Review Paper:

Genetic engineering aided biofortification in rice for improving nutritional status

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Abstract

Biofortification of staple food crops is a new health approach to control vitamin A, iron and zinc deficiencies. Biofortification is a process of escalating the concentration of these elements, as well as nutritional quality of essential nutrients in the economic parts of the staple crops following traditional crop breeding with biotechnology. To proliferate the levels of iron, zinc and other essential nutrients in the endosperm, it is essential to know the physiological and molecular mechanisms behind the uptake, transportation and efflux of these essential elements in rice.

Various genes namely soyferH1 and soyferH2 genes for enhanced iron storage, NAS genes including OsNAS1, OsNAS2 and OsNAS3 for iron transport, OsYSL2 gene for iron translocation into grains, IDS3 gene for iron uptake and translocation, OsVIT1 and OsVIT2 genes for iron translocation were used to fortify the iron level in the white grains. These genes were also reported to play pivotal role in escalating the zinc levels in the endosperm of transgenics. Co-integration of these essential transgenes including phytoene synthase from Daffodil (Narcissus pseudonarsissus), phytoene desaturase from a soil bacterium (Erwinia uredovora) and lycopene cyclase from Daffodil (Narcissus pseudonarsissus) in to the genetic background of a japonica rice cultivar Taipei 309 using Agrobacterium- mediated gene transformation method, escalated β- carotene level in the endosperm.

Keywords: Biofortification, Staple, Transgenes, β- carotene and transformation.

Introduction

Micronutrient malnutrition is the main cause of many diseases and mortality in the children and women in developing countries. This essentiates the application of new technology and methods to improve their nutritional status. Biofortification of staple food crops is a new health approach to control vitamin A, iron and zinc deficiencies[50]. Biofortification is a process of increasing the concentration of these elements as well as nutritional quality of essential nutrients in the economic parts of the staple crops with the adoption of traditional crop breeding and biotechnology[68]. Biofortification differs from normal fortification, as the later mainly aims at value addition through extrinsic application of nutrients during processing of edible portion of a crop.

Rice is one of the most consumed staple food crops across globe especially in Asia and Africa. In developing countries, rice is the main source of nutrition. In rice, the white grains, formed after polishing, contain limited amounts of essential nutrients for good health and development[30] due to which various deficiency related health issues arise[11].

These problems are more prominent with regard to shortage of iron, zinc and provitamin A which are usually lost due to polishing. Due to lack of sufficient genetic variations in the endospermic nutrient level among the germplasms as well as wild types, the conventional breeding approaches to develop improved lines with higher nutrient accumulation in the endosperm are limited[18,21]. The limitations of the conventional breeding can be overcome by genetic engineering techniques that will aid in biofortification of vital nutrients in rice and other important cereals. To escalate the level of iron, zinc and other essential nutrients in the endosperm, it is essential to know the physiological and molecular mechanisms behind the uptake, transportation and efflux of essential elements into the grains.

Iron biofortification in Rice

More than two billion people suffer from anaemia and more than half of these cases were due to the Fe deficiency[5]. The Fe deficiency anaemia (IDA) affects mostly the Asian and African continents. According to the studies made by WHO, children and women of the developing countries are particularly more vulnerable, with 300 million of children and more than 500 million of women suffering from iron deficiency anaemia[70]. The consequences of this type of malnutrition lead to severe problems including poor growth and mental development, lower cognitive ability in preschool children and increased mortality of mother and child at birth[45,61,69].

Minerals concentration gradually reduced in rice endosperm after the post-harvest processing of paddy and in case of iron, the reduction in concentration is more prolific. Paddy (rough rice) contains 38 ppm of iron which is reduced to 8.8 ppm in brown rice and finally to 4.1 ppm in milled rice after post-harvest processing[15]. Polished grains of most of the commonly grown rice varieties provide 2µg/g dry weight of iron against the recommended i.e. 15 µg/g dry wight[12]. Hence, there is a greater scope to alleviate iron malnutrition through the biofortification of rice.

Molecular strategies of iron biofortification

With the advancement of genetic engineering, genes involving in the various biochemical and physiological processes can be traced and isolated with ease. Initially efforts are made for isolating these genes from the cultivated varieties of the crop plants and if not, then the closely-related species would be targeted.

After isolating the gene sequence, molecular characterization and expression analysis studies are to be performed with the help of genetic engineering tools. Hence, it is essential to identify the genes involved in iron uptake, transportation, efflux into the grains. In rice, these genes/ transgenes under the control of seed- specific or constitutive promotors can be suitable expressed to escalate iron concentrations in the endosperm many folds.

Various genes namely *soyferH1* and *soyferH2* genes for enhanced iron storage[19,39,52,56,66], NAS genes for iron transport[30,36,37], *OsYSL2* gene for Iron translocation into grains[27,34], *IDS3* gene for iron uptake and translocation[62], *OsVIT1* and *OsVIT2* genes for iron translocation[9,37] were used to fortify the iron level in the white grains. These genes were also reported to play pivotal role in escalating the zinc levels in the endosperm of transgenics[7,10,41,65].

Table 1
Iron biofortification strategies in rice by using genes responsible for iron uptake, transport and translocation into the grains

Biotechnological Approaches	Genes	Promotors	Plant species/cultivars	Elevated level of Fes/Zn conc. In white/Brown grains
Enhancement of Iron storage via *soyferH1* and *soyferH2* genes	*soyferH1*	*OsGluB-1*	*O. sativa* ssp. *japonica* cv. Kita-ake	3-fold in Fe (white)[19]
	Osfer2	*OsGluA2*	*O. sativa* ssp. *indica* cv. Pusa sugandhi II	2.09- fold in Fe and 1.37-fold in Zn (white)[54]
	soyferH1	*OsGluB-1*	*O. sativa* ssp. *japonica* cv. Kita-ake	3-fold or 16 µg/g Fe (white)[56]
	soyferH1	*OsGluB-1*	*O. sativa* ssp. *indica* cv. BR29	9.2 µg/g of Fe (white)[31]
	soyferH1	*OsGluB-1*	*O. sativa* ssp. *indica* cv. IR68144	37 µg/g or 3.7- fold Fe (white) and 55.5 µg/g Zn (Brown)[66]
	soyferH1	*OsGluB-1*	*O. sativa* ssp. *japonica* cv. Taipei309	2.2- fold Fe (white)[39]
	soyferH1	*OsGluB-4*	*O. sativa* ssp. *indica* cv. IR 64	3.4- fold Fe (white)[52]
Enhancement of Iron transport via NAS genes	*HvNAS1*	*OsActin 1*	*O. sativa* ssp. *japonica* cv. Tsukinohikari	4.5- fold Fe and 2.5- fold- Zn (white)[44]
	OsNAS2	*Maize-ubiquitin*	*O. sativa* ssp. *japonica* cv. Kita-ake	2.9- fold Fe (white)[37]
Enhancement of Iron efflux into seed via *OsYSL2* gene	*OsYSL2*	*OsSUT 1*	*O. sativa* ssp. *japonica* cv. Tsukinohikari	4.4- fold Fe (white)[27]
Enhancement of iron uptake and translocation via *IDS3*	Barely 20-kb IDS *3*	*CaMV35s*	*O. sativa* ssp. *japonica* cv. Tsukinohikari	1.4- fold Fe (white)[43]
	Barely 20-kb IDS *3*	*CaMV35s*	*O. sativa* ssp. *japonica* cv. Tsukinohikari	1.3- fold Fe (Brown)[62]
Knockdown of *OsVIT1* and *OsVIT2* genes	*OsVIT1* and *OsVIT2*	T-DNA insertional inactivation	*O. sativa* ssp. *japonica* cv. Zhonghua 11 and *O. sativa* ssp. *japonica* cv. Dongjin	1.4- fold in Fe and 1.4- fold in Zn (Brown)[73]
	OsVIT2	T-DNA insertional inactivation	*O. sativa* ssp. *japonica* cv. Dongjin	1.5- fold Fe (white)[9]
Reduction in phytic acid accumulation	*IPK 1*	*Ole 18*	*O. sativa* ssp. *indica* cv. Pusa sugandhi II	1.8- fold Fe (white)[2]

Table 2
An alternative approach of iron biofortification possessing combinational use of multiple genes

Genes- promotors	Plant species/ cultivars	Elevated level of Fe/Zn conc. In white/ Brown grains
soyferH2-OsGlb 1 *soyferH2-OsGlu B1* *HvNAS1-OsActin 1* *OsYSL2-OsSUT1* *OsYSL2- OsGlb 1*	*O. sativa* ssp. *japonica* cv. Tsukinohikari	4.4- fold Fe and 1.6- fold Zn (white)[41]
AtRIT1- MsENOD12B *AtNAS1- CaMV35s* *PvFER- OsGlb 1*	*O. sativa* ssp. *japonica* cv. Nipponbare	10.46 µg/g or 3.8- fold Fe (white)[10]
OsNAS1+ OsNAS2+ OsNAS3- Dual CaMV35s	*O. sativa* ssp. *japonica* cv. Nipponbare	2 to 4.2- fold Fe (White)[30]
soyferH2-OsGlb 1 *soyferH2-OsGlu B1* *HvNAS1-OsActin 1* *OsYSL2-OsSUT1* *OsYSL2- OsGlb 1*	*O. sativa* ssp. *japonica* cv. Paw San Yin	3.4- fold in Fe and 1.3- fold in Zn (white)[7]
SoyferH2- OsGluB1 *SoyferH2- OsGlb1* *HvNAS1, HvNAAT-A-B and IDS-3*	*O. sativa* ssp. *japonica* cv. Tsukinohikari	4- fold Fe (white)[42]
Soyfer H1-GluA2 *OsNAS2-CaMV35S*	*O. sativa* ssp. *indica* cv. IR 64	6- fold Fe (white)[65]

Simultaneous overexpression of various genes including phytoene synthase from daffodil[59], phytoene desaturase from Erwinia uredovora[46] and lycopene cyclase from daffodil[1] in japonica rice cultivar Taipei 309 using Agrobacterium- mediated gene transformation method, proliferated β- carotene level in the endosperm[72]. Also, the use of an endosperm-specific promoter to deposit iron within the endosperm of rice so that it is not milled away is a very good strategy that has been achieved by use of transgenic techniques.

Thus, there are six different transgenic approaches and an alternative approach combing the different transgenic approaches and genes (Table 1 and 2) that are employed to proliferate Fe level in the rice endosperm.

1. Enhancement of Iron storage via *soyferH1* and *soyferH2* genes: Ferritin is the principal intracellular iron storage protein in both prokaryotes and eukaryotes, retaining iron in a soluble and non-toxic configuration. Plant ferritin is a large protein having 24 homologous or heterologous subunits and can store up to 4500 atoms of Fe in a non-toxic form[4,10,64]. The human intestine can efficiently absorb mineral irons from the Ferritin-Fe complexes. Hence, various studies aiming at transfer of the *soyferH1* and *SoyferH2*, candidate genes encoding soybean ferritin protein into rice endosperms were conducted.[19,39,56,52,66] Studies also suggested the use of rice globulin (*OsGlb*) and rice glutelin (*OsGlu B1*) endosperm specific promotors during transfer of *soyferH1* and *soyferH2* into the rice genome[19,39,56,52,54,66].

Over expression of *soyferH1* driven by *OsGluB-1* seed-specific promotor, in a japonica cultivar Kit-ake resulted a 3- fold accumulation of Fe in the polished grains of transgenics[19]. A series of such experiments were conducted by introducing *soyferH1* gene under the control of *OsGluB-*

1 and *OsGlb* promotors in genetic background of japonica cultivar Taipei309[39], indica cultivar IR68144[66], japonica cultivar Kit-ake[56], indica cultivar IR 64[52] and a outcome of 2.2 to 3.7-fold increase in Fe concentrations in the polished grains was noted. Introduction of *soyferH2* into the plant system was found to be advantageous over *soyferH1* as the later one was found to be more susceptible to protease digestion affecting structural changes in transgenic plants.[42,65]

2. Enhancement of Iron transport via NAS genes: Phyto siderophores (PS) are organic substances (Nicotinamine, Mugenic acid, Avenic acid) which are emanated by the plant roots in Fe and Zn deficient soil condition, to form chelates with these metals and thereby increasing their uptake. These phyto siderophores are commonly denominated as iron carriers. Phyto siderophores precisely the MA- family PS have higher affinity towards Fe (III) and readily transport Fe (III) from rhizosphere zones to plant roots[25,51]. In rice, Nicotinamine synthase and Nicotinamine transferase enzymes play a vital role in the secretion of PS into the rhizosphere with the help of *TOM1* (transporter of MAs) transporter[25,51].

Finally, the Fe- MAs complexes enter into roots with the help of yellow stripe 1 (*YS1*) transporter[28]. Nicotinamine synthase gene catalyses the biosynthesis of Nicotinamine from the three molecules of S-adenosyl methionine (SAM)[71]. NAs besides metal translocation, also play a critical role in homeostatic processes inside the plant system[22,34,41,63]. Three NAS genes, *OsNAS1, OsNAS2* and *OsNAS3* are reported in rice through various experiments[30,36,37]. Overexpression of NAS genes enhances the MAs secretion in the rhizosphere zones and thereby, increasing the iron uptake.[30,36,37]

In an experiment, the expression of *OsNAS3* gene was increased in two different activation- tagged mutant lines i.e. OsNAS3-D1 and OsNAS3-D1 with the help of 35S enhancer elements. Seeds of OsNAS3-D1 plants, over expressing *OsNAS3* were found to contain elevated amounts of Fe (2.9-fold), Zn (2.2- fold) and Cu (1.7-fold)[36]. Over expression of *HvNAS1* gene was under the control of *OsActin1* promotor in *O sativa* var. Tsukinohikari (japonica type) resulted in 4.5- fold and 2.5-fold increase accumulations of Fe and Zn respectively in the polished grains of transgenics[44].

A gene cassette having *OsNAS1*, *OsNAS2* and *OsNAS3* gene sequences, driven by a constitutive dual *CaMV35S* promotor, was introduced into *O. sativa* ssp. japonica cv. Nipponbare. Three different population of transgenics, each over expressing a one of the three NAS genes, were generated. Two *OsNAS2* overexpressing lines with 14 and 19 µg/g Fe were confirmed[30].

3. Enhancement of Iron translocation into grains via *OsYSL2* gene: Eighteen putative yellow stripe 1 (*YS1*) transporter like genes (OsYSLs) were recognized in the rice genome[34]. The *OsYSL2* gene, encoding a poly peptide of 674 amino acids, accommodating 14 putative trans-membrane domains, is a functional Fe (II) and Mn (II) – Nicotinamine complex transporter.[34] The *OsYSL2* gene is crucial for phloem transport of iron and manganese including the translocation of iron and manganese into the grain[27,34].

In an experiment, the gene sequence of *OsYSL2* in conjunction with 1.7 Kb sucrose transporter promoter (*OsSUT1*) was introduced into rice genome and six independent transformants expressing *OsYSL2* were confirmed. A higher concentration of Fe in the endosperm was obtained and in some transgenics, an increase up to 4.4-fold in Fe concentration compared to wild types was also reported[27].

Previously, it was reported that the lines with the overexpression of *OsYSL2* gene also expressed *OsIRT1* and *OsNAS1* in the phloem[28,34]. In the transgenic plants developed by using RNAi- mediated *OsYSL2* silencing i.e. OsYSL2i, *OsIRT1* and *OsNAS1* genes although expressed but reduced translocation of Fe in shoot and seed was obtained indicating the sole importance of *OsYSL2* for Fe transport from root to shoot and Fe translocation into the grains[27].

4. Enhancement of iron uptake and translocation via *IDS3* gene: In gramineous crops including rice (*O. sativa*), deoxy mugenic acid (DMA) is synthesized from Nicotinamine (NA) with the involvement of Nicotinamine aminotransferase (*NAAT*) and Deoxymuginic acid synthase (*DMAS*) enzymes (Figure 1).

In barely and some gramineous crops, iron deficiency specific clones no. 2 (*IDS2*) and no. 3 (*IDS3*) being deoxygenage genes, are involved in the synthesis of mugenic acid (MAs) from Deoxy mugenic acid (DMA). Barely has got a series of MAs biosynthetic genes including *NAS*, *NAAT*, *DMAS*, *IDS2* and *IDS3*, the expressions of which are up- regulated in barely roots in Fe deficient soil condition[25,43,49,63]. Rice is unable to produce MAs due to the absent of *IDS2* and *IDS3* in its genome and hence, barely is more tolerant to Fe deficiency as compared to rice[33].

Over expression of barely 20-kb IDS 3 driven by *CaMV 35S* constitutive promotor in *O. sativa* ssp. *japonica* cv. Tsukinohikari, led an increase of 1.4- fold Fe in white rice compared to wild type[43]. Similar work was carried in the same Tsukinohikari cultivar and an elevated level of Fe up to 1.3- fold was reported in the unpolished grain[62].

5. Enhancement of iron translocation via *OsVIT1* and *OsVIT2* genes: In rice, some vascular Fe transporter *OsVIT1* and *OsVIT2* are reported to play critical role in subcellular Fe transport[73]. The polypeptides, encoded by *OsVIT1* and *OsVIT2* genetic sequences, consist of 252 and 247 amino acids respectively showing nearly 80% of amino acids sequence similarity with the putative protein, encoded by

Arabidopsis thaliana VIT (*AtVIT1*)[73]. These genes were found to be expressed ubiquitously at lower levels in different parts of the plants but significantly higher expression was noticed in flag leaves[9,73].

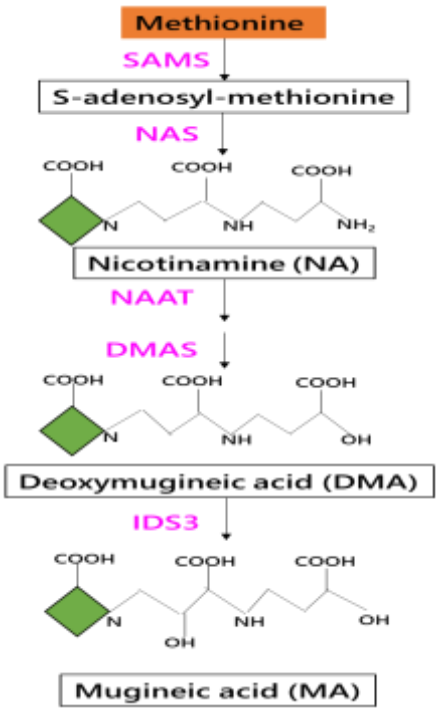

Figure 1: Expression of enzymes viz., *NAS, NAAT, DMAS* and *IDS 3* in biosynthetic pathway of Mugineic acid synthesis

These genes play a vital role in transportation of Zn (II) and Fe (III) into vacuoles through tonoplast[32]. In rice, T-DNA insertional mutants were produced by targeting *OsVIT1* and *OsVIT2* genetic sequences in the genetic background of Zhonghua 11 and Dongjin respectively. Knockdown of these two genes led to a considerable increase in Fe and Zn accumulation in seeds and simultaneous decrease in the flag leaves[73].

Another work aiming at the reduction of *OsVIT2* expression was performed by isolating the T-DNA insertional lines with an integration of T- DNA to the upstream of start of start codon of *OsVIT2* gene. In the same study, *MIT* (Mitochondrial iron transporter) knock down (mit-2) plants were used, where the expression of *OsVIT2* was upregulated. A nearly 1.5- fold increase of Fe accumulation was reported in the polished grains of osvit-2 mutants and mit-2 seeds accumulated less Fe in the endosperm as compared to wild type or control[9]. However, more than 60% increase in the Cd (II) concentration in rice endosperms in osvit1 and osvit2 mutants, as compared to wild types i.e. Zhonghua 11 and Dongjin, in Cd (II) polluted rice field, indicated an unseen disadvantage in the use of these vacuolar membrane transporters in iron fortification[73].

6. Reduction in phytic acid accumulation: Myo-inositol hexa phosphate known as phytic acid (PA) is one of the most prominent and abundant forms of phosphorus present in the seeds[40,55]. It reduces the bioavailability of the bivalent cations by forming chelates with them and hence, it is considered as an anti- nutritional factor in some of the major crops. One of the ways to develop low PA crops is to identify the lpa mutants. A reduction of 45-95% in PA accumulation was reported through various experiments by the use of the lpa mutants in rice. Although, the mutants are effective in reducing the PA accumulation but they compromise in yield and other agronomic performances.

RNAi- mediated gene silencing can be effectively used as an alternative in reducing the PA accumulation by targeting the genes involving in the biosynthetic pathway of the phytic acids. The first step of PA biosynthesis is mediated by the 1D- myo- inositol 3- phosphate synthase (*MIPS*) enzyme. Various researches were conducted to silence the expression of *MIPS* gene under the control of constitutive or seed specific promotors[2,40].

In case of the use of constitutive promotors i.e. *CaMV35S*, the expression of the *MIPS* gene was also lowered in vegetative tissues in addition to the seeds, leading to subversive impacts on the plant system. Hence, seed-specific promotors like Glutelin B-I (*Glu B-1*) and oleosin 18 (*ole 18*) were used to regulate the suppression in the seeds only[2].

However, unintended change in the myo-inositol content in the seed was not considered and might have a pessimistic impact on the plant inositol metabolism, as myo-inositol-3 phosphate, the product of *MIPS* gene is known to be the only antecedent for the de novo synthesis of myo-inositol. Hence, attempts were made to silence the enzymes involved in the later stages of the biosynthetic pathway of Phytic acid to foreshorten the phytic acid content in seeds without impeding concomitant pathways.

In Pusa sugandhi II cultivar, inositol- 1,3,4,5,6-pentakisphosphate 2- kinase (*IPK 1*) gene was silenced under the control of seed- specific oleosin 18 (*ole 18*) promotor with the aid of RNAi- mediated gene silencing. A noticeable 3.85- fold down regulation of *IPK1* transcripts in the subsequent T_4 transgenic lines was obtained. RNAi-mediated gene silencing of *OsMRP5* driven by oleosin 18 (*ole 18*) seed- specific promotor resulted in 35.8- 71.9% decrease of phytic acid accumulation in brown rice of transgenics[2].

An alternative approach of combinational use of multiple genes

Various studies also reported the complementing nature of these candidate genes for Fe biofortifications in rice (Table 2). Masuda et al[41] reported simultaneous overexpression of plant Ferritin genes driven by rice globulin (*OsGlb*) and rice glutelin (*OsGlu B1*) endosperm specific promotors , NAS genes under the control of *OsActin 1* promotor and *OsYSL2* gene driven by *OsGlb 1* and sucrose transported (*OsSUT1*)

promoter in the japonica cultivar Tsukinohikari, resulted 4.4- fold elevation in Fe and 1.6- fold elevation in Zn in polished grains of T_3 transgenics.

AtIRT 1 gene, an iron regulated transporter gene from Arabidopsis, having affinity towards Fe (II), was driven under the control of Medicago sativa EARLY NODULIN 12B (*MsENOD12B*) promotor, expressed in previously developed NFP high- iron rice expressing both *FERRITIN* and NICOTINAMINE SYNTHASE (*AtNAS1*). A significant increase of Fe concentration of 9.6 µg/g in the polished rice grains was recorded[10]. Aung et al[7] reported simultaneous overexpression of plant Ferritin genes driven by *OsGlb* and *OsGlu B1*, NAS genes under the control of *OsActin 1* and *OsYSL2* gene driven by *OsGlb 1* and *OsSUT1* promoters in the japonica cultivar Paw San Yin, led a 3.4-fold increase in Fe in the polished grains of transgenics.

Overexpression of *soyferH1* under the control of *OsGluA2* promotor along with *OsNAS2* gene driven by *CaMV35S* constitutive promotor in indica cultivar IR 64, led an increase of 6- fold Fe in the polished grains[65]. Overexpression of gene cassette consisting of *OsNAS1*, *OsNAS2* and *OsNAS3* gene sequences under the control of a dual *CaMV35S* constitutive promotor in *O. sativa* ssp. *japonica* cv. Nipponbare, led a 2 to 4.2- fold increase in Fe concentration in polished grains[30]. Hence, a combinational use of various genes for biofortification is equally effective in increasing Fe, Zn and other metals in the rice endosperms. A considerable success in the rice can open the door for biofortification in other graminaceous crops mostly cereals.

Golden rice: A β- carotene fortified rice

Vitamin A deficiency leads to serious health related issues including childhood blindness and also increases the risk of death due to common childhood illness such as diarrhoea[17,20,67]. It is estimated that 124 million children are deficient in vitamin A world-wide. Since mammals cannot manufacture vitamin A, diet is the only source of human vitamin A and provitamin A. Rice does not contain β-carotene in the endosperm naturally and being one of the mostly consumed cereals worldwide, leads to health issues associated with VAD. The immature rice endosperm is capable of producing geranylgeranyl diphosphate (GGPP), the precursor of β- carotene biosynthesis in the plastids[13].

Co-integration of these essential transgenes including phytoene synthase (from *Narcissus pseudonarsissus*[59]), phytoene desaturase (from *Erwinia uredovora*[46]) and lycopene cyclase (from *Narcissus pseudonarsissus*[1]) into the genetic background of a japonica rice cultivar Taipei 309 using Agrobacterium- mediated gene transformation method, escalated β- carotene level in the endosperm[72] (Figure 2).

Genetics of Golden rice: Transgenic japonica cultivar (Taipei 309) expressing daffodil (*Narcissus pseudonarsissus)* phytoene synthase in the endosperm, accumulated phytoene is key precursor of carotenoid biosynthesis. Phytoene synthase (*psy*) from daffodil was linked to *CaMV 35S* and *Gt1* promotors in the constructs pCPsyF and pGtPsyH respectively and delivered into the plant genome by microprojectile bombardment method[13].

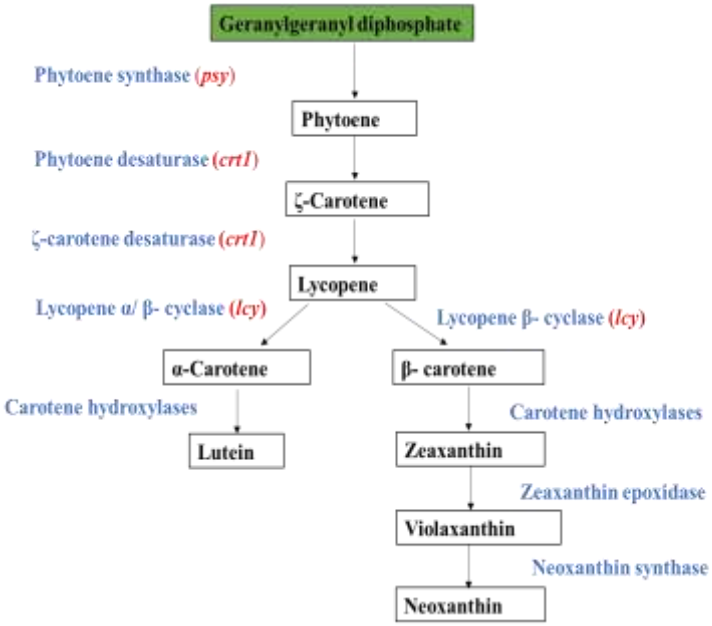

Figure 2: Pathway for β- carotene biosynthesis in plants. The first step in carotenoid biosynthesis is the condensation of two molecules of GGPP to produce phytoene by the catalytic activity of phytoene synthase (psy). Phytoene desaturase and ζ- carotene desaturase (crt1) catalyse synthesis of lycopene from phytoene. The cyclization of lycopene by two different classes specific for α- and ε- inone end groups of lcy marks a branching point in the pathway where one branch leads to α- carotene and its oxygenated derivatives lutein, while other forms β- carotene and the derived xanthophyll, such as zeaxanthin, violaxanthin and neoxanthin

Three different gene Cassettes, with the first one harbouring *psy* (phytoene synthase) sequence from daffodil with *crt1* (phytoene desaturase) sequence from *Erwinia uredovora* bacterium under the control of endosperm- specific glutelin (*Gt1*) and constitutive *CaMV35S* promotors respectively; the second one with the above described gene sequences but lacking *aph1V*, a selectable marker and the third one providing lycopene β- cyclase (*lcy*) from *Narcissus pseudonarsissus*, were constructed[72]. The total carotenoid content was found to be 1.6 µg/g in the endosperm of transgenics[72]. The first gene cassette was meant for single transformation producing lycopene in the endosperm plastids while the latter two were designed for co-transformation producing β- carotene in the seed endosperm.

Interestingly, the cassette harbouring *psy* and *crt 1*gene sequences, meant for single transformation also led to produce β- carotene instead of lycopene[72]. Next attempts were made to initiate β- carotene biosynthetic pathway in the genetic background of indica cultivars. Consequently, two elite indica cultivars, namely, IR 64 and MTL 250 and one previously used japonica cultivar (Taipei 309) were transformed with a vector construct combining bacterial phytoene desaturase *crt 1*, fused with SSU-tp of pea with phytoene synthase psy from *Narcissus pseudonarsissus* under the control of *CaMV 35S* and *Gt1* promotors respectively[23].

Introduction of phytoene synthase (*psy*) driven under the control of endosperm- specific glutelin (*Gt1*) promotor along with phytoene desaturase (*crt1*) and lycopene β- cyclase (*lcy*) under the control of constitutive *CaMV35S* promotor into seven indica rice cultivars including IR 64, BR 29 etc. was performed[14]. First time a novel cestrum yellow leaf curling virus driven pmi, a selectable marker was used to generate transgenics with β- carotene biosynthesis[14]. Total carotenoids ranging from 0.297 µg/g to 1.05 µg/g were accumulated in the endosperm of T_1 transgenics[14]. The combinational use of these two genes omitting *lcy* was able to complete the entire biosynthetic pathway of β- carotene[23,58,72].

Total carotenoid content was estimated to be 0.4 µg/g (indica) to 1.2 µg/g (japonica) in the transformants. Schaub et al[58] confirmed that the carotenoid pattern in Golden rice is a result of constitutive endogenous expression of ε/ β- LCYs and α/ β- HYDs leading the formation of α- and β- carotene and xanthophyll in the endosperm, indicating that the pathway proceeded beyond the end point that would have predicted based on the enzymatic function of the transgene combination[23,58,72].

Subsequently, the pitfall behind the relatively lower accumulation of β- carotene in the endosperm of transformants was considered. Paine et al[53] considering the use of phytoene synthase (*psy*) sequence from daffodil as the limiting step leading to lower production of β- carotene in the endosperm, used *psy* genomic sequence from five different sources i.e. rice, maize, pepper, tomato and Daffodil. The integration of T-DNA constructs having *psy* sequence from maize genome with *E. uredovora crt1*, fused to pea RUBISCO chloroplast transit peptide, driven under the control rice glutelin promotor (*Glu*) in the Kaybonnet cultivar, resulted in 23- fold (maximum 37 µg/g) compared to the original golden rice[53].

The resulted transformants were called as Golden rice- 2[53]. This increase in total carotenoid and proportion of β- carotene compared to the original Golden rice can be considered to combat with the health issues associated with vitamin A deficiency.

High Zinc Rice: Climate change particularly increasing carbon dioxide concentrations in the globe, reduces Zn accumulation in grains[48]. Deficiency of Zn in the food is one of the major issues related to public health and optimal nutrition[35,47]. One third of the population is under serious threat due to this dietary Zn deficiency[24,47]. The point of biofortification of Zn in the cereal grains, rice particularly, is to increase the accumulation of Zn and consequently, increasing its bioavailability in the food and this is turning out to be the most feasible, economical as well as sustainable way to combat the dietary Zn deficiency[6,57,74].

In rice, over expression of iron storage genes from the genomic fragments of soybean and rice, led to increase accumulation of Zn in the endosperm. Introduction of *Osfer2* under the control of seed- specific promotor (*OsGluA2*) into indica variety Pusa sugandhi II, led a 1.3- fold increase in Zn accumulation in the polished grains[54]. Expression of *soyferH1* under the control of seed- specific promotor (*OsGluB-1*) into indica variety IR68144, resulted in 55.5 µg/g Zn accumulation in the brown grains[66]. Over expression of *HvNAS1* gene under the control of *OsActin1* promotor in *O sativa* var. Tsukinohikari (japonica type) resulted in 2.5-fold increase accumulations of Zn in the polished grains of transgenics[44].

In rice, *OsVIT1* and *OsVIT2* genetic sequences play a vital role in transportation of Zn (II) and Fe (III) into vacuoles through tonoplast[32]. In rice, T-DNA insertional mutants were produced by targeting *OsVIT1* and *OsVIT2* genetic sequences in the genetic background of Zhonghua and Dongjin respectively. Knockdown of these two genes, led to a considerable increase in both Fe and Zn accumulation in seeds and corresponding decrease in the flag leaves[73]. Lee et al[38] reported a 2.7- fold more Zn concentration in the endosperm of *OsNAS2* activation- tagged line, indicating the significant role of Nicotinamine synthase gene in bio fortification.

In an experiment, the expression of *OsNAS3* gene was increased in two different activation- tagged mutant lines i.e. OsNAS3-D1 and OsNAS3-D1 with the help of 35S enhancer elements. Seeds of OsNAS3-D1 plants, over expressing *OsNAS3* were found to contain elevated amounts of Fe (2.9-

fold), Zn (2.2- fold) and Cu (1.7-fold)[36]. Three different populations of transgenics, each over expressing a one of the three OsNAS genes, were produced in japonica cultivar Nipponbare. A two-fold increase accumulation was reported in the OsNAS2 population[30].

Combinational use of various transgenes was also reported to boost Zn application in the polished and unpolished grains. Masuda et al[41] reported simultaneous overexpression of plant Ferritin genes driven by rice globulin (*OsGlb*) and rice glutelin (*OsGlu B1*) endosperm specific promotors , NAS genes under the control of *OsActin1* promotor and *OsYSL2* gene driven by *OsGlb* 1 and sucrose transported (*OsSUT1*) promoter in the japonica cultivar Tsukinohikari and resulted in 1.6- fold elevation in Zn in polished grains of T_3 transgenics.

Conclusion

Aung et al[7] reported simultaneous overexpression of plant *Ferritin* genes driven by *OsGlb* and *OsGlu B1*, NAS genes under the control of *OsActin* 1 and *OsYSL2* gene driven by *OsGlb* 1 and *OsSUT1* promoters in the japonica cultivar Paw San Yin, resulted a 1.3- fold increase in Zn in the white grains of transgenics. Despite an enormous endeavour to escalate the Zn concentrations in the polished grains, the anticipated result of Zn biofortification is still not achieved and the reason may be due to incomplete understanding of the physiological and molecular (or, genetic) mechanisms of Zn uptake and translocation into the grains[3,16,26,29,60].

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