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Molecular tagging of a novel genetic locus linked to accumulation of lutein – A therapeutic carotenoid in rice grains

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Abstract

Lutein is one of the major carotenoids in eye macula and its deficiency is attributed to age-related macular degeneration (AMD) and cataracts. Developing Lutein rich staple food crop will help in supplementing its requirement among rural people through regular diet. The present study was undertaken with a view to tag genetic loci controlling lutein accumulation in rice through Bulked Segregant Analysis (BSA). Estimation of lutein content in the dehusked grains of selected 65 RILs revealed the normal distribution (1.14-285.62 µg/100gm) of lutein accumulation with a significance of p<0.041. Parental polymorphism survey using > 350 genome wide SSR markers detected 30.8% (108 SSR markers) polymorphism between Kavuni and CO 50 rice genotypes. BSA of extreme bulks containing contrasting levels of lutein along with the parents using the genome wide polymorphic SSR markers resulted in the identification of four SSR markers namely RM197 (3.0 Mb), RM204 (3.1 Mb), RM225 (3.4 Mb) and RM19442 (3.7Mb) on chromosome 6 showing clear association with the lutein content. Single-marker linear regression approach using the allelic pattern of all four markers in the region 3.0-3.7Mb showed significant association with lutein content. The regression analysis showed that the SSR markers in the region 3.0-3.7Mb linked QTL accounted for 35.9% of the genetic variation for lutein content.

Key words: Kavuni, lutein, carotenoid, BSA, QTL

Introduction

Carotenoids are natural lipophilic pigments in plants which form an important component of light harvesting complex in photosynthetic tissues. Their nutritional relevance in human diet is attributed to their antioxidant and pro-vitamin A activity (Cunningham and Gantt 1998; Fraser and Bramley 2004). Human beings and animals don't have the capacity to synthesize carotenoids and hence they obtain required carotenoids either directly or indirectly from plant sources. Carotenoids are synthesized through Geranyl Geranyl di-phosphate pathway which finally leads to formation of alpha and beta carotenes (Khurana et al. 2010; Kim and Dellapenna 2006; Cunningham et al. 1996). Lutein is one of the major alpha carotenoids among the 600 naturally occurring carotenoids found predominantly in fruits and green leafy vegetables.

Though there are about 16-20 carotenoids reported to be present in the human blood serum, only lutein and zeaxanthin are reported to be deposited in the cones of the macula, peripheral retina and rods of the human eye and contributes to proper central vision and visual sharpness (Fullmer and Shao 2001). Reduced intake of lutein and zeaxanthin is reported to be associated with the risk of developing age-related macular degeneration (ARMD), cataract and cardiovascular diseases (Bone et al. 2003; Berendschot et al. 2002; Arnold et al. 2013; Moeller et al. 2008; Hughes 2001; Handelman 2001). National Eye Institute, USA has recommended that supplementing lutein through tablets/drops was found to have significant influence on reducing ARMD (Chew et al. 2013). Purified food grade lutein is extracted from flowers of "marigold" and used as a lutein supplements globally (Hui-Hiang Koh et al. 2004). Other sources include, egg yolk, carrot, spinach, greens etc., which are not affordable for regular consumption by the poor people. In this context, identification and exploitation of lutein rich staple food crop like rice will have great impact on health issues of poor people by allowing adequate intake of lutein through regular diet.

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Rice, a staple food for majority of the Asian population contains poor level of carotenoids which warranted the adoption of genetic engineering strategy to manipulate carotenoid accumulation by developing "Golden rice" (Ye et al. 2000). Few black/purple grain rice genotypes were found to accumulate minimal level of β-carotene (Frei and Becker 2004), lutein and zeaxanthin (Gema Pereira-Caro et al. 2013). A traditional blackish purple rice variety Kavuni from Tamil Nadu, India was reported to possess detectable levels of β-carotene, significantly higher levels of lutein and several other therapeutic properties (Valarmathi et al. 2014). But no efforts have been made to unravel genetic and molecular basis of such therapeutic clues including lutein accumulation which will accelerate the development of specialty rice possessing medicinal values through MAS programs. In this study, efforts were made to study the pattern of lutein accumulation in a set of RILs derived between a high lutein containing rice genotype Kavuni and a high yielding white rice variety CO 50 and to tag genes associated with accumulation of nutritionally important carotenoid Lutein through Bulked Segregant Analysis (BSA).

Materials and methods

Genetic materials used

Genetic materials used in this study include a set of RILs (F_8 generation) derived between CO 50 (photo-insensitive, semi-dwarf, high yielding rice cultivar possessing white grains) and a traditional rice Kavuni (photo-sensitive, tall, poor yielding rice possessing blackish purple grains). From a total of about 300 RILs, 65 photo-insensitive RILs (homozygous for photo-insensitive loci) were selected, multiplied and used for estimating lutein content in their grains.

Estimation of lutein content in grains of selected RILs

The lutein content in the dehusked grains of 65 RILs and parental lines was estimated as described earlier (Howe and Tanumihardjo 2006). About 0.6 g of powdered rice grains was taken and 3 mL of ethanol containing 0.1% ascorbic acid (w/v) was added and kept at 85^{o}C for 5min. Then the extract was saponified with potassium hydroxide (120 µL, 80%, w/v) at 85^{o}C for 10 min and placed immediately on ice before adding 1.5ml of cold deionized water. Lutein was extracted twice with hexane (1.5 mL) through centrifugation at 1200g. Aliquots were pooled and dried using a roto-evaporator and redissolved in 50:50 (v/v) dichloromethane/methanol (HPLC grade; Fisher Scientific, New Jersey, USA). The solution was filtered through a 0.22 µm PTFE syringe filter (Millipore, Ireland) and collected directly into sample vials and 20ìl of the filtrate was used for HPLC analysis. The analysis was performed in a Shimadzu HPLC system with LC8A pump and DAD (190-800nm) UV detector. Separation of lutein was carried out in a Phenomenex Luna C30 (2) column (250 x 4.6mm) using a gradient elution with acetonitrile, equal volume of methanol and ethyl acetate. Flow rate was set as 1.0 ml/min with a re-equilibration of 15 min and the absorbance was read at 426 nm and used for generation of chromatograms. Lutein content was calculated using calibration curves drawn by plotting four different concentrations of lutein standards (Sigma, USA) and expressed as micrograms (µg) per 100 g fresh weight.

Molecular tagging of Lutein accumulation in rice through BSA

Extreme RILs possessing contrasting levels of lutein in the dehusked unpolished grains were selected and constituted as bulks. A set of 8 RILs possessing Lutein content of >209.36 µg/100 g were grouped as "High Lutein Bulk" and a set of 8 RILs possessing Lutein content of < 12.3 µg/100g were designated as "Low Lutein Bulk". Genomic DNA was extracted from the seedlings of individual RILs comprising both the bulks along with the parents and used for BSA.

Genomic DNA isolation

Genomic DNA was isolated from fresh leaf tissues of RILs of extreme bulks and both the parents (CO 50 and Kavuni) using modified CTAB method (Ausubel et al. 1994). The quality was assessed by resolving the DNA through agarose gel electrophoresis and quantified using Nanodrop Spectrophotometry by measuring the absorbance at 260 and 280nm. After quantification, equal amount of DNA was taken from individuals comprising both the bulks and pooled respectively (High Lutein bulk and Low Lutein bulk). A final concentration of 20 ng/µl DNA was used for the PCR analysis.

Parental polymorphism survey and BSA

Parental polymorphism survey was carried out between the two parent's *viz*., CO 50 and Kavuni using 350 Simple Sequence Repeat (SSR) markers covering all 12 chromosomes (Table 1). PCR amplification of SSR markers was carried out in a reaction volume of 15 µl with 20 ng of genomic DNA, 10 pmole of SSR primers, 1 mM total dNTPs, 1X Taq polymerase buffer and 1U

Table 1. List of SSR markers surveyed for parental polymorphism

Chromosome no.	No. of markers genotyped	No. of polymorphic markers
Chromosome 1	25	7
Chromosome 2	28	11
Chromosome 3	35	8
Chromosome 4	30	10
Chromosome 5	30	12
Chromosome 6	26	7
Chromosome 7	28	6
Chromosome 8	22	11
Chromosome 9	29	8
Chromosome 10	32	13
Chromosome 11	33	5
Chromosome 12	32	10
Total	350	108

Taq polymerase. The PCR products were resolved using 3% agarose gel electrophoresis and SSR markers exhibiting polymorphism between the parents were used for genotyping the bulks along with the parents. SSR markers co-segregating along with lutein contents were identified. Significance of association between the identified SSRs and lutein content in the F_8 RILs phenotype was tested through single-marker analysis using one way ANOVA (Liu 1997).

Results and discussion

Carotenoids are important secondary metabolites belonging to the class of isoprenoid pigments possessing both pro-vitamin A (beta-carotene, alpha-carotene etc.) and non pro-vitamin A compounds (lutein and zeaxanthin). These carotenoids are reported to be associated with preventing eye disorders, cardiovascular diseases and other age related diseases through their antioxidant property and/or as regulators of the immune system (Krinsky et al. 2003). Generally, all cereals except maize are reported to contain no or very low levels of carotenoids. Rice, one of the major cereal food crops was reported to be devoid of carotenoids and hence the strategy of genetic engineering is employed to manipulate carotenoid biosynthetic pathway which led to developing “Golden Rice” (Ye et al. 2000). Though not much information is available on the pro-vitamin A activity of lutein, research on its accumulation, genetic manipulation and bio-digestibility is gaining importance due to its potential role in maintaining proper vision and preventing age related macular degeneration.

In our earlier study, we have reported that a traditional landrace “Kavuni” was found to possess significantly higher level of dietary fibres, phenolic acids and Lutein when compared to white rice genotypes. The traditional rice variety Kavuni was reported to possess 221.6 µg/100gm of Lutein which is almost 50 fold higher than the white rice varieties (Valarmathi et al. 2014). Similar observations have been made by Frei and Becker 2004 that several black/purple rice genotypes of Philippines were found to possess about 0.38 mg/kg β-carotene, while there was no detectable level of β-carotene in white rice varieties. The same trend for the presence of Lutein and Zeaxanthin was also reported in coloured rice genotypes of Malaysia, Vietnam, Japan, Korea and Thailand (Kim et al. 2010; Gema Pereira-Caro et al. 2013). The average lutein content in the coloured rice genotypes ranged between 1.6-5.86 µg/gm, while Zeaxanthin levels varied between 0.08-0.65 µg/gm (Frei and Becker 2004; Gema Pereira-Caro et al. 2013).

In the present study, attempts were made to tag genomic region/gene(s) controlling the accumulation of therapeutically important metabolite Lutein through BSA in a segregating population derived between CO50 and Kavuni which will enable us to manipulate lutein accumulation in rice grains through MAS. About 300 RILs developed between CO 50 and Kavuni were forwarded upto F_8 generation and selection was made based on agronomic traits *viz*., plant height, no. of tillers, photoperiod sensitivity, grain colour and yield. A total of 65 progenies (semi-dwarf, high yielding and photo-insensitive lines) were selected and used for identifying molecular markers linked to Lutein content through Bulked Segregant Analysis.

Lutein contents in the grains of RILs

Lutein content in grains of 65 RILs showed continuous variation and most of the lines were found to contain intermediate levels of Lutein when compared to the parents. Results of Shapiro-Wilk normality test showed a probability of normal frequency distribution with a significance of $p<0.041$. Lutein content among the 65 RILs ranged between 1.14-285.62 µg/100gm (Fig. 1). Seven individual RILs were found to possess low level of Lutein (lesser than CO 50), whereas 10 individual RILs were found to possess high level of Lutein (higher

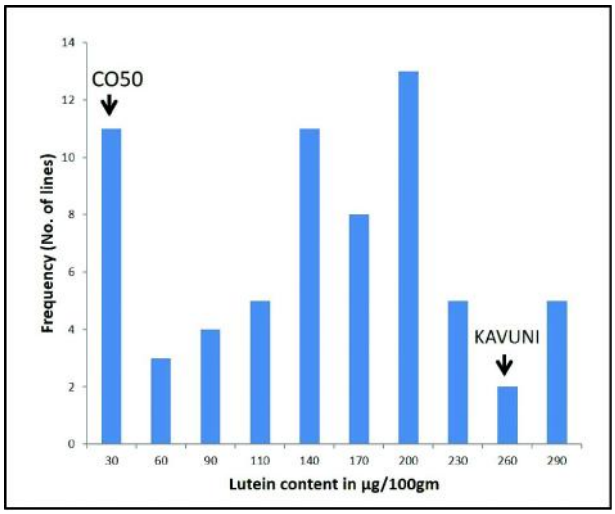

Fig. 1. Frequency distribution of Lutein content in 65 RILS derived between CO50 x Kavuni (p<0.041)

than Kavuni). About 48 RILs were found to be with intermediate levels of lutein between the parents (12.3-205µg/100gm).

Molecular tagging of Lutein accumulation in rice through BSA

The availability of whole genome sequence information in rice accelerated the discovery and use of molecular markers for mapping Quantitative Trait Loci and thus enabled genetic manipulation of complex traits through MAS (Cho et al. 1999; Rae et al. 1999; Flandez-Galvez et al. 2003). With a view to identify polymorphic SSR markers between the parent's CO 50 and Kavuni, a total of 350 SSR markers covering all 12 rice chromosomes were used (Table 1). Out of 350 SSR markers screened, 108 markers were found to be polymorphic between the parents which accounts for 30.8 % of polymorphism between Kavuni and CO 50 (Table 1).

Genetic mapping of complex traits through classical linkage mapping involves the development, genotyping and phenotyping of a mapping population which is laborious and time consuming process. Alternatively, BSA has been developed as an efficient alternate strategy for identifying markers linked to major genes controlling the target trait (Brauer et al. 2006). A simple BSA strategy was developed for the rapid identification of molecular markers linked to major gene(s) controlling target traits (Michelmore et al. 1991). This method was further fine tuned for tagging QTLs of large effects by pooling DNA samples (bulks) of contrasting phenotypes (Wang and Paterson 1994).

In the recent past, BSA has been successfully used in rice and other cereals for identifying markers linked to QTLs associated with several traits *viz*., drought tolerance (Venuprasad et al. 2009), grain yield (Vikram et al. 2011; Ghimire et al. 2012), quality parameters and disease resistance (Yang et al. 2009; Lima et al. 2007; Nguyen thi Lang and Buu 2005; Govindaraj et al. 2005) and heat tolerance (Zhang et al. 2009). In the present study, BSA was performed in the extreme bulks of RILs (possessing contrasting levels of Lutein) by pooling the DNA isolated from the contrasting RILs. Genomic DNA isolated from 8 individual RILs possessing very low level of Lutein (Low Bulk; < 12.3 µg/100g) was bulked as "Low Lutein bulk" and 8 individual RILs possessing very high level of Lutein (High Bulk; >209.36 µg/100g) was bulked as "High Lutein Bulk". The DNA bulks were genotyped along with the parents using the 108 SSR markers identified to be polymorphic between the parents. Out of 108 polymorphic SSRs, four markers namely RM197 (3.0 Mb), RM204 (3.1 Mb), RM225 (3.4 Mb) and RM19442 (3.7Mb) on chromosome 6 were found to discriminate the bulks of extreme phenotypes similar to the parents in the BSA (Fig. 2). To further validate the association

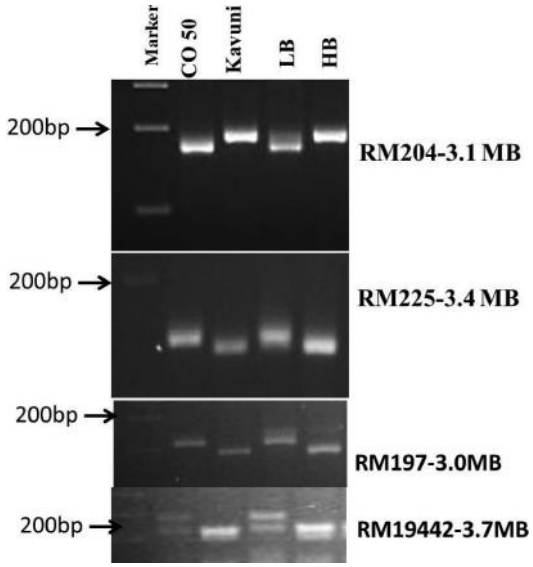

Fig. 2. Agarose gel electrophoretic pattern of three different SSR markers on chromosome 6 showing co-segregation along with Lutein content through Bulked Segregant Analysis. LB-Low lutein Bulk, HB-High lutein Bulk

of these four SSR markers with the lutein content, we have carried out selective genotyping of individual RILs comprising the extreme bulks. Results of genotyping revealed that all the 8 RILs comprising the High Lutein

Bulk possessed the "Kavuni" allele for all the four markers (RM197, RM204, RM225 and RM19442) and all the 8 RILs comprising the Low Lutein Bulk except the line L6 possessed CO 50 allele. Only the low Lutein line # L6 was found to contain "Kavuni" allele for all the four markers. The segregation pattern of all the four SSR markers residing between 3.0-3.7 Mb region on chromosome 6 showed tight genetic linkage with lutein content. However genotyping of bulks using SSR markers *viz*., RM588 (1.6 Mb) and RM402 (6.4Mb) on chromosome 6 showed recombination and it was not found to co-segregate with the phenotype.

Marker-trait association analysis

All the 65 RILs were genotyped by using the above SSRs to establish marker-trait association through one-way ANOVA. The SSR markers *viz*., RM204, RM225, RM197 and RM19442 located between 3.0-3.7 Mb on chromosome 6 showed similar allelic pattern with a recombinant frequency of 6.15%. Single marker linear regression analysis revealed significant association of P<0.041 indicating a linkage between the four SSR markers and Lutein content (Table 2).

Table 2. Regression analysis of lutein content with the markers mapped at chromosome 6

Marker and position	Recombination on frequency	Source	df	SS	MS	F value	P value	R^2 value
RM588-1.6MB	26.15%	Regression	1	2.70043	2.70043	13.08058	p<0.431	0.167
		Residual	65	13.41897	0.206446			
		Total	66	16.1194				
RM197-3.0MBR	6.15%	Regression	1	5.542538	5.542538	36.53637	p<0.041	0.3598
M204-3.1MB								
RM225-3.4MB		Residual	65	9.860447	0.151699			
RM19442-3.7MB		Total	66	15.40299				
RM402-6.4MB	23.07%	Regression	1	2.460927	2.460927	11.71143	p<0.371	0.152
		Residual	65	13.65848	0.21013			
		Total	66	16.1194				

The R^2 value of 0.359 suggested that the QTL (3.0 - 3.7Mb) on chromosome 6 may contribute upto 35.9% of genetic variation for lutein content. Genotyping of all the 65 RILs using the SSR marker RM588 at 1.6Mb and RM402 at 6.4Mb revealed a recombination frequency of 26.15% and 23.07% respectively.

The abundant distribution of carotenoids from archea to higher plants/animals indicates the diverse role(s) of these important compounds. Pathways involved in carotenogenesis have been established through mutation/genetic engineering in model organisms *viz*., Arabidopsis, cyanobacteria, tomato, pepper, maize etc., (Hirschberg 2001; Della Penna and Pogson 2006). In rice, only few Cytochrome P450 genes involved in carotenoid biosynthesis have been validated for their function (Ming-Zhu Lv et al. 2012; Wei et al. 2010; Wurtzel et al. 2001). Even though molecular markers associated with genes/QTL controlling several traits of economic importance have been identified in rice, not much initiative(s) have been taken towards tagging genes/QTLs controlling nutritionally/therapeutically important compounds in rice. This study represents the first of its kind in rice in which efforts were made to exploit the natural genetic variation for mapping QTLs/genes linked to accumulation of an important carotenoid 'Lutein". Our results clearly indicated that RM204, RM197, RM225 and RM19442 (3.0-3.7 Mb on Chromosome 6) were found to be significantly linked to Lutein content in rice grains. Intensive efforts are needed through QTL-Seq and gene expression profiling for identification of putative candidate genes controlling Lutein accumulation in rice. Overall, results of this study demonstrate the potential of a traditional rice genotype "Kavuni" as a source for supplementing therapeutically important carotenoid "Lutein" to combat an emerging eye disorder(s) like age related macular degeneration, cataract and other chronic diseases.

Authors' contribution

Conceptualization of research (MR); Designing of the experiments (RV, MR); Contribution of experimental materials (RV); Execution of field/lab experiments and

data collection (MR, RV); Analysis of data and interpretation (RV); Preparation of manuscript (MR).

Declaration

The authors declare no conflict of interest.

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