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Identifying molecular targets for modulating carotenoid accumulation in rice grains

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ABSTRACT

Carotenoids are potential antioxidants offering extensive human health benefits including protection against chronic diseases. Augmenting the supply of health-benefiting compounds/metabolites through dietary supplements is the most sustainable way for a healthy life. Our study compares the traditional rice cultivar Kavuni and the white rice variety ASD 16. RNA-Seq analysis was carried out in the maturing panicles of Kavuni, which are enriched with antioxidants such as the therapeutic carotenoid lutein, polyphenols, and anthocyanins, along with "ASD 16", a popularly eaten white rice variety, to elucidate the molecular networks regulating accumulation of health benefiting compounds. Systematic analysis of transcriptome data identified preferential up-regulation of carotenoid precursors (*OsDXS*, *OsGGPS*) and key carotenoid biosynthetic genes (*OsPSY1*, *OsZ-ISO*) in the maturing grains of Kavuni. Our study also identified enhanced expression of *OsLYC-E*, *OsCYP97A,* and *OsCYP97C* transcripts involved in the alpha-carotenoid biosynthetic pathway and thereby leading to elevated lutein content in the grains of Kavuni. Kavuni grains showed preferential down-regulation of negative regulators of carotenoid metabolism viz., AP2 and HY5 and preferential up-regulation of positive modulators of carotenoid metabolism viz., *Orange*, *OsDjB7,* and *OsSET29*, thus creating a favorable molecular framework for carotenoid accumulation. Our study has unearthed valuable gene control points for precise manipulation of carotenoid profiles through CRISPR-based gene editing in rice grains. Perturbation of carotenoid biosynthesis holds unprecedented potential for the rapid development of the next generation of 'Golden rice'.

1. Introduction

Carotenoids (C_{40}) are terpenoid compounds having diverse roles in photosynthesis, photomorphogenesis, and photoprotection. Based on the functional groups, carotenoids are broadly classified into two classes: (1) hydrocarbon carotenes with hydrogen and carbon groups (alpha-carotene, β-carotene, and lycopene), (2) oxygenated carotenes called Xanthophylls. The Xanthophylls are again subjected to several chemical modifications like hydroxylation (Lutein, Zeaxanthin), epoxidation/de-epoxidation (violaxanthin, neoxanthin, fucoxanthin), ketolation (astaxanthin, canthaxanthin), and methoxylation (Spirilloxanthin) to form specialized carotenoids with specific functional roles in downstream pathways [1]. Carotenoids and their derivatives function as antioxidants, hormones, and signalling molecules that mediate plant growth, development, and other stress responses [2]. Carotenoids offer extensive human health benefits to prevent chronic diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, night blindness, and other age-related eye disorders through their provitamin-A activity and antioxidant potential [3,4].

Vitamin A deficiency (VAD) is an important health concern that affects 200 million population with various health complications, such as impaired vision, cardiovascular diseases, weak immunity and high mortality rates in young children and pregnant woman [5]. Carotenoids are primary source of Pro-Vitamin A and dietary antioxidants for Human. Carotenoid biosynthetic machinery stops at the early stages of geranyl geranyl diphosphate (GGDP) in the endosperm of widely consumed white rice varieties and it does not accumulate carotenoids. Hence, VAD is highly prevalent in developing countries of Africa and south-east Asia where rice is the staple food [6]. Through biotechnological interventions, beta-carotene enriched 'Golden Rice' has been developed by introducing the carotenoid biosynthesis pathway into the endosperm of white rice cultivars by ectopic expression of bacterial

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phytoene synthase and phytoene desaturase enzymes [7,8].

The endosperm of white rice lacks carotenoids, while, traditional pigmented landraces accumulate low to traceable levels of carotenoids like β-carotene, Lutein, Zeaxanthin, Lycopene, and Phytoene in their coloured endosperm [9]. Cultivar-specific differences in the carotenoid composition of rice, maize, and sorghum can be attributed to genetic variation in the expression of endogenous MEP and carotenogenic genes [10–12]. Transcriptome profiling coupled with metabolite analysis identified higher expression of *PSY1*, *PDS*, and *LCY-B* transcripts as the key players behind high carotenoid accumulating phenotypes of purple rice cultivars compared to brown and white rice cultivars [13]. Similarly, comparative transcriptomics in mature seeds of non-pigmented and pigmented rice varieties identified notable genetic clues for nutrition quality traits in pigmented rice [14].

Traditional rice cultivars are excellent reservoirs that hold clues to resolve the bottlenecks of carotenoid biosynthesis in rice endosperm. The grains of traditional pigmented rice Kavuni accumulated high levels of bioactive compounds such as Flavonoids, Anthocyanin, phenolic acids, lutein, and traceable levels of beta-carotene compared to white rice variety [15,16]. We conducted a comparative transcriptome profiling in grains of traditional pigmented rice Kavuni and the widely consumed white rice ASD 16 to elucidate key gene clues involved in the biosynthesis and regulation of carotenoids. Our goal was to identify key up-regulated and down-regulated genes associated with carotenoid biosynthesis, and utilize them for pro-vitamin A biofortification of rice endosperm.

2. Materials and methods

2.1. Plant materials, RNA extraction, and RNA quality assessment

Two contrasting rice genotypes viz., ASD 16 (white grain that lacks grain carotenoids) and Kavuni (pigmented rice with high lutein and traces of beta-carotene) were chosen for the study. Seedlings of both rice genotypes were planted in 30 × 20 cm spacing and maintained till maturity in Paddy Breeding Station, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. The maturing panicles of both 'Kavuni' and 'ASD16' genotypes were collected at 15 days after flowering and stored at −80 °C. Total RNA was extracted from maturing panicles using TRIzol method (TaKaRa, Kusatsu, Japan) with certain modifications. Genomic DNA contaminations were removed by DNase treatment. The quality of RNA samples was assessed using formaldehyde agarose gel electrophoresis, and then quantified by Nanodrop ND-1000 spectrophotometer (M/s. Thermo Fisher Scientific, Wilmington, DE, USA).

2.2. cDNA library preparation and transcriptome sequencing

RNA Integrity Number (RIN) of the samples were ascertained using bioanalyzer and high-quality RNA samples possessing RIN score = > 8.0 were processed for cDNA library construction. Equimolar concentrations of total RNA from the three replications of a genotype were pooled and subjected to rRNA depletion, cDNA library construction, and adaptor ligation as described by the manufacturer (Illumina Inc., USA). The cDNA fragments were then purified, quantified and the final libraries were sequenced using Illumina HiSeq platform at M/s. Agri-Genome Labs Pvt. Ltd., Cochin, Kerala, India. The raw reads were deposited in the NCBI short archive reads bearing Accession number # PRJNA787761.

2.3. Pre-processing of raw data, mapping of reads, and analysis of differential gene expression

The paired end reads were subjected to pre-processing and quality checks to trim ambiguity reads, adaptor sequences and poor-quality reads using CLC Genomics Workbench 11.0.1 (M/s. QIAGEN Denmark, USA). The raw reads were trimmed with a minimum PHRED score of Q ≥ 30 and the low-quality reads were discarded. Reference-based transcript assembly and differential expression analysis were carried out using the RNA-Seq DEG analysis pipelines available in CLC Genomics Workbench 11.0.1. For each sample, high-quality reads were aligned to the rice reference genome (*O. sativa*; MSU Rice Genome Annotation Project Version 7.0; www.tigr.org) using default parameters. Raw read counts were normalized to Reads per Kilobase per Million (RPKM) values to measure the transcript abundance in each genotype. Gene loci with RPKM >0.1 were considered as "expressed" to eliminate false-negative and false-positive transcripts at the rate of 5 % [18]. Differentially Expressed Genes (DEGs) across the cultivars were identified in a rigorous comparison of log2 fold change ≥1.5 for upregulation, ≤−1.5 for downregulation at statistical significance of p-value ($p < 0.05$) calculated using Baggerley's test implemented in CLC genomic Workbench suite. DEGs were functionally annotated and mapped to their definite pathways using the KEGG database (http://www.genome.jp/kegg/pathway.html) and MapMan (http://mapman.gabipd.org) tools. Transcription factors in our datasets were identified from the annotations available in Plant Transcription Factor Database.

2.4. Annotation of DEGs related to carotenoid pathway

Our earlier study identified unique upregulation of genes involved in carotenoid biosynthetic pathway in the traditional genotype Kavuni compared to the white grain rice genotype ASD16 [17]. To have a deeper understanding of the carotenoid accumulation in Kavuni grains and to shortlist molecular targets for channelizing carotenoid pathway towards the accumulation of β-carotene, a comprehensive list of structural genes, transcription factors, and other carotenoid modulators involved in carotenogenesis were enumerated from various literature sources and their expression levels were compared between Kavuni and ASD 16. The carotenoid pool is significantly influenced by upstream mechanisms providing carotenoid precursors and the downstream pathways that catabolize the available carotenoids. Based on their phenotypic significance, genes affecting carotenoid homeostasis were primarily categorized under five pathways *viz.,* MEP Precursor pathway, Carotenoid biosynthesis, Xanthophyll biosynthesis, Carotenoid catabolism, and Carotenoid modulators. Furthermore, the transcript abundance of carotenoid pathway genes between rice genotypes ASD16 and Kavuni, were visualized as heat maps.

2.5. Validating differential expression of putative candidates using qRT PCR

The expression level of four key carotenoid genes *OsLYC-E*, *OsLYC-B*, *OsBCH1*, and *OsCCD4a* was validated using Quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the maturing panicles (10th day after flowering and 20th day after flowering) of Kavuni and ASD16, and first-strand cDNA was synthesized (Kit # 6110A, Takara, India) as per the manufacturer's instructions. An equal amount of first strand cDNA was used for qRT-PCR reactions in CFX96TM real-time system (Bio-Rad, Hercules, CA, USA) using SYBR™ Green PCR Master Mix (#43-091-55, Applied Biosystem, Thermo Scientific, USA) and gene-specific primers listed in Table 1. The relative expression levels of each gene were normalized to the expression level of an endogenous reference gene *Ubiquitin3* following the $2_{T}^{-\Delta\Delta C}$ method [19]. Validation was carried out with two independent biological replicates and two technical replicates.

3. Results and discussion

Carotenoid biosynthesis is a complex, dynamic, and highly regulated metabolic process. Grains of white rice do not accumulate any carotenoids, while grains of pigmented rice (purple, black, red, and brown) accumulate traceable levels of different carotenoid intermediates [9].

Table 1
Details on genes and gene-specific primers used for validation of RNA-seq data through qRT-PCR.

Target gene	Primer Name	Nucleotide sequence (5'-3')	G + C (%)	T_m (°C)	Amplicon size (bp)	Reference
OsLYC-E	RT-LYCE-F2	TACGGTGTGTGGGAGGATGA	55.0	60.3	185	Present Study
	RT-LYCE-R2	GTGACTCCAGCGTCATAGCA	55.0	59.8		
OsLYC-B	RT-LYCB-F	GCATCCTCCCTCCTCATCT	57.9	57.8	192	Present Study
	RT-LYCB-R	CCAGTCCATGAACAGCATCTT	47.6	58.0		
OsBCH1	RT-BCH1-F	GCCCACCAGATACATCACAT	50.0	57.4	147	Present Study
	RT-BCH1-R	CGCATCTAAGGTCTCTTTCCTC	50.0	58.3		
OsCCD4a	RT-CCD4a-F	TCCCTCCTGCTGCTTCT	58.8	57.0	165	Present Study
	RT-CCD4a-R	CCAACTGCTCGTCCTCTTG	57.8	58.0		
OsUbiquitin	RT-Ubi-F	AGAAGCGCAAGAAGAAGACG	50.0	56.0	100	[20]
	RT-Ubi-R	GCGTCGTCCACCTTGTAGA	57.9	57.0		

Earlier research identified that grains of traditional pigmented rice Kavuni accumulated lutein and traceable levels of beta-carotene compared to the white rice variety [15,16]. A comparative transcriptome analysis between pigmented rice Kavuni and non-pigmented white rice ASD16 was carried out to elucidate differences in transcript expression patterns of candidate genes that affect the carotenoid accumulation in pigmented rice grains. Multiple bottlenecks coexist in rice carotenogenesis and focusing on 'multiple gene overarching' strategies will help us to elucidate its complex regulatory mechanism and provide new clues for targeted metabolite engineering.

3.1. Specific up-regulation of carotenoid precursor genes in the grains of kavuni

Glycolysis-derived pyruvate (C3) and glyceraldehyde 3-phosphate (GAP, C3) are condensed into MEP (C5) by the sequential action of two rate-limiting enzymes *viz.,* Deoxy-xylulose 5-phosphate synthase (DXS) and Deoxy-xylulose 5-phosphate reductor-isomerase (DXR). In our study, Kavuni grains showed nearly 5.5-fold upregulation of two gene homologs encoding DXS (*OsDXS1* - LOC_Os05g33840; *OsDXS3* - LOC_Os06g05100) resulting in high carotenoid accumulation compared to the white rice ASD16 (Fig. 1A). Previous studies demonstrated that

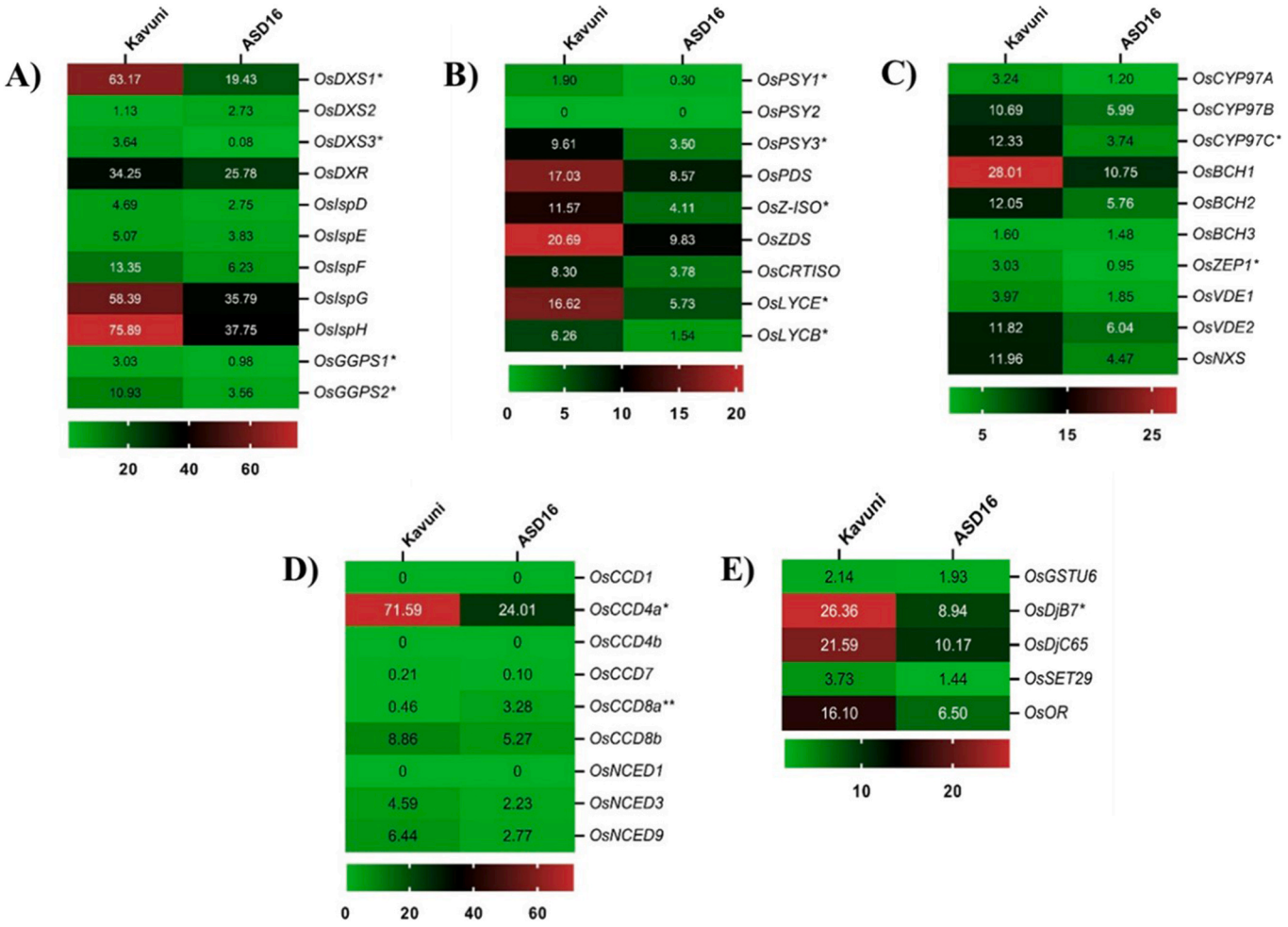

Fig. 1. Heatmap visualization of the expression pattern of genes related to the carotenoid pathway in rice genotypes ASD16 and Kavuni. A) Precursor pathway; B) Carotenoid biosynthesis; C) Xanthophyll biosynthesis; D) Carotenoid catabolism; E) Carotenoid modulators; The scale green to red indicates lower to higher RPKM values (Gene with RPKM>0.1 expressed; <0.1 NA). * indicate upregulated DEGs and ** indicate downregulated DEGs in Kavuni grains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

overexpression of *AtDXS* transgene increased the supply of upstream isoprenoid precursors and boosted the overall carotenoid flux in rice [21,22]. The MEP molecules are sequentially condensed and modified by enzymes IspD, IspE, IspF, IspG, and IspH to produce two isoprene precursors, isopentenyl diphosphate (IPP, 5C) and dimethylallyl diphosphate (DMAPP, C5). In subsequent reactions, IPPs and DMAPP precursors are condensed by GeranylGeranyl diphosphate synthase (GGPS) to produce Geranylgeranyl diphosphate (GGPP, C20). GGPPs are considered the building blocks/substrates for carotenoid (C40) biosynthesis. In maize and ornamental pepper, the higher expression level of GGPS positively correlated with increased accumulation of carotenoid contents [23,24]. Similarly, we observed a notable upregulation of GGPS gene homologs (*OsGGPS1* - LOC_Os07g39270; *OsGGPS2* - LOC_Os02g44780) in grains of Kavuni compared to white rice ASD16 (Fig. 1A). Our study has identified that high expression of precursor pathway genes overcomes the first bottleneck of carotenoid biosynthesis and it is a prime contributor towards high carotenoid accumulating phenotypes of pigmented Kavuni grains (Fig. 2).

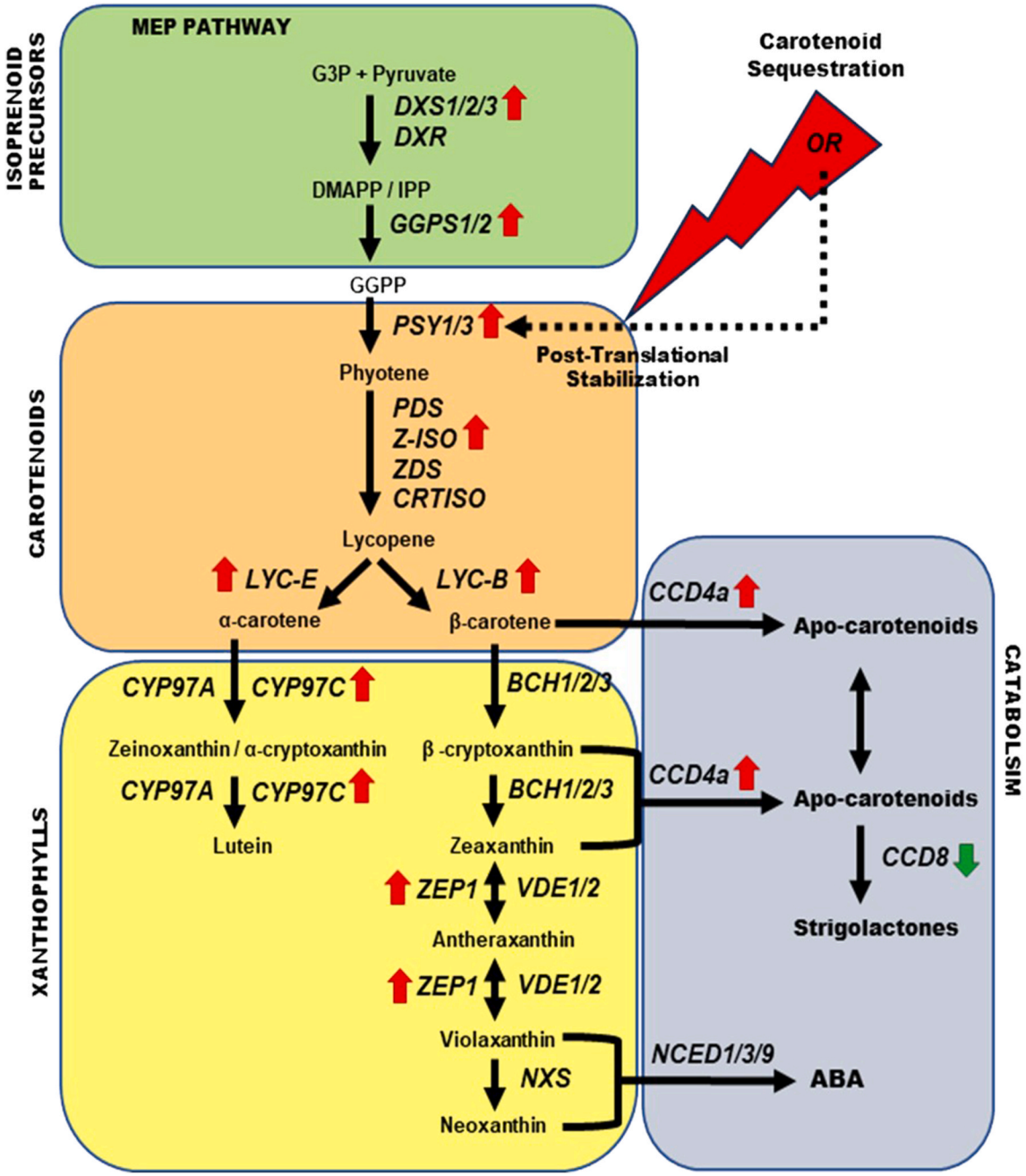

Fig. 2. Schematic Illustration of Carotenoid Metabolic Pathway in Kavuni rice grains. Red arrow indicates upregulated DEGs and green arrow indicates down-regulated DEGs in Kavuni grains. [Abv: MEP, methylerythritol 4-phosphate; G3P, glyceraldehyde 3-phosphate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYC-B, lycopene β-cyclase; LYC-E, lycopene ε-cyclase; CYP97, cytochrome P450 carotene hydroxylase; BCH, β-carotene hydrolase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase; CCD, carotenoid cleavage dioxygenases; NCED, 9-cis-epoxycarotenoid dioxygenase; OR, ORANGE protein; ABA, abscisic acid]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Up-regulation of carotenoid biosynthesis genes in kavuni

Phytoene synthase, the first rate-limiting enzyme of the carotenoid biosynthesis pathway, is involved in the conversion of two GGPP molecules into 15-cis-phytoene (C40). Phytoene synthase closely associates with GGPS to channel the endogenous flux toward carotenoid biosynthesis [25–27]. We observed that two homologs of Phytoene synthase (*PSY1* - LOC_Os06g51290; *PSY3* - LOC_Os09g38320) were significantly upregulated in Kavuni, while the third homolog (*PSY2* - LOC_Os12g43130) was not detected in grains of both rice genotypes (Fig. 1B). The *OsPSY1* and *OsPSY2* homologs possess light-responsive *cis*-regulatory elements which indicate their role in rice carotenogenesis, on the other hand, *OsPSY3* has ABRE binding elements that trigger stress response modules in vegetative tissues [28]. A series of carotene desaturase enzymes *viz.*, *OsPDS*, *OsZDS*, *OsCRTISO* and *OsZ-ISO* are synergistically involved in the conversion of phytoene (C40) to lycopene (C40). The *OsZ-ISO* transcripts of the carotene desaturase family showed nearly 3-fold upregulation in Kavuni grains. Our study demonstrates that higher expression of *OsPSY1* and *OsZ-ISO* transcripts signifies the existence of an active carotenoid pathway which overcomes the second bottleneck of carotenoid biosynthesis in pigmented Kavuni grains (Fig. 2).

Genes encoding key carotenoid branching enzymes, such as *lycopene epsilon-cyclase* (LOC_Os01g39960) and *lycopene beta-cyclase* (LOC_Os02g09750) involved in the conversion of lycopene (C40) to alpha-carotene(C40) and beta-carotene (C40) respectively, were found to be up-regulated in Kavuni grains compared to white rice ASD16 (Fig. 1B). Molecular synergism between Lyc-b and Lyc-e regulates the lycopene flux towards the synthesis of β, β-carotenoids and β, ε-carotenoids respectively [29]. Kavuni grains show relatively high expression of *Lyc-e* transcripts compared to *Lyc-b* transcripts (Fig. 1B) which is attributed to high lutein (β, ε-carotenoid) accumulating capability of Kavuni grains, as reported by Ref. 16. High lutein accumulation and high *Lyc-e* transcript expression marks the third bottleneck for beta-carotene accumulation in grains of pigmented kavuni rice. Previous studies suggest that targeted suppression of *Lyc-e* could potentially block the β, ε-carotenoid pathway and redirect the lycopene precursors towards biosynthesis of β, β-carotenoid pathway in potato, brassica, sweet potato, and Banana [30–33]. Our study has identified *Lyc-e* as a candidate gene target for metabolic engineering of selective carotenoid profiles in Kavuni rice grains (Fig. 2).

3.3. Up-regulation of genes involved in xanthophyll biosynthesis in kavuni

Carotene hydroxylases are important regulatory enzymes that convert pro-vitamin A carotenoids into non-provitamin A xanthophylls. Carotenoids are hydroxylated into their oxygenated derivatives 'xanthophylls' by the action of two families of hydroxylases: (1) heme-type Cyt-P450 monohydoxylases; (2) non-heme-type di-iron monooxygenases. Rice carotenoid machinery comprises three homologs of Cyt-P450 monohydoxylase (CYP97A, CYP97B, CYP97C) and three homologs of BCH di-iron monooxygenase (BCH1, BCH2, BCH3). A member of the CYP97 family of carotene hydroxylases, *OsCYP97C* (LOC_Os10g39930) specifically involved in ε-ring hydroxylation of α-carotene to form lutein was significantly up-regulated in Kavuni grains (Fig. 1C). Lutein biosynthesis requires co-expression and synergistic interaction between CYP97A (β-ring hydroxylase) and CYP97C (ε-ring hydroxylase) enzymes [34]. Similarly, in our study high lutein accumulating Kavuni grains showed high transcript expression of *OsCYP97A* and *OsCYP97C* compared to ASD16. Rice carotene hydroxylase enzymes encoded by *OsBCH1* and *OsCYP97A4* catalyze β-ring hydroxylation of carotenoids to form lutein and zeaxanthin [35]. Earlier studies reported a higher expression of *OsBCH2* and *OsBCH3* homologs at seedling stages and *OsBCH1* during the panicle development stage [36]. Similarly, we observed a high accumulation of *OsBCH1* transcripts compared to *OsBCH2* and *OsBCH3* transcripts in grains of both rice genotypes (Fig. 1C). High expression of carotene hydroxylases marks the fourth bottleneck for carotenoid accumulation in grains of kavuni rice. Previous reports demonstrated that targeted suppression of one or more BCH gene homologs resulted in substantial increase in beta-carotene content as well as total carotenoid contents in Rice, Potato, Maize, and Sweet Potato [36–39]. Our study identified BCH homologs as key gene targets that may be genetically manipulated to enhance carotenoid accumulation in kavuni rice grains (Fig. 2).

Zeaxanthin epoxidase (*OsZEP1* - LOC_Os04g37619), a key enzyme involved in the epoxidation of Zeaxanthin to Antheraxanthin (C40)/ Violaxanthin (C40) was preferentially upregulated in Kavuni. No differential expression was observed for enzymes Violaxanthin de-epoxidase (*OsVDE1* - LOC_Os04g31040; *OsVDE2* - LOC_Os01g51860) and Neoxanthin synthase (*OsNXS* - LOC_Os01g03750) involved in the conversion of Violaxanthin to Zeaxanthin and Neoxanthin respectively (Fig. 1C). Natural variation in *ZEP* gene expression is a major contributor that regulates carotenoid content, stability, and composition in Arabidopsis seeds [40]. ZEP acts as an upstream checkpoint in carotenoid homeostasis that epoxidates carotenoids and provides precursors for Abscisic Acid (ABA) biosynthesis [40,41].

3.4. Differential expression of genes involved in carotenoid catabolism

Carotenoid-cleavage dioxygenases (CCDs) and 9-cis-epoxy carotenoid dioxygenases (NCEDs) are negative regulators of carotenoid metabolism that degrade/catabolize carotenoids into apo-carotenoids. CCDs are ubiquitous carotenoid cleavage proteins with broad substrate specificities ranging from phytoene to neoxanthin. CCD gene family comprises six homologous genes namely, CCD1, CCD2, CCD4, CCD7, CCD8a, and CCD8b [42]. In our study, kavuni grains showed significant upregulation of *OsCCD4a* (LOC_Os02g47510) and down-regulation of *OsCCD8a* (LOC_Os01g38580), while CCD1 and CCD4b were not expressed in both genotypes. NCED homologs (*OsNCED1*, *OsNCED3*, *OsNCED9*) showed no significant difference between the two genotypes (Fig. 1D). The carotenoid catabolism machinery plays a crucial role in regulating carotenoid turnover and it marks the fifth bottleneck for carotenoid accumulation in grains of pigmented kavuni. Previous reports demonstrated that targeted suppression of carotenoid catabolism genes, namely CCD1 and CCD4 remarkably enhanced carotenoid accumulation in rice, wheat, sweet potato, and banana [42–45]. Our study identified *OsCCD4a* as a key gene target within catabolism pathway that may be genetically manipulated to enhance beta-carotene levels in kavuni rice grains (Fig. 2).

3.5. Differential expression of genes encoding key carotenoid regulators

Carotenoid biosynthesis is a complex metabolic process regulated at multiple nodes of transcription, post-transcription, post-translation, and epigenetic controls. Transcription factors such as Phytochrome-Interacting Factors (PIFs), *LONG HYPOCOTYL5* (HY5), and AP2/ERF TFs bind and repress the upstream regulatory elements of *PSY*, and *PDS* thereby blocking the carotenoid pathway at its first committed step [46–48]. In our study, AP-2 and AP2/ERF family of transcription factors are either not expressed in both rice genotypes or notably down-regulated in purple rice genotype Kavuni. Another negative regulator, *OsbZIP48/HY5* (LOC_Os06g39960) showed 1.38-fold down-regulation in Kavuni grains (Table 2). Reports suggest that homeobox-leucine zipper protein and MADS-box TFs are co-expressed with carotenoid biosynthetic genes [13,49,50]. Kavuni grains showed more than 3-fold upregulation of a transcription factor *OsMAD26* (LOC_Os08g02070), and 1.4-fold upregulation of a homeobox associated – leucine - zipper protein (LOC_Os08g04190) that positively promotes carotenoid accumulation.

Carotenoid accumulation is regulated by several carotenoid modulators. Earlier reports identified a SET-domain-containing histone methyltransferase (SDG8) that epigenetically regulate the expression of

Table 2
Expression pattern of transcription factors with regulatory elements of genes involved in carotenoid biosynthesis pathway in rice genotypes ASD16 and Kavuni.

Family	Feature ID	Transcription factors	Crops Studied	Reference	RPKM value		Fold change (Log_2 ratio)	P value
					Kavuni	ASD16		
bZIP	LOC_Os06g39960	*OsbZIP48* (HY5)	*Arabidopsis thaliana, Solanum lycopersicum, Zea mays*	[55–57]	2.96	7.84	−1.38	0.000
Homeobox	LOC_Os08g04190	Homeobox associated -leucine-zipper protein	*Oryza sativa*	[50]	1.30	0.51	1.38	0.001
MADS	LOC_Os08g02070	*OsMAD26*	*Oryza sativa, Citrus sinensis*	[13,49, 58]	19.80	2.25	3.16	0.000
MYB-related	LOC_Os06g10350	R2R3-MYB	*Actinidia deliciosa; Oryza sativa; Citrus reticulate*	[13,59, 60]	2.45	5.40	−1.12	0.000
AP2 & AP2/ ERF	LOC_Os09g35020	AP2 & AP2/ERF	*Oryza sativa, Solanum lycopersicum, Arabidopsis thaliana*	[13,48, 61]	0.18	0.84	−2.17	0.030
	LOC_Os08g36920				1.14	2.48	−1.09	0.010
	LOC_Os05g49010				0	0.5	–	0.025
	LOC_Os03g09170				41.14	17.91	1.22	0.000
	LOC_Os06g09370				0.81	2.75	−1.74	0.000
	LOC_Os08g33150				0.38	0.07	–	0.060
	LOC_Os10g23050				0	0.43	–	0.008
	LOC_Os10g38820				1.59	0.75	1.11	0.020

CRTISO enzyme, resulting in increased carotenoid accumulation in Arabidopsis [51]. Similarly, our study identified 1.4-fold upregulation of a SET domain-containing carotenoid modulator *OsSET29 (*LOC_Os08g14660*)* in Kavuni grains. Additionally, cysteine-rich DnaJ proteins are highly conserved molecular chaperones that positively regulate carotenoid accumulation and maintains plant homeostasis under stress conditions. Previous research showed that higher expression of DnaJ transcripts positively correlated with increased carotenoid sequestration in grains of a purple rice cultivar [13]. Similarly, in our study, we observed that the transcripts of DnaJ-domain-containing proteins *Orange* (LOC_Os02g33149) and *OsDjB7* (LOC_Os05g48810) were upregulated by 1.3-fold and 1.6-fold respectively in Kavuni grains (Fig. 1E). Orange gene increases carotenoid accumulation by improving carotenoid sequestration through differentiation of additional chromoplast substructures and post-translational stabilization of PSY activity [52]. Earlier reports have demonstrated an increased carotenoid accumulation in orange gene mutants carrying a golden SNP (Arg to His) or differentially spliced OR transcripts [53,54]. Our study has identified orange gene as a valuable gene target for enhancing carotenoid accumulation without directly altering the expression of structural genes involved in the carotenoid pathway (Fig. 2).

3.6. Validation of differentially expressed genes through qRT-PCR

RNA-Seq-based transcriptome profiling was further confirmed through qRT-PCR experiments. Randomly four DEGs were selected from the carotenoid biosynthetic pathway for real-time quantification of their differential gene expression using qRT-PCR analysis. A high degree of consistency was confirmed in the expression pattern of the selected DEGs between RNA-Seq data and RT-PCR data at different developmental stages studied (Fig. 3). Temporal variations in the expression of carotenoid genes are key players behind differential carotenoid accumulation across different stages of rice grain development. In our study, a progressive increase in target genes expressions were observed across the three developmental stages (10 DAP, 15 DAP, and 20 DAP), particularly higher expression levels during the dough stage (20 DAP) of grain development in Kavuni genotype (Fig. 3). Previous research reported an increasing trend in the expression of carotenoid pathway genes at three stages (15 DAP, 25 DAP and 40 DAP) in purple rice grains. On the contrary, the expression pattern of the same genes were highly variable between different timescales in both brown rice and white rice grains [13]. Similarly, in our study, purple coloured Kavuni grains showed progressive increase in expressions of *OsBCH1*, *OsLYC-B*, *OsCCD4a* genes at 10 Days, 15 Days and 20 Days after flowering, compared to the white rice ASD16. However, *OsLYC-E* expression was slightly low at 15 days (Fig. 3). Differences in the activities of carotenoid

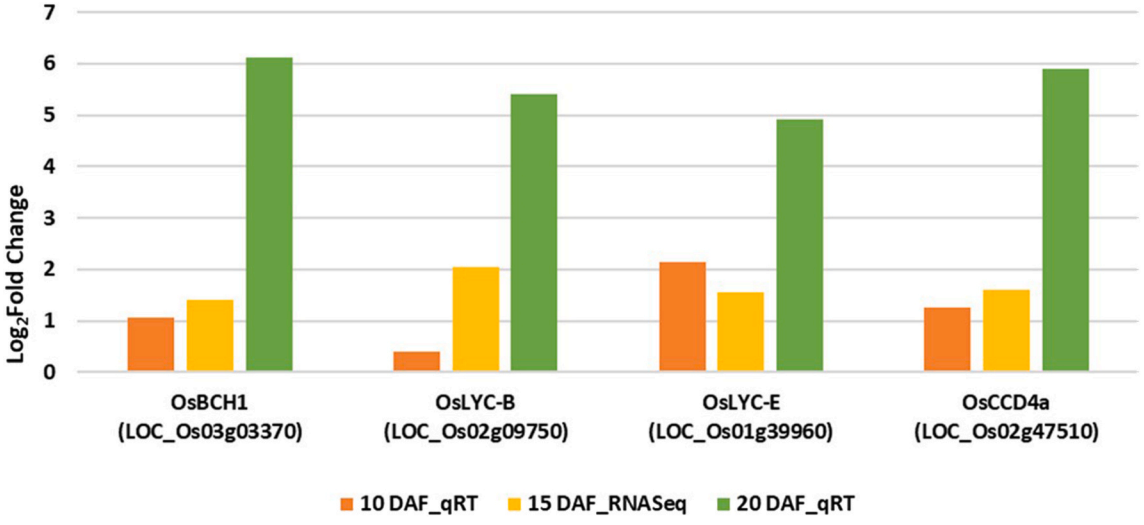

Fig. 3. qRT-PCR validation of selected genes in the carotenoid biosynthetic pathway. [10 DAF_qRT and 20 DAF_qRT indicates qRT-PCR results; 15 DAF_RNASeq indicates RNASeq results].

cyclases *OsLYC-E* and *OsLYC-B* are crucial in determining the overall carotenoid composition and the ratios of β, β-carotenoids to β, ε-carotenoids [29,62]. The high expression of *OsLYC-E* and *OsLYC-B* can be attributed to increased conversion of lycopene to form alpha-carotene/lutein and beta-carotene/zeaxanthin respectively. As a result, high OsLYC-E expression leads to increased lutein accumulation in Kavuni grains, while the highly active downstream enzymes OsBCH1 and OsCCD4a are rate-limiting for beta-carotene accumulation. Furthermore, subsequent changes in gene expression patterns during the later stages of grain development can impact the carotenoid turnover and overall accumulation of carotenoids in rice grains.

4. Conclusion

Alleviation of beta-carotene deficiency is crucial to prevent diseases such as night-blindness and age-related macular degeneration in young children and pregnant woman. Nutrient supplementation through 'nutritive pills' and 'specialty foods' is cost-intensive and unsustainable, especially for the poor in rural households who dependent on agriculture for income and livelihood. Fortifying the regular staple food with beta-carotene through genetic improvement will benefit women and children with better health and development at low cost. However, understanding the complex regulation of rice carotenoids is crucial for successful biofortification. Our study has identified several bottlenecks that can impede beta-carotene accumulation in rice endosperm, such as the notable upregulation of four key genes, namely LYC-E, BCH1/2/3, CCD4a, and OR, which play a vital role in limiting the accumulation of beta-carotene during the grain-filling stages of Kavuni rice. These genes are important regulators in the rice carotenoid pathway and could be targeted for suppression or knockout using precise genome editing techniques such as CRISPR/Cas9 to enhance beta-carotene accumulation in kavuni rice grains. Furthermore, novel haplotypes and favorable genetic variations in the key genes could be identified from the diverse panel of 3K-RGP (3000 rice genome project, IRRI) and utilized for fortification. However, our study has few limitations. Firstly, a more comprehensive biochemical profiling of the carotenoid pathway would have been advantageous. Secondly, Kavuni has an active alpha-carotenoid pathway and only minimal activity in the beta-carotenoid pathway. If we had used a line with high beta-carotene content, our results may have been more informative.

Recent advances in synthetic biology have opened promising opportunities for the rapid development of the next generation of 'Golden rice' using non-transgenic methods. Our study has unearthed novel insights into the genomic structural diversity and cellular regulatory mechanisms that could be harnessed for pro-vitamin A biofortification of rice endosperm. Furthermore, our research findings have shed light on the complex regulatory framework of the carotenoid pathway, which has the potential to revolutionize crop engineering and biotechnology.

CRediT authorship contribution statement

Rakshana Palaniswamy: Writing – original draft, Software, Investigation, Data curation. **Rohit Kambale:** Software, Data curation. **Vignesh Mohanavel:** Investigation, Data curation. **Veera Ranjani Rajagopalan:** Software, Data curation. **Sudha Manickam:** Investigation, Data curation. **Raveendran Muthurajan:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

All the authors have declared no conflict of interest.

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101815.

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