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RESEARCH ARTICLE

Identification and validation of genetic locus linked to flavonoid and anthocyanin content in rice using Bulk Segregant Analysis

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Abstract

A study was undertaken to identify the genetic locus linked with the therapeutic traits, *viz*., flavonoid and anthocyanin contents in rice grains by Bulk Segregant Analysis using the F2 population derived from the cross, ADT(R)48/*Kavuni*. The donor for high anthocyanin and flavonoid content was *Kavuni*, one of the popular landraces of Tamil Nadu, which exists in various forms with respect to grain pigmentation. Through this study, three linked SSR markers, namely, RM228 (Chromosome 10) for flavonoid and anthocyanin content, RM5348 (Chromosome 10) for flavonoid content, and RM297 (Chromosome 1) for anthocyanin content, were identified. Among these markers, RM5348 was identified as a novel marker, lies in the genomic region of the gene encoding the enzyme flavonoid 3'-monooxygenase indicating more reliability. The association of these identified markers was further validated through Single Marker Analysis (SMA), and the results confirmed the strong association of these markers with the respective traits in various forms of *Kavuni*, other pigmented landraces, and improved cultivars. However, the validation must also be done in a population with another genetic background to prove the stern association of these markers with the traits.

Keywords: Rice, *Kavuni*, flavonoid, anthocyanin, Bulk Segregant Analysis, linked marker, association

Introduction

Biochemical profiling of traditional landraces, particularly pigmented ones, had shown the existence of higher levels of bioactive and antioxidant compounds such as phenols, flavonoids, anthocyanin, saponins, carotenoids, saturated fatty acids besides proteins and vitamins (Verma and Srivastav 2017). One such traditional landrace of Tamil Nadu known for its anti-diabetic properties is *Kavuni* which is reported to possess enormous curative properties (Valarmathi et al. 2015)due to its high flavonoid and anthocyanin content (Raveendran et al. 2020) and different types of *Kavuni* have been reported which vary in their caryopsis pigmentation pattern. However, most of the landraces are photosensitive and poor yielders. So, due to the introduction of high-yielding modern rice varieties, many of these landraces went out of cultivation, leading to genetic erosion. But owing to the increased awareness among consumers about the health benefits of traditional landraces, they are gaining more importance recently. Hence, to combat present-day health conflicts and satisfy consumer demand and farmers' needs, improving the therapeutic properties, plant type, photo insensitiveness, and yield in our staple food will be an ideal solution. In this regard, *Kavuni* remains an excellent donor for plant breeders looking to create novel rice cultivars with high therapeutic value thereby reducing the cost of natural antioxidants so that it can reach a common person. To hasten the process, unraveling the genetic and molecular basis of such therapeutic traits will be very useful for exploration through marker-assisted selection

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programmes. But the research programme on therapeutic traits in rice is still at a primitive stage. Therefore, the present study was undertaken to identify the genetic locus linked to flavonoid and anthocyanin using Bulk Segregant Analysis in F2 population derived using therapeutic traditional landrace *Kavuni* and an improved cultivar ADT(R)48. The identified markers were further screened in various forms of *Kavuni*, germplasm lines, and improved cultivars to validate the association of markers with flavonoid content and anthocyanin content in rice.

Materials and methods

Materials used

The F_2 population derived using ADT(R)48 (an early maturing, semi-dwarf, erect plant type with long slender white grains variety) and *Kavuni*(tall plant type with medium bold blackish brown grains) was raised along with the parents during rabi, 2020 at Department of Rice, Centre for Plant Breeding and Genetics, TNAU, Coimbatore. A total of 121 F_2 segregants were randomly tagged from the entire population and were subjected to analysis of two therapeutic traits *viz*., flavonoids and anthocyanins in brown rice. Powderedbrown rice samples of twenty-four germplasm lines, various forms of *Kavuni* and improved cultivars were also employed for estimation of flavonoid and anthocyanin contents (Table 1). Total flavonoid content (TFC) was analysed using the protocol advocated by Woisky and Salatino (1998) with slight modifications. The pH differential method recommended by Fuleki and Francis (1968) was adopted for the assay of total anthocyanin content (TAC).

Identification of genetic loci linked with flavonoid and anthocyanin content through BSA

Bulk Segregant Analysis (BSA) was performed for flavonoids and anthocyanin content using the F_2 segregants of ADT(R)48/*Kavuni* for the identification of markers linked with the trait. Based on phenotypic data, eight individuals exhibiting total flavonoid content < 65mgQE/100g and eight individuals with flavonoid content > 200mgQE/100g were used for the constitution of low and high flavonoid bulks. Similarly, segregants with an anthocyanin content of> 0.6mgCGE/100g and < 0.14mgCGE/100gmade up the high and low anthocyanin bulks. The genomic DNA of the parents and the segregants were isolated for constituting the bulks and to perform BSA.

Genomic DNA isolation

Leaf samples of 10-15 days old seedlings of F_2 segregants, parents, germplasm lines, and improved cultivars were subjected to DNA isolation by adopting the method suggested by Doyle (1991). After quantification, an equal amount of DNA was taken from individuals comprising both the bulks (high and low) for flavonoid and anthocyanin content and pooled, respectively. A final equimolar concentration of 60 ng DNA was used for PCR analysis. Parental polymorphism was surveyed using 370 SSR primers spanning all twelve chromosomes. The markers that exhibited polymorphism between the parents ADT (R) 48 and *Kavuni* were utilised for performing BSA (Table 2). The high and low DNA bulks for flavonoid and anthocyanin as well as parental DNA were tested using polymorphic markers. The resulting banding pattern was used to identify the co-segregating polymorphic markers. The co-segregating SSR markers identified were then utilized for selective genotyping of individual lines constituting the bulk. The co-segregating SSR markers identified were used for genotyping the entire 121 F_2 segregants. SMA was performed using single factor ANOVA and regression to validate the marker-trait association.

Validation in various forms of Kavuni, germplasm lines, and improved cultivars

The markers linked to flavonoid and anthocyanin content in rice were used for genotyping 24 germplasm lines with pigmented rice, including various forms of *Kavuni* and white riced improved cultivars (Table 1). SMA was performed using single factor ANOVA and regression to validate the association between the marker and the trait.

Table 1. Germplasm used for the validation of markers

Type	Identity of the germplasm
Various forms of *Kavuni*	Karuppu *Kavuni*, Sivappu *Kavuni*, Burma *Kavuni*, Thailand *Kavuni*, Improved *Kavuni*, *Kavuni* ADT and *Kavuni* CBE
Landraces	Chitthan Samba, Muthuvellai, Ponni (130 days), T256 Puthupatty Samba, Illupam Poo Samba, Mappillai Samba, Kala Namak, Purpleputtu, Burmablack, Mutrina Kannam, T1509 Valasamudon, Kitchalli Samba, and Seeraga Samba
Improved cultures and cultivars	CB19107, CB20131, CO50 and ADT(R)48

Results and discussion

The phenolic compounds such as flavonoids and anthocyanins present in pigmented and traditional rice varieties confer immunity against several lifestyle-related diseases due to their antioxidant properties. They have immense health benefits and therapeutic values (Mbanjo et al.2020). One such pigmented rice is *Kavuni*, a traditional variety of Tamil Nadu with extensive health benefits due to its abundant phenolic compounds (Kowsalya et al., 2022, Valarmathi et al., 2015). So, to unravel the genetic locus governing health benefits in *Kavuni*, the F_2 generation of ADT(R)48/*Kavuni* was used. Tagging of genomic regions associated with flavonoid and anthocyanin content will enable us to manipulate its accumulation in rice grains

through marker-assisted selection. *Kavuni* possessed TFC and TAC of 301.75mgQE/100g and 0.668mgCGE/100g, respectively, which was nearly 5-fold times higher when compared with improved cultivar ADT(R)48. This is in accordance with the results of Shen et al. (2009) and Zhang et al. (2015) for flavonoid content in white and black rice. Asem et al. (2015) stated parallel findings for anthocyanin content. The segregating individuals exhibited flavonoid content in a range of 57.14mgQE/100g to 231.13mgQE/100g with a mean value of 137.71mgQE/100g and recorded an average anthocyanin content of 0.34mgCGE/100g ranging between 0.1mgCGE/100g- 0.65mgCGE/100g (Fig. 1). A platykurtic nature of frequency distribution was observed for both the traits (Fig.2) which indicated the quantitative inheritance of these two traits. None of the segregants exhibited a higher range of flavonoids and anthocyanins than the donor parent *Kavuni* whereas 13 segregants were found to possess lower flavonoid content [lesser than ADT(R)48] and 20 segregants for anthocyanin content.

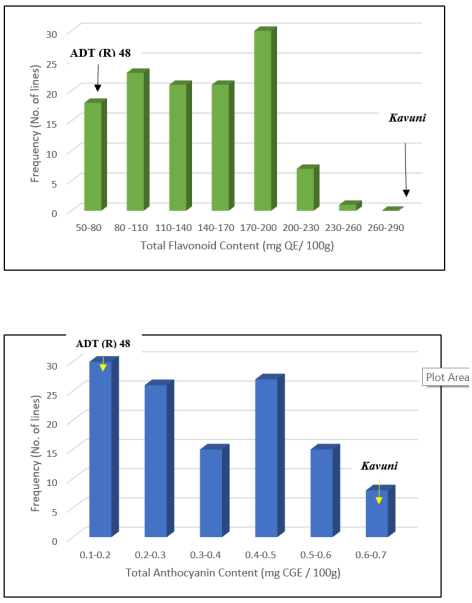

Fig. 1. Range of flavonoid and anthocyanin contents in grains of F2 segregants along with parents

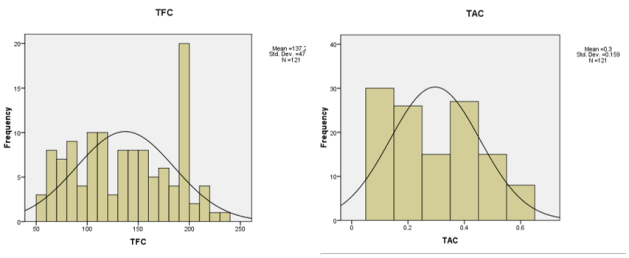

Fig. 2. Frequency distribution for flavonoid and anthocyanin content in F2 population of ADT (R) 48 / *Kavuni*

BSA is a rapid innovative method to determine the relationship between a marker and a trait in a segregating population which is obtained by crossing genetically contrasting parents for trait of interest. The polymorphic percentage found in genomic regions determines the significance of BSA (Michelmore et al. 1991). A total of 371 SSR markers encompassing all the twelve chromosomes were employed for the parental polymorphism survey, revealing a polymorphic percentage of 20.22 with 75 SSR markers. Polymorphic percentage exhibited a range of 4.76% in chromosome 12 to 27.27% in chromosome 7 (Table 2). A similar trend was reported between the two rice varieties Kasturi and Chaw Khao varying for their drought tolerance by Venkateshwarlu and Kole (2019). BSA performed for flavonoid content using high and low bulks for flavonoid content resulted in co-segregation of markers RM228 and RM5348 on chromosome 10 between the parents and the high and low flavonoid bulks. In contrast, between the parents and the anthocyanin bulks constituted using segregants with extreme values for anthocyanin content, the co-segregation of markers RM297 on chromosome 1 and RM228 on chromosome 10 were observed. Co-segregation of markers RM228 and RM5348 for flavonoid content and RM297 and RM228 for anthocyanin content indicated that these markers are putatively linked with the trait.

SMA revealed a significant association of $p < 0.05$, indicating a strong linkage between identified markers and therapeutic traits. The R^2 value of 0.8431 suggested that the marker RM228 on chromosome 10 may contribute up to 84.31 % of genetic variation for flavonoid content. The

Table 2. Chromosome-wise polymorphism percentage between the parents ADT(R)48 and *Kavuni*

Chromosome	Total number of primers screened between parents	Number of polymorphic primers identified	Polymorphism percentage
1	44	8	18.18
2	35	6	17.14
3	25	6	24.00
4	23	4	17.39
5	35	8	22.86
6	41	9	21.95
7	22	6	27.27
8	39	8	20.51
9	24	3	12.50
10	25	6	24.00
11	37	10	27.03
12	21	1	4.76
Total	371	75	20.22

Table 3a. SMA for flavonoid content

Markers	Source	df	SS	MS	F value	P value	R2 value
RM228	Regression	2	231357	115678.5	323.35	P<2.2e-16***	0.8431
	Residual	118	42214	358			
	Total	121	273531				
RM5348	Regression	2	44313	22156.6	11.494	P<2.97e-05***	0.1478
	Residual	118	229258	1942.9			
	Total	121	273571				

Table 3b. SMA for anthocyanin content

Markers	Source	df	SS	MS	F value	P value	R2 value
RM228	Regression	2	0.46026	0.230132	10.792	P<4.963-05***	0.1403
	Residual	118	2.51627	0.021324			
	Total	121	2.97653				
RM297	Regression	2	0.32252	0.161258	7.1697	P<0.001152**	0.0932
	Residual	118	2.65402	0.022492			
	Total	121	2.97654				

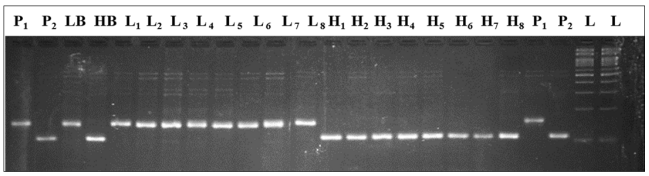

Fig. 3a. BSA of high and low flavonoid bulks along with parents using SSR marker RM228

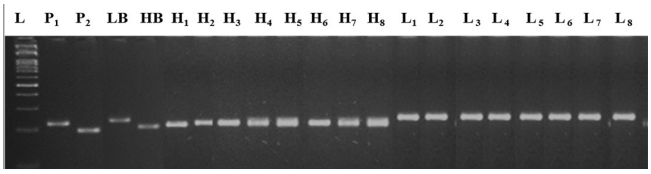

Fig. 3b. BSA of high and low flavonoid bulks along with parents using SSR marker RM5348

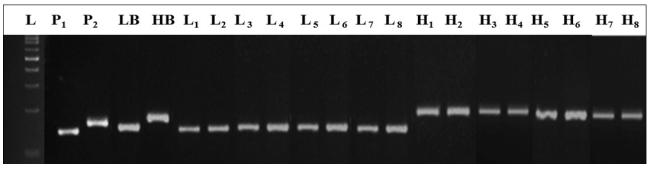

Fig. 3c. BSA of high and low anthocyanin bulks along with parents using SSR marker RM297

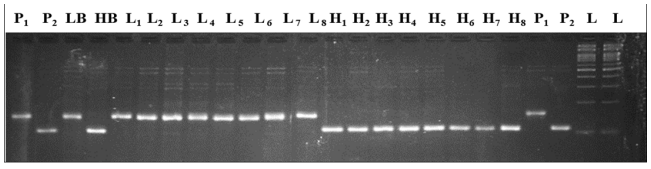

Fig. 3d. BSA of high and low anthocyanin bulks along with parents using SSR marker RM228

L – Ladder, P1 - ADT (R) 48, P2 – *Kavuni*, LB - Low Bulk, HB - High Bulk

Fig. 3, SSR markers showing co-segregation through BSA

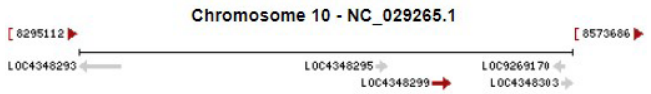

Fig. 4. Physical location of the gene near to RM5348 (retrieved from NCBI)

Table 4. SMA for flavonoid and anthocyanin content in various forms of *Kavuni*, germplasm lines, and improved cultivars - validation

Markers	Physical Position	Chromosome	p-value (F)	R^2
RM228	22,243,157-22,243,349bp	10	0.0496	0.1640
RM5348	8,485,607-8,485,799bp	10	0.0300	0.2825
RM297	32,099,56-32,099,760bp	1	0.0176	0.0851

Plate 1: Variation observed for grain colour in brown rice of F2 segregants

R^2 value of 0.140 and 0.093 for markers RM228 and RM297 on chromosomes 10 and 1 may contribute up to 14 % and 9.3 % of the variation for anthocyanin content (Tables 3a and 3b). The present finding was in accordance with the earlier report of a significant association of SSR marker RM228 on chromosome 10 and RM297 on chromosome 1 for therapeutic traits by Shao et al. (2011). To validate the significant association of markers with the therapeutic traits, RM228 and RM5348 were further genotyped in individual lines of flavonoid bulks in which eight lines of high bulk showed banding patterns similar to *Kavuni* and the banding pattern of eight low bulk lines were similar to that of ADT(R)48. Similar results were observed for the markers RM297 and RM228 for anthocyanin content (Fig. 3). The co-segregated markers were evaluated in the entire F_2 population comprising of 121 segregants and SMA was performed to determine the linkage of markers RM228 and RM5348 with flavonoid content and RM297 and RM228 with anthocyanin content. These results were in accordance with Shao et al. (2011) indicating the significant association of SSR marker RM228 on chromosome 10 with flavonoids and pericarp colour and RM297 for pericarp colour i.e., anthocyanin content (Plate 1).

RM5348 which is located in chromosome 10 at a physical position of 8,485,607-8,485,799 bp lies in the genomic region of the gene encoding the enzyme flavonoid 3'-monooxygenase (LOC4348299:Chr10:8,493,253-8,505,253) (Fig.4) which is present in the pathway of flavonoid biosynthesis. Hence, the identified novel marker may be considered reliable, and its significant association with the trait is well identified through SMA.

Validation of the identified markers *viz*., RM228, RM5348, and RM297 in various forms of *Kavuni*, germplasm lines, and improved cultivars for flavonoid and anthocyanin content revealed the significant association of marker RM228 and RM5348 on chromosome 10 with flavonoid content explaining the variance of 16.4 and 28.25% and RM297 on chromosome 1 with anthocyanin content explaining a variation of about 8.51%, respectively. A similar range of variation was reported in an F_2 population of rice by

Mebeaselassie et al. (2018) for false smut resistance. SMA revealed a significant association of $p < 0.05$, indicating a strong linkage between identified markers and therapeutic traits in the evaluated germplasm lines (Table 4).

In the present study, the genomic regions identified to be linked with flavonoid and anthocyanin content should be explored further or fine mapped for identifying candidate genes or putative QTLs governing the pathway underlying the therapeutic traits for utilization in marker assisted selection programmes. In addition, the new marker RM5348 identified to be associated with flavonoid content explaining 14.78% of phenotypic variation in segregating population of ADT(R)48/*Kavuni* needs to be validated in subsequent generations of ADT(R)48/*Kavuni* and in diverse genetic backgrounds to obtain more clear-cut results for its association with flavonoid content. The results of this study also strongly indicated that pigmented rice, like *Kavuni* is a valuable patron for combating lifestyle-related diseases, which is a serious problem of global concern. It is also suggested that extensive studies on the possibilities of the potential use of the SSR marker, RM5348 identified and validated through this study lies in the genomic region of the gene encoding the enzyme flavonoid 3'-monooxygenase could further be explored for improving the flavonoid content in high yielding rice varieties.

Authors' contribution

Conceptualization of research (SG); Designing of the experiments (SG, KA, MAF); Contribution of experimental materials (SG, KA, LAL); Execution of field/ lab experiments and data collection (MAF, MH, GA); Analysis of data and interpretation (SG, KA, MAF, MH, GA, ND); Preparing of the manuscript (SG, KA, MAF, MH).

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