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Marker assisted biofortification of rice with pro-vitamin A using transgenic Golden Rice® lines: progress and prospects

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Abstract

Rice (*Oryza sativa* L.) is the major staple food crop which feeds more than half of the world's population. However, rice endosperm lacks pathway to synthesize provitamin A. Therefore, rice eaters particularly children and pregnant women suffer due to vitamin A deficiency (VAD) which, besides causing night blindness, also makes them more prone to a range of ailments. With the aim of overcoming VAD, Golden Rice® lines were developed by connecting the missing links of β-carotene pathway in the rice endosperm through genetic transformation. We present an overview on the origin of Golden Rice® concept, development of prototypes, different generations of Golden Rice®, various events and their characteristics, the progress in marker assisted development of Golden Rice® and future prospects in India.

Key words: Golden rice®, Provitamin A, Transgenics, Event characterization, Marker assisted backcross breeding

Introduction

Micronutrients are nutrients required in small quantities by humans and other organisms throughout their life to orchestrate a range of physiological functions [1]. These include micro minerals like iron, zinc, iodine, cobalt, molybdenum etc., and vitamins. Among these, Vitamin A, iron and zinc are important in terms of global public health. Although required in minuscule amounts, they can be called as "magic wands" as they enable the body to produce enzymes, hormones and other substances essential for proper growth and development and the consequences of their absence are severe.

Vitamin A and its multifaceted roles

Vitamin A, also called as retinol is a fat soluble organic compound synthesized in the body from provitamin A carotenoids *viz*., β-carotene, α-carotene and β-cryptoxanthin. Structurally vitamin A is one half of the β-carotene molecule which is the most potent and also most widespread provitamin A carotenoid. α-carotene and β- cryptoxanthin exhibit only 50 % of the vitamin A activity of the β-carotene [2]. Plants and algae are capable of synthesizing provitamin A carotenoids whereas humans and other animals cannot. Herbivorous animals synthesize vitamin A from the plant derived β-carotene and after meeting their daily requirements, store the remaining in their tissues and organs like liver, egg yolk and milk. Hence the animal foods are direct sources of vitamin A. Plant foods, mainly green leafy vegetables, dark orange fruits (apricots, cantaloupe) and vegetables (carrot, winter squash, sweet potato, pumpkin) are rich in provitamin A carotenoids, predominantly β-carotene [3].

Vitamin A produces retinol pigments, which is very important for night vision. It also maintains the normal functioning of glandular and epithelial tissues lining the alimentary canal, respiratory and urinary tracts, skin and eyes. It is also essential for the growth of skeletal tissue. Vitamin A is also important for embryonic development and regulation of adult genes [4].

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Vitamin A deficiency

Prolonged VAD results in a condition known as nictalopia or blindness, in which one is unable to see well in dim light and particularly after exposure to a bright light. VAD also causes xerophthalmia or dry eyes where in the tear glands stop producing tears which keep the eye moist and as a result the cornea becomes dry, wrinkled, lusterless, hazy and pigmented [5]. Finally it leads to softening and destruction of eyeball leading to complete blindness. VAD makes infants and preschool children (1-5 years of age) vulnerable to infection and increases susceptibility to diseases like measles, diarrhoea and other respiratory disorders [6]. VAD is also a common problem in pregnant women and lactating mothers. The poor vitamin A status of the mother has been identified as the major reason for VAD in infants who are exclusively breastfed [7]. It has been established that the maternal VAD leads to infant and maternal mortality and makes the lactating mothers vulnerable for diseases [8, 9]. VAD is a serious public health problem in at least 26 countries including highly populated areas of Asia, Africa and Latin America. According to World Health Organization (WHO) estimates, about 250 million preschool children are vitamin A deficient and among which a substantial proportion is pregnant women. An estimated 250,000 to 500, 000 vitamin A-deficient children become blind every year, half of them dying within 12 months of losing their sight. VAD is a severe problem in India which causes 40% of all blindness [10]. About 13,000 people, mostly children, go blind each year from xerophthalmia in India. For every case of total blindness, two more people are either blind in one eye or have corneal scars so that their sight is seriously impaired [11].

Approaches to alleviate VAD

Vitamin A supplementation, industrial food fortification, and biofortification form the three major approaches to alleviate VAD. Inspite of considerable successes with the traditional public health interventions and food fortification, they are not preferred choice for the alleviation of VAD as their requirement for infrastructure to produce Vitamin A supplements and food fortification makes them expensive and unaffordable to the poor people. As a result these vitamin A supplements and fortified foods are not accessible to people living in remote rural areas [12]. Biofortification is development of micronutrient-dense staple crops using the best traditional breeding practices and modern biotechnology. Unlike supplementation and industrial food fortification, genetic fortification is a one-time investment to develop seeds that fortify themselves, recurrent costs are low and germplasm may be shared internationally [13]. Furthermore, biofortification implicitly targets low-income households as staple foods predominate in the diets of the poor.

Rice as a choice crop for biofortification

Although there are 50,000 edible crops in the world, only three of them *viz*., rice, wheat and maize account to 60 per cent of worlds food energy intake [14]. Rice (*Oryza sativa* L.) is the major staple food crop which feeds more than half of the world's population. Countries in Southeast Asia are heavily reliant upon rice with nearly 90% of the world's rice produced and consumed in these countries. Rice cultivation forms the main source of income and employment for more than 100 million households in Asia and Africa. About 80% of the world's rice is produced on small farms, primarily to meet family needs, and poor rural farmers account for 80% of all rice producers [15]. In developing countries, rice accounts for 715 kcal/caput/day i.e., 27 per cent of dietary energy supply, 20 per cent of dietary protein and 3 per cent of dietary fat [16]. A typical milled rice kernel contains 90% carbohydrate, 6-7% protein and 1.5-1.7% fat [17]. However, it is very low in micronutrients like iron and zinc and almost no provitamin A carotenoids, as they are removed along with aleurone and embryo during milling and polishing [18]. Considering the fact that rice is the staple food for poor and low income groups who cannot afford vitamin A rich non-vegetarian foods or costly fruits and vegetables, biofortification of rice is one of the best options to address to VAD. Furthermore, food- and diet-related factors like the food matrix, food-preparation techniques, dose of β-carotene, amounts of fat, fiber, preformed vitamin A, or other carotenoids in the diet affect the bioavailability of β-carotene [19, 20, 21]. Owing to complex foods matrices, the bioavailability of β-carotene tends to be lower in dark green leafy vegetables that are rich sources of provitamin A carotenoids [22]. In contrast, the simple food matrix of rice compared to vegetables and fruits enhance the bioavailability of β-carotene from rice.

Development of Golden Rice®

Though β-carotene is synthesized in the green tissues of the rice plant, no known cultivar synthesizes and accumulates in the endosperm, making recombinant DNA technology the only option to fortify rice endosperm with provitamin A carotenoids [18]. The elucidation of various enzymes involved in the carotenoid biosynthetic

pathway was the major breakthrough that created platform to think of the possibility of provitamin A rich rice endosperm with yellow colour, now known as Golden Rice® [23]. Carotenoid biosynthesis requires an isoprenoid substrate derived from the plastid-localised 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [24]. Isoprenoids, also called as terpenoids are naturally occurring organic compounds that serve as precursors for the synthesis of a diverse range of compounds such as tocopherols, chlorophylls, gibberellins, abscisic acid, phylloquinone, monoterpenes and plastoquinone [25] . The first step in the carotenoid biosynthesis pathway is the condensation of two geranyl geranyl pyro phosphate (GGPP) molecules, the product of MEP pathway, catalysed by the phytoene synthase (PSY) enzyme to produce the first colourless carotenoid, 15-*cis*-phytoene. Phytoene then undergoes four sequential desaturation steps, mediated by two desaturases *viz.,* phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), introducing two double bonds each and carotenoid isomerase (CRTISO) which catalyses the *cis–trans* reactions to isomerize the four *cis*-bonds introduced by the desaturases [26]. Lycopene is further converted to α and β-carotene by the enzymatic action of two lycopene cyclases, eLCY and bLCY. It has been suggested that the phytoene synthase (*Psy*) gene is not transcriptionally active in rice endosperm blocking the carotenoid biosynthesis in the tissue**.** However, the discovery of the presence of GGPP, the initial precursor molecule in rice endosperm formed the major breakthrough in the development of Golden Rice® [27]. This suggested the possibility of diverting the precursor towards β-carotene biosynthesis by complementing the defunct pathway with the missing genes. Transformation of the cultivar Taipei 309 with *psy* gene from a flowering plant daffodil (*Narcissus pseudonarcissus*) demonstrated the accumulation of the colorless carotenoid phytoene in rice endosperm. Complementation of PSY enzyme with bacterial carotene desaturase (CRTI) which can substitutes for the two plant carotene desaturases, phytoene desaturase and ζ-carotene desaturase, as well as for the carotene-isomerase (CRTISO), resulted in the conversion of phytoene to lycopene and subsequently to β-carotene [28]. The first prototype of β-carotene rich rice lines developed in the background of a *japonica* rice variety, Taipei 309 demonstrated the feasibility of Golden rice® [29]. β-carotene accumulation occurred even in the absence of *Lcy* gene suggesting that the endogenous *Lcy* gene is active in rice endosperm and thus further transformations involved only *Psy* and *CrtI* genes. However, the carotenoid content of these prototypes was very low (1.6 μg/g of endosperm) and these were developed using antibiotic selectable marker gene *aph IV*. The low level of carotenoids were gravely criticized by the anti-GM protestors saying that an average 2-year-old would need to eat 3 kilos and a breastfeeding mother more than 6 kilos of Golden Rice® a day to reach the recommended daily intake of vitamin A. Further, the feasibility of this technique in *indica* lines was to be proved. Several transformations were made both in the public sector and private sector partner Syngenta to address these issues. The public sector transformation events, called *pCaCar* events were made in the background of *indica* rice variety, IR64 replacing the antibiotic selectable marker with phosphomannose-isomerase (Positech®) sugar-based selection system, the public sector events could accumulate total carotenoids only up to a maximum of 1.6 μg/g of endosperm [30, 31]. In parallel, Syngenta scientists produced transgenic Golden rice® lines in the *javanica* cultivar Cocodrie (called SGR1 lines), which could accumulate significantly higher amounts of carotenoids (6 μg/g of endosperm). Further research showed that in the carotenoid biosynthesis pathway, the biochemical reaction catalyzed by the PSY enzyme is rate limiting, ultimately determining the amount of carotenoids accumulated in the tissue. Therefore, *psy* genes from various plant sources other than daffodil *viz*., pepper, tomato, maize and rice were tested and it was found that *psy* gene from maize (*Zmpsy*) complemented by the bacterial *crt1* gene lead to the accumulation of higher carotenoids content in the rice endosperm. This led to the development of second generation Golden Rice® lines called as Syngenta Golden Rice2 (SGR2) in the genetic background of American long grain rice genotype Kaybonnet wherein, both the maize *psy* and bacterial *crtI* genes were driven by endosperm specific Glutelin 1 (Gt1) promoter of rice. SGR2 lines accumulated higher level of total carotenoids ranging from 11.4 -37μg/ gram of endosperm tissue [32]. The different generations of Golden rice® lines along with the details of gene construct, promoters, selectable markers, varieties used and carotenoid content is presented in Table 1.

Humanitarian Board (HumBo) on Golden Rice®

In the beginning, the development of Golden Rice® was funded by the Rockefeller Foundation and the European Community. Although, funding from the Rockefeller Foundation was free of obligations, European Community had a clause in its funding stating that its industrial partner, Grenovation, a small biotechnology company linked to German University, would hold rights

Table 1. List of various generations of Golden Rice® lines

GR line	Genes in the construct	Promoters	Selectable marker	Method of transformation	Variety	Total carotenoid content (µg/g)	Reference
Prototype lines	Daffodil *psy*	Endosperm specific Glutelin (Gt1)	*aphIV* (for Hygromycin resistance)	Agrobacterium mediated co-transformation	Taipei 309 (*japonica*)	1.6	[29]
	Erwinia uredovora crtI	CaMV 35S					
	Daffodil *lcy*	Gt1					
pCaCar lines	Daffodil *psy*	Gt1	Phophomannose-isomerase (*pmi*)	Agrobacterium mediated	IR64 & MTL250 (*indica*)	0.8-1.2	[30, 31]
	E. uredovora crtI	CaMV 35S	(Positech®) sugar based selection	co-transformation	Taipei 309 (*japonica*)		
SGR1 lines	Daffodil *psy*	Gt1	*aphIV*	Agrobacterium mediated co-transformation	Cocodrie (*javanica*)	6.0	[28]
	E. uredovora crtI	Gt1					
SGR2 lines	Maize *psy*	Gt1	*pmi*	Agrobacterium mediated co-transformation	Kaybonnet (*javanica*)	37.0	[32]
	E. uredovora crtI	Gt1					

on the inventions developed during the research. Grenovation transferred the rights to Zeneca Agrichemicals, which then licensed back to the inventors 'non-commercial rights and undertake to help them improve the grain, deal with patenting issues and guide Golden Rice® through the costly testing and regulatory process. In exchange, Zeneca wanted to commercialize the Golden Rice® in developed countries among a group of increasingly health conscious population. The inventors planned to distribute the rice free to public sector breeding centers and agriculture institutes in India, China and other rice-dependent Asian nations with initial condition that the Golden Rice® will be made available free of cost to farmers with income less than $10,000 per annum. Later on, as Zeneca merged with Novartis to form Syngenta which thus received an exclusive license for the commercial use of the technology in developed nations. Simultaneously, Syngenta granted back to the inventors Prof. Ingo Potrykus and Prof. Peter Beyer, the exclusive license and the right to grant sublicenses for its noncommercial use [18]. A Humanitarian Board (HumBo), comprising of internationally recognized experts belonging to various reputed institutions of Philippines, Vietnam, India, Indonesia, China, Germany and Bangladesh was established with a view to make available transgenic Golden Rice® lines for utilization in the national breeding programmes to transfer the provitamin A trait in locally adapted and widely cultivated rice varieties so that the technology reaches the poor and needy farmers. The HumBo still continues to provide strategic guidance to the project.

Indian Network on Golden Rice®: Development of Golden Rice® lines in the genetic background of Indian rice varieties

An Indian Network on Golden Rice® was established with three institutions namely, Indian Agricultural Research Institute (IARI), New Delhi, Directorate of Rice Research (DRR), Hyderabad and Tamil Nadu Agricultural University (TNAU), Coimbatore through the funding from Department of Biotechnology, Government of India. Initially, the prototype transgenic Golden Rice® line in the genetic background of IR64 and Taipei 309 and SGR1 line in the Cocodrie background were used as donors in the backcross breeding programme. However, later this programme was discontinued owing to low carotenoids in these donors. Further, Syngenta through HumBo, made available six SGR2 events (G1, R1, L1, E1, T1 and W1) for use in public sector breeding programmes in India and other countries. The chromosomal location of transgene and β-carotene content of different events in Kaybonnet background is presented in Table 2. The development of *indica*

Table 2. Transgene location and β-carotene content in different events of SGR2 lines

Event	Transgene location		β-carotene content (µg/g)
	Chromosomal	Physical (Mb)	
G1	5	28.04	12.8
R1	1	38.75	19.7
L1	2	36.32	13.8
T1	3	18.26	23.0
W1	10	17.58	25.4
E1	3	24.65	11.4

versions of Golden Rice® utilizing the transgenic Kaybonnet as a donor is under progress at all the three aforesaid centers. At the Indian Agricultural Research Institute (IARI), Swarna, a mega rice variety grown in India on approximately 5 million ha area was chosen as recurrent parent (RP), while Improved Samba Mahsuri and MTU1010 were used as RPs at Directorate of Rice Research, Hyderabad and ASD 16 and ADT43 were utilized as RPs for incorporation of provitamin A trait at Tamil Nadu Agricultural University, Coimbatore.

Marker assisted backcross breeding (MABB) for incorporation of provitamin A traits in genetic background of Swarna

At IARI, MABB approach was adopted to introgress the provitamin A trait in the background of Swarna and its flash flood tolerant version Swarna Sub1. Initially, SGR2-G1 event was selected as an event for deregulation; however, it was later discontinued on account of certain regulatory issues. At present SGR2 R1 event is being used as donor for public sector breeding programs and advanced backcross derived lines (BC_3F_4 and BC_4F_4) having total carotenoid content in the range of 6.5 to 26.7 µg/g of endosperm in the freshly harvested samples and recurrent parent genome recovery in the range of 89.47 to 96.42% are available.

Inheritance and stability of transgene

Stability in transgene inheritance and expression is an important consideration in utilizing transgenic germplasm for the development of commercially viable transgenic crops. The pattern of transgene inheritance was studied through PCR analysis in F_2, BC_1F_1, reciprocal BC_1F_1 and BC_2F_2 populations derived by crossing a pCaCar event of golden rice line developed in the genetic background of IR64 with Swarna. Segregation distortion was observed for the transgene in F_2 and BC_1F_1 (Swarna × F_1) populations where as in the reciprocal BC_1F_1 (F_1 × Swarna) cross, the transgene showed normal Mendelian segregation ratio (1:1). Similar trend was also observed in the BC_2F_2, where the transgene followed an expected segregation ratio of 3:1 [33]. Transgene inheritance was also analyzed in the F_2, BC_1F_1 and BC_1F_2 families derived using SGR2 events in the Swarna background and segregation distortion was observed BC_1F_2 and BC_2F_2 families derived using SGR2 G1, T1 and R1 events. The inheritance pattern of provitamin A transgenes in SGR2 L1, W1 and E1 events was found to show normal Mendelian segregation [34]. The observed segregation distortion was explained due to preferential transmission of transgene either through male or female gametes or both the sides. Since, SGR2 R1 event was selected as candidate event for deregulation, a detailed phenotypic characterization of backcross derived lines in Swarna background using SGR2R1 event as donor, was carried out, which is presented as under:

Phenotype of transgene homozygous plants: Disruption of endogenous OsAux1 in SGR2 R_1 *event*

Phenotypic characterization of the backcross derived lines using SGR2 R1 as donor, revealed that the transgene homozygous plants, when compared to their hemizygous and null siblings, showed reduced plant height and shortened internodes, pale green leaves arranged in the form of whorls, elongated nodal buds giving rise to short panicles that remain partially enclosed within the leaf sheath [35]. This kind of phenotype is a serious limitation in the development of a commercially viable product and hence necessitated to explore the reasons behind this altered phenotype of transgene homozygotes. Nucleotide BLAST analysis using the SGR2 R1 event specific primers revealed that the transgene integration on chromosome 1 in Kaybonnet had disrupted the exon 1 of endogenous *OsAux1* gene there by disrupting its coding sequence and affecting its function. The absence of the expression of *OsAux1* in homozygous lines was further confirmed using real time expression analysis. In the nulls, with two intact copies of the gene on the homologous chromosomes the transcript levels were two fold higher to hemizygous lines with only one intact copy of the gene (Data not presented).

Quantification of beta carotene content in the backcross derived lines

The β-carotene concentration of the transgene homozygous lines was in the range of 6.5 to 26µg/g

when analyzed from the seeds harvested at physiological maturity and quantified using High-performance liquid chromatography. However, there is a gradual reduction of carotenoid content upon storage to an amount of 70 per cent in four to five months (Data not presented). These results suggest that there is a need to understand the mechanisms of carotenoid degradation and ways to counter this limitation to make Golden Rice® a commercially viable product.

Are the extreme regulatory precautions, sole cause for the delay in Golden Rice® release?

Being the product of recombinant DNA technology, Golden Rice® does not form any exception to the regulatory precautions which a genetically modified (GM) crop is ought to undergo. In fact, it is being more rigorously tested for safety as it is meant for direct human consumption. Although it is being said that the product development was complete by the year 2002, Golden Rice® had not reached the plates of targeted group of people. From 2002 to till date it is still fighting to come out of the stringent regulatory mechanisms. It fulfilled several requirements of GMO opponents like sustainable, cost free solution, complements traditional interventions to prevent VAD, avoids negative side effects of green revolution, not for the benefit of industry, does not create new dependencies, does not reduce agricultural biodiversity and does not affect natural biodiversity are few among them [36]. However, the issues like the withdrawal of G1 event, *Aux1* disruption in R1 event leading to a phenotype not amenable for commercial cultivation and the carotenoid degradation upon storage leaves behind several questions, which need to be addressed through rigour of science, so that the commercialization of Golden Rice® becomes a reality.

Conclusion

Golden Rice® is a powerful technology and a scientific breakthrough that demonstrated the feasibility of activating a defunct β-carotene biosynthetic pathway by introducing the missing genes in rice endosperm. Thanks to the inventors whose intellect and endeavors deserve universal applause and the contribution of Syngenta, which donated the technology for the benefit of poor and underprivileged group of population in the developing countries is praiseworthy. However, the product developed so far in the genetic background of Indian rice variety Swarna has several limitations. The results from our study also show that sufficient care should be taken in the characterization of the transgenic events and their selection for utilization in breeding programmes. The transgenic events are to be analyzed not merely for the target trait but for their overall phenotype and agronomic performance. We hope to see the Golden Rice in the plates of the target group, fulfilling the wishes of its inventors and serving the purpose behind its creation *i.e.,* elimination of VAD.

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References

1. **UNICEF.** 2002. Vitamin A global initiative (available at www.unicef.org/vitamina; accessed end May 2003).
2. **Olsen J. A.** 1989. Provitamin A function of carotenoids: the conversion of beta-carotene into vitamin A. J. Nutr., **119**: 105-108.
3. **Olson J. A.** 1990. Vitamin A. In: Brown ML *et al*., eds. Present knowledge in nutrition. 6th ed. Washington, DC: International Life Sciences Institute-Nutrition Foundation, 96-107.
4. **Wolf G.** 1984. Multiple functions of vitamin A. Physiol. Rev., **64**: 873-937.
5. **Groff J. L., Gropper S. S. and Gropper S. M.** 1995. Advanced Nutrition and Human Metabolism. 2nd ed. Minneapolis, MN, USA: West Publishing Company.
6. **Jones G., Richard W. S., Robert E. B., Zulfiqar A. B. and Saul S. M.** 2003. How many child deaths can we prevent this year? The Lancet, **362**: 65-71.
7. **Melissa M., Jean H., Elizabeth., Edmore M., Ron B. and Joanne K.** 2002. Why Do Children Become Vitamin A Deficient? J. Nutr., **132**: 2867-2880.
8. **Christian P. and West K. P.** 1998. Interactions between zinc and vitamin A: an update. Am. J. Clin. Nutr., **68**: 435-41.
9. **Christian P., Keith P., West J., Subarna K. K., Joanne K., Steven C., Elizabeth L., Pradhan K. and Sanu M. D.** 2000. Vitamin A or β-Carotene Supplementation Reduces Symptoms of Illness in Pregnant and Lactating Nepali Women. J. Nutr., **130**: 2675-2682.
10. **Chouhan B. S.** 1977. Kajal eye staining for early detection of vitamin A deficiency in the field. Trop.

Geogr. Med., **29**: 278-282.

11. **World Health Organization. 1997.** Vitamin A supplements: a guide to their use in the treatment of vitamin A deficiency and xerophthalmia. Geneva, Switzerland: WHO, 1997.
12. **Mayer J.E., Pfeiffer W.H. and P. Beyer.** 2008. Biofortified crops to alleviate micronutrient malnutrition. Curr. Opin. Plant Biol., **11**: 166-170.
13. **Nestel P., Howarth E. B., Meenakshi J. V. and Wolfgang P.** 2006. Biofortification of staple food crops. J. Nutr., **13**6: 1064-1067.
14. **FAO.** 1995. Dimensions of need: An atlas of food and agriculture.
15. **FAO**. 2004. Rice is Life. http://www.fao.org/newsroom/en/focus/200436887/index.html.
16. **Kennedy G., Nantel G. and Shetty P.** 2003. The scourge of "hidden hunger": global dimensions of micronutrient deficiencies fna ana 32.
17. **Juliano B. O.** 1993. Concerns for quality maintenance during storage of cereals and cereal products. In: Proceedings of 6th International working conference on stored product protection, **2**: 662-665.
18. **Beyer P., Al-Babili S., Ye X. D., Lucca P., Schaub P., Welsch R. and Potrykus I.** 2002. Golden rice: Introducing the beta-carotene biosynthesis pathway into rice endosperm by genetic engineering to defeat vitamin A deficiency. J. Nutr., **132**: 506S-510S.
19. **Castenmiller J. J. and West C.** 1998. Bioavailability and bioconversion of carotenoids. Annu. Rev. Nutr., **18**:19-38.
20. **Vanderhof K. H., West C., Weststrate J. and Hautvast J.** 2000. Dietary factors that affect the bioavailability of carotenoids. J. Nutr., **130**: 503-6.
21. **Grune T., Lietz G., Palou A., Ross A. C., Stahl S., Tang G., Thurnham D., Yin S. and Biesalski H. K.** 2010. â-Carotene is an important vitamin A source for humans. J. Nutr., **140**: 2268-85.
22. **Yeum K. J. and Russell R.** 2002. Carotenoid bioavailability and bioconversion. Annu. Rev. Nutr., **22**: 483-504.
23. **Cunningham F. X. and Gantt E.** 1998. Genes and enzymes of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Mol. Biol., **49**: 557-583.
24. **Rodriguez-Concepcion M.** 2010. Supply of precursors for carotenoid biosynthesis in plants. Arch. Biochem. Biophys., **504**: 118-122.
25. **Cordoba E., Salmi M. and Leon P.** 2009. Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. J. Exp. Bot., **60**: 2933-2943.
26. **Cazzonelli C. I., Yin K. and Pogson B. J.** 2009. Potential implications for epigenetic regulation of carotenoid biosynthesis during root and shoot development. Plant Signal Behav., **4**: 339-341.
27. **Burkhardt P. K., Beyer P., Wunn J., Kloti A., Armstrong G. A., Schledz M., Von L. V. and Potrykus I.** 1997. Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus psecudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. Plant J., **11**: 1071-1078.
28. **Al-Babili S. and Beyer P.** 2005. Golden Rice — Five years on the road — five years to go? Trends Plant Sci., **10**: 565-573.
29. **Ye X., Al-Babili S., Klöti A., Zhang J., Lucca P., Beyer, P. and Potrykus, I.** 2000. Engineering the provitamin A (beta carotene) biosynthetic pathway into (Carotenoid-Free) rice endosperm, Science, **287**: 303-305.
30. **Hoa T.T.C., Al-Babili S., Schaub P., Potrykus I. and Beyer P.** 2003. Golden indica and *japonica* rice lines amenable to deregulation. Plant Physiol., **133**: 161-169.
31. **Datta K., Baisakh N., Oliva N., Torrizo L., Abrigo E., Tan J., Rai M., Rehana S., Al- Babili S., Beyer P., Potrykus I. and Datta S.K.** 2003. Bioengineered golden indica rice cultivars with beta-carotene metabolism in the endosperm with hygromycin and mannose selection systems. Plant Biotech. J., **1**: 81-90.
32. **Paine J. A., Shipton C. A., Chaggar S., Howells R. M., Kennedy M. J., Vernon G., Wright S. Y., Hinchliffe E., Adams J. L., Silverstone A. L. and Drake R.** 2005. A new version of Golden Rice with increased pro-vitamin A content. Nat. Biotechnol., **23**: 482-487.
33. **Nandakumar N., Tyagi N. K., Balram M., Chikkappa G. K., Aprajita G., Mohapatra T., Prabhu K. V. and Singh A. K.** 2008. Molecular analysis of transgene (psy) inheritance in a golden rice line developed in the genetic background of IR64. J. Plant Biochem. Biotech., **17**: 127-132.
34. **Chikkappa G. K., Tyagi N. K., Venkatesh K., Ashish M., Prabhu K. V., Mohapatra T. and A. K. Singh.** 2011. Analysis of transgene(s) (*psy+crtI*) inheritance and its stability over generations in the genetic background of indica rice cultivar Swarna. J. Plant Biochem. Biotech., **20**: 29-38.
35. **Bollineni H., Gopala Krishnan S., Prabhu K.V., Singh N.K., Madan Pal, Mishra Sushma, Khurana, J.P. and A.K. Singh.** 2014. Biofortification for improvement of nutritional quality: the journey of Golden Rice. In: Proc. National Symposium on Crop Improvement for Inclusive Sustainable Development, Nov 7-9, 2014, PAU, Ludhiana, India. 8.
36. **Potrykus I.** 2001. Golden Rice and beyond. Plant Physiol., **125**: 1157-1161.