutated riboswitch (A515G in the 3′-UTR) that can no longer bind TPP tightly, into a Arabidopsis *THIC* knockdown mutant line, increased thiamine in seeds by 20% with no increase in TMP or TPP (Bocobza et al., 2013). However, these transgenic lines showed chlorosis, retarded growth and delayed flowering. In another study, constitutive over expression of *THIC* under Ubi promoter resulted in moderate rise in TMP and TPP levels in leaves with increased oxidation of carbohydrates. Thus these strategies seem to be inapt for vitamin B1 biofortification. Subsequent work pointed out that the first two steps of the thiamine biosynthesis pathway (e.g., those catalyzed by THIC and THI1) are important to increase thiamine content in plants. A balance of the two key precursors, i.e., HMP and HET, will result in the accumulation of most bioavailable thiamine (Pourcel et al., 2013). Further, the strategy of targeting thiamine binding protein, which accumulates during seed maturation on the periphery of the seed to the rice endosperm, needs to be explored (Rapala-Kozik, 2011; Pourcel et al., 2013).

Folate. Folate, also known as vitamin-B9, is essential for numerous functions such as acting as a cofactor in certain biological reactions like the transfer of carbon units (C1 metabolism) which in turn is important for the biosynthesis of some nucleotides, synthesis of vitamin B5, methionine or Met formyl-Met-tRNA in all living forms (Roje, 2007). A lack of dietary folates can lead to several diseases, including neural tube defects in developing embryos such as anencephaly and spina bifida (Pitkin, 2007), megaloblastic anemia, birth anomalies, cardiovascular diseases, certain types of cancers, and cognitive deficits (Blancquaert et al., 2013). Detailed information on folate biosynthesis, turnover, and transport in plants can be utilized for further enhancement of folate content in rice (Hanson and Gregory III, 2011). However, less is known about the genes involved in folate catabolism, turnover, transport, and subcellular localization. Folate biofortification via metabolic engineering has been achieved by the use of various enzymes involved in the para-aminobenzoate and pterin branches of the folate biosynthetic pathway such as GTP cyclohydrolase I (GTPCHI) along with *Arabidopsis thaliana* aminodeoxy chorismate (ADC) synthase (Diaz de la Garza et al., 2007; Storozhenko et al., 2007; Blancquaret et al., 2013), hydroxymethyldihydropterin (HMDHP), pyrophosphokinase (HPPK), and dihydropteroate (DHP) synthase (HPPK/DHPS) (Gillies et al., 2008).

To avoid negative feedback regulation from GTPCHI from plant sources, mammalian GTP cyclohydrolase I (GTPCHI) along with *Arabidopsis thaliana* ADC synthase, the first enzymes in pterin and p-ABA biosynthesis, respectively, were utilized for folate biofortification in tomato leading to an enhancement of folate content by 25-fold (Diaz de la Garza et al., 2007). Storozhenko et al. (2007) specifically overexpressed *Arabidopsis thaliana* GTPCHI (G- line) and ADCS (A-line) genes in rice seeds using endosperm-specific globulin promoter. Plant GTPCHI was used to avoid accumulation of undesirable intermediates. ACDS overexpression resulted in 49-times higher PABA content than untransformed plants. However, the increased PABA level had an inhibiting effect on folate synthesis, as a result of which, folate content was reduced to six-fold than control plants. Seeds of G-lines had almost the same amount of folate as control plants. Seeds of the GA lines (overexpressing both *Arabidopsis thaliana* GTPCHI and ACDS) had a staggering 15–100 times increase in folate amount ranging from 6.0 up to 38.3 nmol/g. Blancquaret et al. (2013) investigated the effect of overexpression of 6*Arabidopsis thaliana* GTPCHI and ACDS on rice seed metabolism aspects including seed developmental and plant stress/defense related responses. Further, the expression of the endogenous level of biosynthetic genes of folate was not affected by folate biofortification. Gillies et al. (2008) utilized a bifunctional enzyme, HPPK/DHPS, from wheat to enhance folate content in rice seeds using a maize ubiquitin promoter. There was an increase of folate level by 1.2- to 2-fold as compared to control plants. Thus, enzyme(s) of folate biosynthesis pathway from a closely related species can also be utilized for folate improvement in rice.

Vitamin E. Vitamin E refers to eight lipid soluble antioxidant compounds of tocopherol and tocotrienol family that are collectively known as tocochromanols. All eight lipid-soluble antioxidant compounds function as important component of human defence, providing protection against oxidative damage thereby decreasing the risk of various diseases such as cancers,

cardiovascular disorders, and neurodegenerative disorders. The entire biosynthetic pathway of vitamin E has been well characterized in the model organisms, *Arabidopsis thaliana* and *Synechocystis* sp PCC6803. The genes of its biosynthetic pathway have been cloned and employed to modify the single and multiple pathway steps for the manipulation of its content and composition (DellaPenna and Pogson, 2006). Transgenic rice constitutively overexpressing Arabidopsis p-hydroxyohenylpyruvate dioxygenase (HPPD) has been shown to accumulate marginally higher levels of total tocochromanol in grains mostly due to slightly higher tocotrienol content. However, the tocopherol content was not changed but the grains showed a marked shift from the γ- to the α-isoform and thereby increasing the vitamin E activity of the grain (Farre et al., 2012). The work on an another cereal, maize, has shown that the simultaneous expression of different genes involved in tocopherol biosynthesis in combination is the promising strategy to increase vitamin E content and composition (Naqvi et al., 2011). This is yet to be confirmed in rice. *Constitutive expression of Arabidopsis* γ-TMT (AtTMT) resulted in a rise in the α-tocotrienol content as most of the γ-isomers were converted to α-isomers. However, there was no significant effect on α-tocopherol level or the absolute total content of either tocopherols or tocotrienols. This was the first demonstration that showed the shift in the tocotrienol synthesis in rice on overexpression of a foreign γ-TMT (Zhang et al., 2013). Zhang et al. (2013) showed that overexpression of GmTMT2a resulted in significant increase in α-tocopherol content (4–6-fold in transgenic *Arabidopsis,* 3-4.5-fold in transgenic maize seed). This strategy can be employed to increase α-tocopherol content in rice.

Vitamin C. Ascorbic acid is an important water-soluble vitamin owing to its antioxidant, antiatherogenic, immunity boosting, and anticarcinogenic properties. It performs an array of functions which involve biosynthesis of collagen, muscle carnitine, catecholamines and neurotransmitters (Naidu, 2003). Humans are unable to synthesize this important vitamin as they lack gulonolactone oxidase enzyme. Different pathways for the biosynthesis of ascorbic acid have been reported in plants although not much is known about this pathway in monocots. Holler et al. (2015) generated knock-out mutants for ascorbic acid biosynthetic genes in rice to study the influence of ascorbic acid on stress tolerance and overall plant development. Since ascorbic acid is an important nutrient, its values can be enhanced in staple crops such as rice to harness the wide variety of health benefits it offers.

Iron and zinc. Iron deficiency affects about 2 billion of the world population, making it the most widespread micronutrient deficiency (Zimmermann and Hurell, 2007) causing 0.8 million casualties per year world-wide. Iron deficiency affects immunity, causes fatigue, low-work productivity, higher chances of pregnancy mortality, insufficient psychomotor and mental development in infants, and chronic hypoxia (Goto and Yoshihara, 2001). Zinc acts as a cofactor essential for the structure and functioning of various proteins. Its deficiency is common in plants as well as animals and has been associated with difficulties in pregnancy and delivery, poor growth of infants, congenital anomalies, and retarded mental and immunological development of the fetus.

The amount of bioavailable iron is influenced in part by iron intake and in part by its absorption. Some crops such as spinach and various leguminous plants are rich in iron. But they contain compounds such as oxalic acid, polyphenols and phytate-like substances which reduce the bioavailability of iron. One of the strategies for iron biofortification involves elevating the iron content of the hydroponic culture media or soil, thereby improving the Fe concentration of the crops. However, it is expensive and does not allow the desirable targeted accumulation to a specific plant part. Another approach involves the use of foliar sprays (Yuan et al., 2012).

A more sustainable approach is biofortification via plant breeding or genetic engineering. One of the strategies involves increasing natural seed ferritin (Goto et al., 1999; Qu et al., 2005; Oliva et al., 2014). Goto et al. (1999) employed soybean ferritin gene to elevate iron levels in rice using an endosperm specific promoter, *GluB-1*. The iron content was increased up to three-fold (38.1 $\pm$ 4.5 μg/g DW) as compared to nontransformed seeds (11.2 $\pm$ 0.9 μg/g DW). The meal-size portion of this ferritin enriched rice would be able to provide around 30–50% of the daily recommended iron dose (approximately 13–15 mg-Fe). However, the increase in iron content achieved using ferritin overexpression is upto a limit. Attempts to further increase iron concentration through ferritin overexpression cannot be achieved. Besides, rice milling involving the removal of outer layers of rice leads to dramatic reduction in iron levels of the grains as majority of the iron is stored in the aleurone layer. Another method to alleviate iron deficiency involves introduction of metal chelators like nicotianamine and mugineic acid (Lee et al., 2012; Masuda et al., 2013).

Although Fe and Zn are essential micronutrients, they are toxic at higher concentrations. Thus, their amounts must be regulated by balancing their uptake, utilization and storage in order to maintain proper metal homeostasis. Alternatively, Fe and Zn levels have been modified using these transporters to increase their uptake as well as their distribution to a specific plant part. OsIRT1, OsIRT2, OsZIP4, OsYSL15, OsMTP1 OsZIP1, OsZIP3, OsZIP4, and OsZIP5, *OsFRDL1* and OsYSL2, OsYSL2, OsYSL15, and OsYSL18 are some of the examples of such transporters which help in uptake of iron and zinc from the soil (Bughio et al., 2002; Vert et al., 2002; Ramesh et al., 2003; Koike et al., 2004; Ishimaru et al., 2006; Ishimaru et al., 2007; Aoyama et al. 2009; Lee et al., 2009; Yang et al. 2009; Yokosho et al., 2009; Inoue et al. 2009; Ishimaru et al. 2010; Lee et al. 2010; Menguer et al., 2013).

Coenzyme Q10. Coenzyme Q (CoQ), also known as ubiquinone, is an important electron transfer molecule in the respiratory chain which produces ATP and has fundamental role in cellular bioenergetics (Kawamukai, 2002). CoQ_{10} is now widely used as a food supplement due to its beneficial effect in cardiovascular, neurodegenerative, and mitochondrial conditions, diabetes, periodontal disease and male infertility and some other diseases as suggested in a number of preclinical and clinical studies (see Parmar et al., 2015). Accumulation of increased levels of CoQ_{10} has been achieved in transgenic tobacco, rice and *Panicum meyerianum* (Ohara et al., 2004; Takahashi et al.,

2006, 2009 and 2010; Seo et al., 2011). Indica rice which has higher grain weight than that of japonica rice can be utilized to introduce CoQ10 biosynthesis which would tend to result in even higher accumulation.

Recombinant proteins. Plants have been employed for the production of various recombinant proteins as the product can be easily scaled and the production costs are low. Seeds, especially cereal endosperm, have the potential advantages such as high accumulation of products, high stability at ambient temperature, possession of complex post-translational glycosylation capabilities, no contamination of human and animal pathogens and the products do not require further processing and are safe enough for direct oral administration (Lau and Sun, 2009; Stoger et al., 2005; Takaiwa et al., 2007). Recently, the advantages, limitations, production methods, and improvements in yield and quality of the pharmaceutical protein production in rice have been reviewed (Kuo et al., 2013). Till date, maize has been used as a model crop for the production of industrial enzymes such as avidin and β-glucuronidase (Hood et al., 1997; Witcher et al., 1998). Rice has also been used as an alternative for recombinant protein production as it has various advantages like high grain yield, well-developed transformation system, easy scalability, self pollinating, and direct oral administration (Stoger et al., 2005; An et al., 2013; Cabanos et al., 2013; Kuo et al. 2013; Takaiwa, 2013; Ou et al., 2014). Besides, it is hypoallergenic, which makes it an excellent host for pharmaceutical protein production and administration. However, there are certain limitations too. For example, accumulation of several cytokines was found to be as low as less than 1% of the total soluble protein (Sirko et al., 2011). Therefore, improvements are needed to further increase their accumulation. Some of the improvements already in use involve the use of transit peptides to direct the products to a specific plant part, codon optimization of the target gene, and addition of ER-retention signals along with the use of strong tissue-specific promoters (Kawakatsu and Takaiwa, 2010). Mutants with reduced storage protein levels or suppressed seed protein expression have been shown to accumulate higher amounts of recombinant proteins (Schmidt and Herman, 2008). Several potential therapeutic candidates such as 7Crp epitope peptide (Takaiwa et al., 2007), IL-10 (Fujiwara et al., 2010) have been produced in rice and other crops as well. However, further improvement in production of the recombinant proteins at large-scale, their glycosylation concerns for immunogenicity and allergenicity, and biosafety aspects are still challenging (Ou et al., 2014).

Antinutrients

Phytate. In rice, 80% of phosphorus is stored in the form of phytic acid (PA) and its salt (phytate) in protein bodies present in the aleurone layer and embryo of seeds (O'Dell et al., 1972). Phytate is an antinutrient as it forms a complex with the divalent cations (e.g., Fe^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+}) that are not hydrolyzed and absorbed in monogastric animal's gut due to the absence of the digestive enzyme, phytase and are excreted to the environment causing the loss of minerals from animal body and pollution. In view of the adverse effects, attempts have been made to develop low phytate (LPA) cereal crops to utilize stored phytate-P and to increase bioavailability of mineral nutrients for human health (Li et al., 2014). In rice, chemical and/or physical mutagenesis has been employed to generate low phytate mutants, although cloning of the corresponding genes has not been reported (Larson et al., 2000; Liu et al., 2007; Kim et al., 2008 a, b). However, these mutant lines often have inferior yield, reduced seed viability, and emergence than wild type parents (Tan et al., 2013). This may be ameliorated through further breeding of LPA rice lines, as was shown in soybean (Trimble and Fehr, 2010). However, recently a low phytic acid rice mutant without inferior performance has been identified by TILLING (Targeting of Induced Local Lesions in Genomes) (Kim and Tai 2014).

The biochemical pathway and molecular biology of phytate biosynthesis in rice have been studied and 12 genes encoding the enzymes that catalyze intermediate steps in phytate metabolism have been identified (Suzuki et al., 2007). The genes involved in PA synthesis are also expressed in tissues other than seeds, their suppression either by antisense or RNAi or artificial miRNA may affect their functions in various tissues or organs consequently exert negative effects on plant growth and yield, as proven by deriving expression of these genes by constitutive promoters. For efficient knockout of PA synthesis in the seed, these genes must be driven by specific promoters (Ole and Glb) that preferentially express in embryo and the aleurone layer, the site of phytate accumulation in developing seeds. Seed specific silencing of *OsMIPS1* using rice glutelin GluB-1 (Kuwano et al., 2006) or oleosin 18 (Ole18) (Kuwano et al., 2009) or *OsMIPS* and *OsIPK1* (Ali et al., 2013 a, b) resulted in the reduction of PA and increase in inorganic-P (Pi) in seeds without significant negative effect on growth and seed weight. Similar seed specific silencing of *OsMRP5* using amiRNA technology and Ole18 promoter significantly reduced the PA and increased the Pi content in seeds. However, it also lowered seed weight in rice (Li et al., 2014).

Allergens. Rice is gluten-free and is often considered low-allergenic food. However, its ingestion has been reported to cause allergy in only a few cases. The clinical symptoms of rice allergy are atopic dermatitis, asthma, and eczema (Jeon et al., 2011). In contrast to other food allergy, this allergy is more common in adults than children. Several rice seed proteins are responsible for allergy. α-amylase/trypsin inhibitor (14–16 kDa), α-globulin (23 kDa), and β-glyoxylase (33 kDa) have been suggested as main allergens that induce an immune response by alleviating immunoglobulin E (IgE) in patients with rice allergy (Usui et al., 2001; Matsuda et al., 2006). Avoidance of food containing allergens and removal of allergens from food are the only available approaches for the therapy of allergy.

Some processing technologies like enzymatic digestion, alkali hydrolysis and high hydrostatic pressure (100–400 MPa) have been used to remove allergens from rice especially in Japan; however, these technologies are costly and reduce the taste quality of the processed rice (Watanabe et al., 1990 a, b; Kato et al., 2000). It is, therefore, desirable to develop a cost-effective approach for low-allergen (hypoallergenic) rice with good taste. The major allergen (14–16 kDa and 33 kDa) levels in rice have been suppressed by RNAi using a mutant of an excellent taste variety "Koshihikari" that lacked the 26kDa

allergen (GbN-1) as a host. The content of all the three allergens and binding to IgE patient sera were reduced without any apparent effect on the transgenic seed phenotype (Wakasa et al., 2011). However, some people retained allergenic reactivity to the transgenic rice indicating that the presence of additional allergens in rice. Two high molecular weight (HMW) globulin-like proteins of 52 and 63 kDa present in rice seed are also found to act as allergen. The levels of HMW allergens were reduced by RNAi and crossing of RNAi lines with transgenic lines with reduced levels of the major allergens showed substantial reduction in major and HMW allergens suggesting that the crossed lines are effective in therapy of allergenic proteins other than major allergens (Ogo et al., 2014). The transgenic lines with reduced allergen are promising for generating hypoallergenic rice.

Challenges for quality/nutritional enhancement

The following are the major challenges which need to be overcome to improve rice grain and nutritional qualities.

1. Use of genetic/metabolic engineering in nutritional quality improvement requires optimal expression of desired gene(s) in the right compartment using suitable promoter(s) for the production of functional protein(s)/enzyme(s) that effect metabolic pathways of macro- and micronutrients biosynthesis without affecting other endogenous metabolism, plant growth, and development (Farre et al., 2014). However, the lack of basic knowledge of the biosynthetic pathways of metabolites and their complex interactions has limited their manipulation. With the availability of complete rice genome sequence (Feng et al. 2002; Goff et al. 2002; Sasaki et al. 2002; Yu et al. 2002, 2005; The Rice Chromosome 10 Sequencing Consortium 2003), identification, characterization, and regulation of the relevant genes related to a particular trait of interest, be it yield or grain quality, has been undertaken and still requires more attention. Rapid progress in biochemistry, molecular biology, genetic engineering, "-omics" platforms and analytical tools has made quality improvement possible. Various high-throughput untargeted profiling technologies including proteomics, metabolomics, and transcriptomics have been developed to elucidate the proteome, metabolome, and transcriptome to aid in rice functional genomics research. In case of rice proteomics, significant progress has been made in the identification and characterization of different proteins. Recent advances in two-dimensional polyacrylamide gel electrophoresis (PAGE), mass spectrometry along with increased information in various protein databases have revolutionized the area of research involving genome-scale profiling, identification, and characterization of proteins. The role of rice proteomics in crop improvement and food security has been reviewed recently (Kim et al., 2014). Substantial amount of work has been done in the area of rice proteomics including construction of a rice proteome database (Komatsu, 2005), proteome analysis of molecular mechanism of poor grain filling on inferior spikelets (Zhang et al., 2014), and proteomic analysis of rice bran (Wang et al., 2014). Allergenic or toxic proteins produced as a result of some undesirable changes due to genetic engineering can also be screened using proteomics. Rice metabolomics is the quantification of the metabolites produced in rice. Some of these metabolites include health promoting phytochemicals, which makes their study quite important for improving human health and welfare. Recently, the primary and secondary metabolites composition in the kernel as well as other aerial parts of the cultivated rice with the help of sophisticated techniques such as GC-MS, LC-MS (gas and liquid chromatography-mass spectrometry), and capillary electrophoresis (CE)–MS has been presented (Kusano et al., 2015). Metabolomics has also made identification of metabolite biomarkers possible which are associated with abiotic stress tolerance in rice (Degenkolbe et al., 2013; Maruyama et al., 2014) and nutrition starvation (Masumoto et al., 2010; Okazaki et al., 2013). Most of the metabolomics research has been focused on metabolic profiling of colored rice, rice bran, and bran oil. Transcriptomics, the study of the transcriptome, is another useful method which can be used in improving rice grain quality along with the help of other tools such as oligoarrays, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), and sequence-by-synthesis (SBS) sequencing. SBS sequencing has certain perks over other techniques, viz., it is cheaper and can generate large sequencing output (Venu et al., 2010). Besides, promoter analysis can also help in finding certain *cis* regulatory elements in the upregulated genes in the rice cultivars with superior milling and eating quality (Venu et al., 2011). More work is needed to synchronize the progress made and to enable the queries of these -omics studies against databases in order to further improve the rice grain quality. Further, in recent years, techniques for direct imaging of elements in biological tissues and cells are rapidly developed to provide information for concentration and distribution of elements with more accuracy and high sensitivity. These techniques include mass spectrometric methods such as laser ablation inductively coupled plasma mass spectrometry (La-ICP-MS), secondary ionization mass spectrometry (SIMS), X-ray fluorescence spectroscopy based on synchrotron radiation (SRXRF), proton/particle-induced X-ray emission (PIXE), scanning or transmission electron microcopy with energy dispersive X-ray analysis (SEM-EDX or TEM-EDX) (Wu and Becker, 2012).
2. Engineering of multiple genes to simultaneously target several steps of a biosynthetic pathway or of different pathways to introduce several traits is challenging till today. Thus, transformation technology requires: (1) development of high-capacity binary vector, (2) development of a wide range of efficient monocot promoters, and (3) transfer of multiple genes as a single locus. Some work has been done to transfer multiple transgenes using high-capacity binary vector based on bacterial artificial chromosomes (BIBAC), bacteriophage PI-derived transformation competent artificial chromosome (TACs) (Farre et al., 2014), and plant artificial chromosome

(PAC) (Yang et al., 2015). Artificial chromosome (mini-chromosome) approach is considered to be superior to the existing techniques of randomized integration of one single or a few genes transferred at one time either by *Agrobacterium* or biolistic-mediated genetic transformation. Development of a range of promoters in rice to introduce multiple transgenes under the control of different promoters reduces homology-based transcription gene silencing. Besides, the constitutive promoters, ubiquitin (OsUbi) or actin 1 or actin 2 overexpress a transgene in most or all tissues at all the times which may result in abnormalities in transgenic plants such as delayed growth, dwarfism, and low yield (Wong et al., 2015). These problems can be overcome by the use of developmental-specific or tissue-specific or inducible (activated by external physical or chemical signals) promoters. Several promoters of seed storage protein genes, glutelin (Glu B1, Glu B2, Glu B4), 13 kDa, and 16 kDa prolamin genes and globulin (Glb1) direct endosperm-specific expression in peripheral layers of endosperm while rice embryo globulin gene (REG2), and a rice oleosin gene (Ole18) directs expression in embryo and aleurone layer and AGPase small subunit promoter expresses in whole seed have been identified. Stacking of multiple genes usually takes several years but still the possibility of segregation in later generation cannot be ruled out. Rice artificial chromosome (mini chromosome) is a genome independent vector that does not integrate into host genome but can inherit stably (Xu et al., 2012). It has unlimited capacity to accommodate multiple genes as a single locus, independent of all other genes in the genome, thus linkage drag and gene segregation can be avoided. They are useful in stacking multiple transgenes of a biochemical pathway or pathways to improve yield and quality (Kazuki et al., 2013; Yang et al., 2015) but it is yet to be experimentally proved in rice. Rightly targeted and efficient application of all these advancements may result into development of a "super fortified rice" having high vitamin A with high iron, high folate, added thiamine or having all macronutrients, and micronutrients together making rice or any other cereal, a complete food.

3. Most of the research on nutrient enhancement is focused on the total amount of nutrient produced rather than its bioavailability. But there is no advantage of having high nutrient rice if the nutrients aren't bioavailable. The key factors that affect the bioavailability of nutrients are: (1) chemical form of the food, (2) presence of certain compounds that positively such as cysteine, ascorbic acid, etc., or negatively such as phytate and tannins, etc., affect absorption and bioavailability (Welch and Graham, 2004), and (3) processing of food (Gibson et al., 2006). The limitations with bioavailabilty are that the bioavailability studies require special animal feeding trials which are expensive and cannot be applied to every GM crop (Graham et al., 2001). Moreover, there are differences in the mechanism of nutrient absorption between animals and humans. Thus, bioavailability is a critical issue which needs to be studied in detail. Different reviews have been recently published focusing on dosage and bioavailability of nutritionally enhanced foods developed by conventional breeding and genetic engineering (Graham et al. 2001; Tang et al. 2012; Bhullar and Gruissem 2013; Jain et al., 2013; Sanahuja et al., 2013; Zhu et al., 2013; Morris et al., 2014).
4. Acceptance of nutritionally enhanced rice or other foods globally has been a matter of discussion for long due to biosafety concerns. Though a lot of risk assessment studies have been undertaken to gratify the consumers' concerns, no adverse effects on health and environmental have been observed so far. The various emerging "omics" tools can be employed for assessing any unintended effects of genetically engineered crops. Proteome profiles of GM rice by 2-DE differential in-gel electrophoresis (2D-DIGE) were not significantly different from that of non-GM controls (Gong et al., 2012). There is a lack of research regarding proper epidemiological studies comparing food safety profiles of crops developed though genetic engineering. A case-by-case research is needed to thoroughly study the biosafety of the GM products using these high throughput technologies. Techniques to remove the selectable marker genes and strategies to circumvent the expensive and lengthy regulatory procedures are essential for commercial acceptance of the GM rice crops. Several strategies such as co-transformation, use of site-specific recombination, transposition systems, multi-autotransformation vectors (MAT), and use of positive selectable markers (mannose phosphoisomerase (PMI), and xylulose isomerase (xyl) have been used to either eliminate or as alternative selectable marker genes in GM rice and other crops (Jaiwal et al., 2002; Sripriya et al., 2011; Yau and Stewart, 2013). Recently, artificial site specific nucleases (SSN), zinc finger nucleases (ZFN), transcription activator like effectors' nucleases (TALENS), and CRISPR/cas9 have been identified with the potential to direct mutagenesis of specific genes for silencing, transgene targeting or stacking of multiple genes at single loci, targeted replacement, and chromosome rearrangement in rice (Li et al., 2012; Zhang et al., 2013; Xu et al., 2014). These techniques are efficient tools for targeted and marker-free genome engineering for manipulation of multiple genes with less labor and time consumption and avoid the regulatory issues associated with transgenic plants (Lusser et al., 2012). These approaches can further accelerate functional genomics and metabolic engineering (Endo et al., 2015; Jakociunas et al., 2015).
5. Climatic factors that adversely affect rice production and grain quality involve rise in temperature, CO_2 levels, drought, salinity, and flooding. Several authors have investigated the association of changing climatic conditions with plummeting yield and adverse grain quality (Madan et al., 2012; Shi et al., 2012; Zhao and Fitzgerald, 2013; Kim et al., 2013; Wang et al., 2014; Coast et al., 2015). Adverse environmental conditions cause an increase in chalkiness, reduced fertility and affects grain quality (head rice and milling yield) with apparent decrease in grain weight and reduced grain filling (Ndindeng et al., 2014; Sreenivasulu

et al., 2015). These effects are due to the mutilation in starch accumulation and inhibition of biosynthetic pathways of other storage metabolites in the grain. Only a few studies are dealt with effect of climate change on the quantification of micronutrients and phytochemicals (Goufo et al., 2014). More research is required to explore the climate change effects and to design climate-resilient rice varieties with better grain quality.

Conclusions and future prospects

Limited nutritional quality of rice and nonavailability of diversified diets have emerged as a major problem for a large number of poor populations solely dependent on rice as a staple food crop especially in developing countries. Lack of balanced diet leads to micronutrient deficiencies and hence, negative consequences on people's nutrition and health. More than 200 million children are hungry and about 5 million of them die every year due to macro- and micronutrient deficiency. Micronutrient deficiencies affect 925 million people worldwide (Ronald, 2014). Thus fortifying most important staple crops such as rice and wheat are extremely necessary. Genetic engineering represents a rapid way of developing plants with improved nutrient content. It works by overexpression of the gene(s) of biosynthetic pathway of the essential nutrient or by downregulation of the gene(s) of the competing or catabolic pathway. However, transgenic approach like other technologies has its own limitations. The biggest challenge is the public concern over the acceptance and biosafety of genetically engineered plants, but there is no scientific evidence (Raven, 2010). The strategies employed to avoid the escape of transgenes into the environment and to address public safety concerns results in an increase in expenditure for production of transgenic plants (Ronald, 2014). To counter some of these issues, methods like intragenic bioengineering techniques or cisgenesis, which transfer genes within the same species, and site specific nucleases [Zinc finger nucleases, CRISPER/Cas9 and Transcription activator like effector nucleases (TALENS)] can circumvent regulatory mechanisms associated with GMOs.

Various nutrients such as vitamins, minerals along with some secondary metabolites have already been targeted in rice. But in near future, other nutrients such as thiamine, magnesium and calcium should also be fortified. The lack of extensive knowledge about the concerned biosynthetic and regulatory pathways of the essential nutrients along with the genes concerned and transcription factors involved are few of the challenges facing the further improvement of rice. However, the gap is minimizing with the recent advances in technology centered on the "omics" and use of artificial chromosomes for future manipulations which involves multiple combinatorial approaches for gene stacking in rice. Future work should avoid use of selectable markers for public-safety concerns. Further research is needed to evaluate and consider various strategies to be employed for developing rice according to local and/or national preferences and multidisciplinary complementation for better understanding along with public–private partnerships to distribute the gained benefits systematically at a wider scale.

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