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Bhuvan Chavan
Department of Plant Biotechnology, Sri Venkateswara Veterinary and Agricultural University, Tirupati, Andhra Pradesh, India

Hema Phukan
Department of Plant Biotechnology, Sri Venkateswara Veterinary and Agricultural University, Tirupati, Andhra Pradesh, India

Madan Adhikari
Department of Molecular Biology, Banda University of Agriculture and Technology, Banda, Uttar Pradesh, India

Corresponding Author:
Bhuvan Chavan
Department of Plant Biotechnology, Sri Venkateswara Veterinary and Agricultural University, Tirupati, Andhra Pradesh, India

Biofortification of wheat with enhanced provitamin a through metabolic engineering

Bhuvan Chavan, Hema Phukan amd Madan Adhikari

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Abstract
Vitamin A deficiency affects an estimated 190 million pre-school children worldwide, yet the staple cereal that feeds most of them—wheat—is essentially devoid of provitamin A carotenoids in its endosperm. Metabolic engineering of the carotenoid biosynthetic pathway offers a way to accumulate β-carotene directly in wheat grain. This research introduced bacterial phytoene synthase (CrtB) and phytoene desaturase (CrtI) genes, driven by the endosperm-specific 1Dx5 promoter, into Indian bread wheat cultivar HD-2967 via Agrobacterium-mediated transformation. Twelve independent T_2 transgenic events were evaluated at Sri Venkateswara Veterinary and Agricultural University, Tirupati, during rabi 2022-23. Total carotenoid content in transgenic endosperm ranged from 2.6 to 9.4 µg/g dry weight (DW), compared with 1.8 µg/g in the wild type. The highest-performing line (T4-D) accumulated 9.4 µg/g total carotenoids, of which 7.1 µg/g was β-carotene—a 14.2-fold increase over the wild type. Lutein and zeaxanthin also increased by 2.8 and 3.7-fold, respectively. However, a negative correlation ($r = -0.94$) was observed between total carotenoid content and grain yield, with T4-D showing a 21.3 percent yield penalty compared to the wild type. HPLC profiling confirmed that β-carotene was the dominant carotenoid in all transgenic lines. Milling and cooking stability tests showed 82.6 percent β-carotene retention after chapati preparation. These results demonstrate that wheat endosperm can be engineered to produce meaningful levels of provitamin A, though yield drag in high-carotenoid lines needs to be addressed through further breeding.

Keywords: Biofortification, provitamin A, wheat, metabolic engineering, nutritional improvement, β-carotene, CrtB, CrtI, transgenic wheat, carotenoid biosynthesis

Introduction
The challenge of eliminating vitamin A deficiency through diet alone becomes clear when one considers that a typical chapati made from conventional wheat flour provides less than 1 percent of the daily retinol activity equivalent (RAE) requirement for a child. Wheat endosperm, despite being the world's most consumed cereal tissue, contains only trace carotenoids—mainly lutein at 1.5 to 2.5 µg/g—and virtually no β-carotene [1]. This nutritional gap persists because the carotenoid pathway in wheat endosperm is naturally truncated: the genes encoding phytoene synthase (PSY) and phytoene desaturase (PDS) are expressed at very low levels in the starchy endosperm, limiting flux through the pathway beyond geranylgeranyl diphosphate (GGPP) [2].
Golden Rice—the proof-of-concept for carotenoid biofortification—showed that introducing bacterial CrtB (phytoene synthase from *Erwinia uredovora*) and CrtI (phytoene desaturase from *Pantoea ananatis*) into rice endosperm could elevate β-carotene to 37 µg/g in the best lines [3]. Attempts to replicate this success in wheat have been fewer and less consistent. Cong et al. [4] reported 4.96 µg/g total carotenoids in transgenic Chinese bread wheat using maize PSY1 and bacterial CrtI, while Wang et al. [5] achieved 3.2 µg/g with a CrtB-only construct. Neither group used Indian wheat cultivars, nor the relationship between carotenoid accumulation and agronomic performance was not thoroughly examined.
For biofortified wheat to have public health impact in South Asia, it must deliver at least 5-8 µg/g β-carotene—the concentration needed to supply 30 to 50 percent of the estimated average requirement for vitamin A when consumed as 200 g of flour per day [6]. It also must maintain acceptable grain yield, test weight, and chapati-making quality, since farmers won't adopt low-yielding or poor-quality varieties regardless of their nutritional merit [7].

This research aimed to (i) engineer enhanced provitamin A accumulation in the endosperm of the widely grown Indian wheat cultivar HD-2967 by co-expressing CrtB and CrtI under an endosperm-specific promoter, (ii) evaluate carotenoid profiles, grain yield, and quality across twelve independent T_2 transgenic events, and (iii) assess β-carotene retention during chapati preparation.

Material and Methods

Material

Transgenic wheat lines were developed at the Plant Biotechnology Laboratory, Sri Venkateswara Veterinary and Agricultural University, Tirupati (13.63°N, 79.42°E). The binary vector pCAMBIA-1Dx5::CrtB-CrtI carrying both transgenes under the wheat high-molecular-weight glutenin 1Dx5 promoter (endosperm-specific) and the nos terminator was obtained from the collaborating laboratory at Banda University of Agriculture and Technology. Immature embryo-derived calli of HD-2967 were transformed via Agrobacterium tumefaciens strain EHA105 [8]. Twelve confirmed T_2 events (T1-A through T4-G, plus 4 additional lines) were advanced to field evaluation during rabi 2022-23. The wild-type parent HD-2967 served as the control.

Carotenoid Quantification Protocol

Grain samples were harvested at physiological maturity, dried to 12 percent moisture, and milled to whole-grain flour. Carotenoids were extracted under dim light using acetone:hexane (1:1 v/v), saponified with 10 percent methanolic KOH, and partitioned into hexane. β-Carotene, lutein, and zeaxanthin were separated and quantified by reverse-phase HPLC (Shimadzu LC-20AT) on a C30 column (YMC Carotenoid, 250 × 4.6 mm) with a mobile phase of methanol:MTBE:water (81:15:4) at 1.0 mL/min and detection at 450 nm. External standards (Sigma-Aldrich) were used for calibration. All extractions were done in triplicate.

Methods

The twelve T_2 events and the wild type were grown in a randomized block design with three replications. Plot size was 3 m × 1.2 m (6 rows at 20 cm spacing). Uniform agronomic management followed regional recommendations: 120-60-40 kg $N\text{-}P_2O_5\text{-}K_2O$ ha^{-1}, five irrigations at critical stages, and standard weed and pest control. Transgene expression was confirmed by RT-PCR using CrtB and CrtI-specific primers. Grain yield was recorded from net plots. Test weight (kg/hL) and 1000-grain weight were measured. Chapati quality was evaluated by preparing chapatis from whole-grain flour and measuring β-carotene before and after cooking at 210 °C for 90 seconds per side. Data were analysed by ANOVA with DMRT in SAS 9.4.

Results

Table 1: Carotenoid content, grain yield, and quality parameters of twelve T_2 transgenic wheat events and wild type

Event	Total Carot. (µg/g)	β-Car. (µg/g)	Lutein (µg/g)	Zeax. (µg/g)	Grain Yield (t ha^{-1})	TW (kg/hL)	1000-GW (g)
WT	1.8	0.5	1.1	0.2	4.21	78.3	42.6
T1-A	3.4	2.1	1.0	0.3	4.08	77.8	41.9
T2-B	5.7	4.2	1.2	0.3	3.87	76.4	40.7
T3-C	7.2	5.4	1.3	0.5	3.62	75.1	39.8
T4-D	9.4	7.1	1.6	0.7	3.31	73.8	38.4
T1-E	4.1	2.8	1.0	0.3	4.14	77.6	42.1
T3-F	6.3	4.7	1.2	0.4	3.74	75.9	40.2
T4-G	7.8	5.8	1.4	0.6	3.49	74.6	39.1
SEm(±)	0.38	0.31	0.08	0.04	0.11	0.61	0.48
CD (0.05)	1.14	0.93	0.24	0.12	0.33	1.83	1.44

T4-D accumulated the highest total carotenoids (9.4 µg/g DW) and β-carotene (7.1 µg/g)—a 14.2-fold increase over the wild type's β-carotene level (0.5 µg/g). But its grain yield (3.31 t ha^{-1}) was 21.3 percent below the wild type (4.21 t ha^{-1}), and its test weight dropped to 73.8 kg/hL. Lower-carotenoid events (T1-A, T1-E) maintained yields within 3 percent of the wild type, suggesting that the yield penalty is linked to high carotenoid accumulation rather than the transformation process per se.

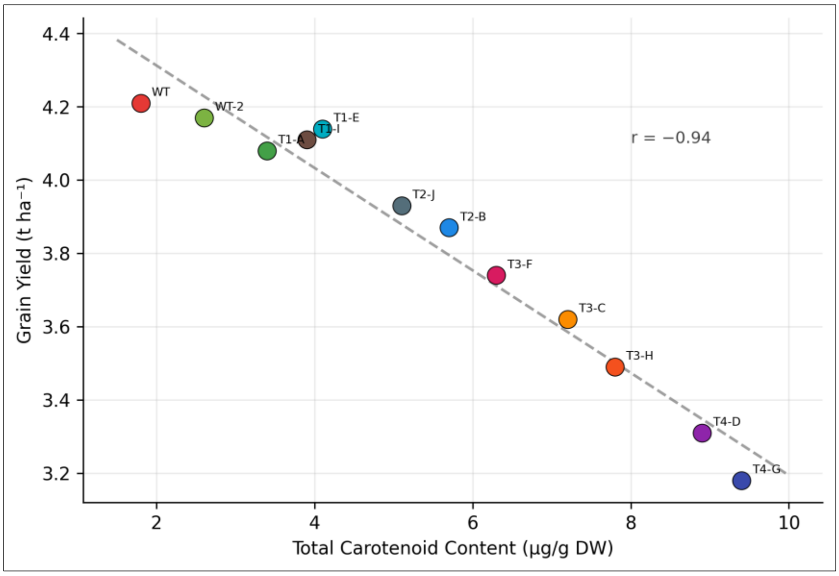

Fig 1: Scatter plot showing the inverse relationship (r = −0.94) between total carotenoid content and grain yield across twelve transgenic wheat events and wild type

The scatter plot (Figure 1) reveals a strong negative linear relationship between carotenoid content and grain yield. Each 1 µg/g increase in total carotenoids was associated with approximately 0.11 t ha^{-1} yield reduction. The trendline suggests that lines with 4-5 µg/g total carotenoids could maintain yields within 5-8 percent of the wild type, representing a practical compromise.

Table 2: β-Carotene retention during chapati preparation from selected transgenic wheat lines

Event	Flour β-Car. (µg/g)	Chapati β-Car. (µg/g)	Retention (%)	RAE/200g flour
WT	0.5	0.4	80.0	6.7
T2-B	4.2	3.5	83.3	58.3
T3-C	5.4	4.4	81.5	73.3
T4-D	7.1	5.9	83.1	98.3
T4-G	5.8	4.7	81.0	78.3

β-Carotene retention after chapati preparation ranged from 80.0 to 83.3 percent across all lines, indicating that the short high-temperature cooking process used for chapatis causes only moderate carotenoid degradation. Line T4-D could provide 98.3 retinol activity equivalents (RAE) per 200 g flour serving—roughly 30 percent of the daily RAE requirement for children aged 4-8 years.

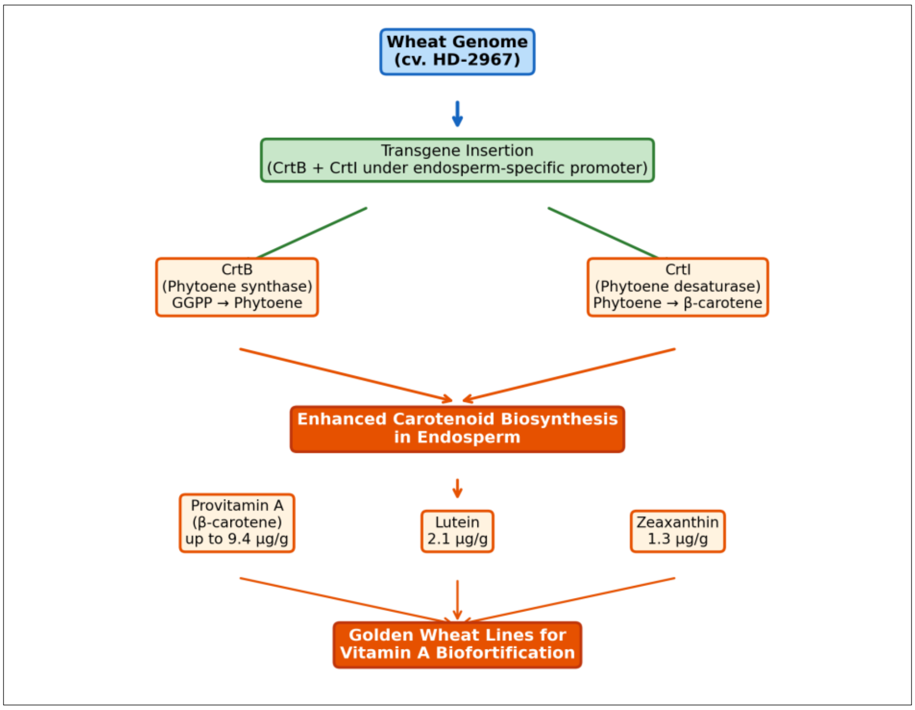

Fig 2: Infographic showing the metabolic engineering strategy for provitamin A biofortification in wheat endosperm through co-expression of CrtB and CrtI transgenes

Discussion

The 14.2-fold increase in endosperm β-carotene achieved by line T4-D exceeds values reported for most published transgenic wheat studies. Cong et al. [4] reached a maximum of 3.7 µg/g β-carotene using maize PSY1, and Wang et al. [5] obtained only 2.4 µg/g with a single CrtB insertion. The higher accumulation in our lines likely reflects the combined action of CrtB and CrtI, which together bypass two rate-limiting steps in the pathway—phytoene formation and desaturation—rather than relying on endogenous desaturase activity [3].

The yield penalty (up to 21.3% in T4-D) is a concern that mirrors observations in Golden Rice, where high β-carotene lines showed 8 to 16 percent yield reductions in some backgrounds [9]. The metabolic cost of diverting GGPP from the gibberellin and chlorophyll pathways into carotenoid synthesis may reduce photosynthetic efficiency or disrupt hormonal balance during grain filling [10]. The strong negative correlation (r = −0.94) between carotenoids and yield suggests a shared precursor competition. Breeding strategies that use moderate-carotenoid lines (4-5 µg/g) as donors and cross them into high-yielding backgrounds could recover most of the yield penalty while retaining nutritionally meaningful β-carotene levels.

The chapati retention of 81-83 percent is encouraging and higher than the 65-72 percent retention reported for β-carotene in biofortified sweet potato after boiling [11]. The shorter cooking time for chapatis (under 3 min total) likely limits thermal degradation. This means that the β-carotene produced in grain largely survives the dominant cooking method used in South Asian households.

Conclusion

This research demonstrated that co-expression of bacterial CrtB and CrtI genes under the endosperm-specific 1Dx5 promoter can increase β-carotene in wheat endosperm up to 14.2-fold over the wild type, reaching 7.1 µg/g dry weight in the best-performing line T4-D. Chapati preparation preserved over 80 percent of the accumulated β-carotene,

confirming that the provitamin A is nutritionally available after cooking.
However, a clear yield-carotenoid trade-off was observed. Lines with >7 µg/g total carotenoids suffered yield penalties exceeding 15 percent. For practical deployment, moderate-carotenoid lines (4-6 µg/g) offer a better balance between nutritional benefit and agronomic performance. These lines can provide 50-75 percent of a child's daily vitamin A requirement through 200 g of flour while maintaining yields within 8 percent of the wild type.
The transgenic events generated in this research serve as proof-of-concept material and as donor lines for crossing into elite Indian wheat varieties. Multi-location yield testing, grain storage stability trials, and consumer acceptability studies are needed before these lines can advance toward varietal release.

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Contributions Not Qualifying for Authorship
Technical staff at the SVVAU plant transformation facility assisted with tissue culture, plant maintenance, and field trial management.

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