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3-S Lines and molecular tagging of the gene for purple apiculus in rice (*Oryza sativa* L.)

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Abstract

Anthocyanin pigments leads to production of purple apiculus in rice (*Oryza sativa* L.). Proper understanding of anthocyanin pathway may lead to the development of a powerful tool in rice genetics and molecular biology. SSR marker-assisted molecular backcrossing approach was utilized to develop single segment substitution lines (3-S Lines) of Hua Jing Xian 74 with substitution segment from Lian Jian 33. The gene for purple apiculus was mapped on distal end of chromosome 6 and was found to be closely linked with RM 253 (0.2cM), PSM 349 (1.5 cM) and PSM 425 (3.8 cM), respectively. The 3-S Lines which contained only one substitution segment of chromosome from Lian Jian 33 on the genome of Hua Jing Xian 74 appeared as a viable alternative population for mapping of the gene (s) in rice.

Key words: Rice, gene mapping, purple apiculus, flavonoids, SSR, 3-S lines

Introduction

Anthocyanin pigments, displaying colors varying from bright red, purple to blue, belongs to the general class of plant pigments called flavonoids, a secondary metabolite. Flavonoids are attributed with many important functions like insect attractant for pollination, protection from UV light, defense against pathogens and insect attack, male sterility and gamatogenesis, response to wounds, etc. [1]. Anthocyanin gene pigment system consists mainly of three basic genes: the C (chromogen), the A (activator), and the P (distributor). The purple or red pigment appears in a variety of organs of the rice (*Oryza sativa* L.) plant except roots and anthers [2].

The anthocyanins are synthesized and regulated by structural and regulator genes, respectively. A powerful tool in rice genetics and molecular biology can be developed if the biosynthetic pathway and its genes are characterized in detail and put in a proper perspective [1, 2]. For such purposes, mapping of the gene with closely linked molecular marker is essential. In rice, using monosomic alien addition lines (MAAL), Jena and Khush [3] identified the gene for purple stigma (Ps) as monogenic dominant and located it on chromosome 3 of *O. officinalis*. Similarly, the gene for purple apiculus (Pa) was also defined to be monogenic dominant gene [3]. Mikarni *et al.* [4] showed that the C (chromogen for anthocyanin) locus is located on the short arm of chromosome 6 and is linked to the *Wx* locus.

For effective mapping, a number of mapping population *viz.*, F_2, RIL, DH, etc. are used. Each of such population arguably has its inherent strength and weaknesses. Here, we used an alternative mapping population called Single Segment Substitution Lines (SSSL) or 3-S Lines, in short. Such lines were developed through Simple Sequence Repeats (SSR) marker-assisted backcrossing approach [5]. The unique feature of the 3-S Line is that unlike Introgression Lines (ILs) [6], it contains only one substitution segment of chromosome from the donor (here, Lian Jian 33) on the genetic background of the recipient (here, Hua Jing Xian 74). So, any differences between the 3-S Lines and the recipient parent can be attributed to the gene(s) located on the substituted segment.

Materials and methods

Plant materials: Hua-Jing-Xian 74, a popular rice variety of Southern China, was crossed and backcrossed with Lian Jian 33 - a black rice variety. The population was advanced to BC_3F_2. In each generation, whole genome of the plants was surveyed with 258 polymorphic SSR markers and plants with fewer numbers of substitution segments were selected. In BC_3F_2, some plants were found to contain only one substitution segment of chromosome from the donor. Such plants were selfed to produce lines with homozygous substitution segment. Since such plants contained only one segment of chromosome from the donor on the genetic background of the recipient, hence these were called Single Segment

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Substitution Lines (SSSLs) or 3-S Lines, in short. One such line (W14-13-9-19) contained in heterozygous condition (BC_3F_1) a segment of chromosome from Lian Jian 33 on chromosome 6. SSR Marker RM253 defined the substitution segment. Seeds of the plant were multiplied (BC_3F_2) to develop the mapping population segregating for the locus of interest. The population was raised in the experimental farm of South China Agricultural University, Guangzhou, China. Single seedling was planted for a population of 152 plants and standard package was practiced for management of the crop.

Molecular genotyping: For molecular genotyping, leaf samples were collected from every single plant separately and DNA was extracted following the Mini Scale DNA extraction procedure [7]. Whole genome survey was conducted with 258 SSR markers polymorphic to the parents and located the substituted segment of chromosome in recurrent genome. PCR was carried out as per Panaud *et al.* [8] with little modifications in 20µl reaction volume in PTC 100 Programmable Thermal cycler (MJ Research Inc.). For PCR, the template DNA was subjected to denaturation at 94°C for 5 min, followed by 35 cycles of PCR amplification (denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min.), and finally incubation at 72°C for 5 minutes for extension completion. PCR products were run on 6% denatured polyacrylamide gel using vertical electrophoresis tank. After electrophoresis, the gels were subjected to silver staining and bands were scored accordingly. Gel pictures were taken with Gel-1000 photo system (Bio-Rad).

Designing of SSR primers: The selected substitution line contained one chromosomal segment introgressed from the donor at the marker locus RM253 on chromosome 6. Nearest markers on either side of RM253 on Cornell map [9] were monomorphic to the parents. Hence, Position Specific Microsatellite (PSM) primers were designed with Primer Premiers 5.0 from the published rice genome sequences downloaded from Monsanto (http://www.rice-research.org) and International Rice Genome Sequencing Project (IRGSP) (http://rgp.dna.affrc.gov.jp/IRGSP/) and used for mapping the gene. The sequences of the primer pairs are given in Table 1.

Map construction: Co-segregation of microsatellite markers for purple apiculus trait was observed on 152 segregating plants (F_2). Apiculus phenotypes (purple or normal brown) and markers genotypes on every plant were recorded and used to determine linkage between the markers and the gene for purple apiculus. The linkage map of the gene and SSR markers was constructed using MAPMAKER version 3.0 [10].

Table 1. List of newly designed primers along with original clone ID and polymorphic status

Primer	Primer sequence[a](5'-3')	Clone	Polymorphism[b]
PSM 349	F-GATCTGGTGGTTTGTTTTACC	AP003458	P
	R-AAGGAGTCGTCGTTTGGTTTA		
PSM 350	F-ACTTGAAACGAGGGAGGATAG	AP003525	M
	R-GTTTCGTTGACTTGTTTTACCC		
PSM 424	F-GTTTCGTTGACTTGTTTTACCC	AP004741	M
	R-CCGACTTGAAACGAGGGA		
PSM 425	F-AGGTCCTCCATCGAGCAGTAT	AP003488	P
	R-CGTTGACGCCTTGTGCCTA		

[a]F: Forward sequence; R: reverse sequence, [b]P: polymorphic; M: Monomorphic

Results and discussion

Mapping of genes with closely linked marker facilitates cloning which in turn helps understanding product and function of a gene. Well-characterized anthocyanin pathway in maize, barley, *Petunia* and *Antirrhinum* has been extensively utilized for gene isolation, expression, regulation, etc. Similar utilization can be achieved in rice too, provided the pathways as well as its intermediates are properly understood. In the current investigation, attempt was made to map the gene responsible for anthocyanin pigmentation on the apiculus of rice grain for identifying linked markers.

It was observed that the plants of the mapping population segregated for brown (normal) and purple apiculus. The ratio of purple apiculus to brown (normal) apiculus was 109:43 (χ^2 = 0.71) (Table 2), which showed a good fit to 3:1 ratio indicating the gene for purple apiculus to be monogenic dominant. The result was in good agreement with Jena and Khush [3]. The phenotypic observations were validated with the molecular genotype of the segregating plants. For this purpose, the molecular genotypes of the plants were determined based on the banding pattern of the PCR products on the polyacrylamide gel. It was observed that the genotypes of the polymorphic markers *viz.*, RM 253, PSM 349 and PSM 425 exhibited a true Mendelian inheritance (1:2:1) (Table 3). It also showed absence of any segregation distortion conforming suitability of the population for gene mapping purpose. The banding patterns of the marker RM 253 have been depicted in Fig. 1.

The molecular genotype and the apiculus phenotypes of the plants were subjected for the establishment of the linkage relationship. The marker RM 253, PSM 349 and PSM 425 was found to co-segregate with the gene for purple apiculus with occasional recombinations. Two of the newly designed primer pairs (PSM 424 and PSM 350) failed to produce

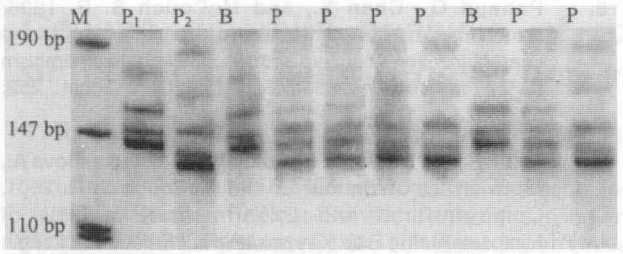

Fig. 1. Banding pattern of RM 253 in rice plants segregating for purple apiculus. M: marker ladder (pUC19/Msp I); P_1: *HJX* 74; P_2: *Lian jian* 33; P: Purple colour; B: Brown colour

Table 2. Chi-square test of plants segregating for purple apiculus

Phenqtypic class	Observed value	Expected value	χ^3 (3:1) value
Purple apiculus	109	114	0.71
Brown (normal) apiculus	43	38	
Total	152	152	

any polymorphism between the parents and hence could not be used for mapping purpose (Table 1). The linkage map constructed with the MAPMAKER showed that the gene for purple apiculus (named as *Pa*-6) was closely linked with SSR marker RM 253 (0.2cM) on the short arm of chromosome 6. The newly designed markers PSM 349 and PSM 425 clustered in one side of the gene and were located at a distance of 1.3 and 3.6cM, respectively. The map constructed with MAPMAKER is depicted in Fig. 2. It was observed that the order of the markers on the linkage map of *Pa*-6 constructed here was in good agreement with the original map [9] except variation in map distance between markers. Such variations might be attributed to the use of smaller size of the mapping population; utilization of a large size segregating population is likely to overcome this discrepancy.

Mikami *et al.* [4] has mapped a candidate C (chromogen for anthocyanin) gene to a clone located between RZ 588 (18.6 cM) and G 200 (34.3cM) on chromosome 6. In present study, *Pa*-6 (the gene producing purple apiculus) was mapped to a clone located between 19.1 and 31.4 cM on the chromosome

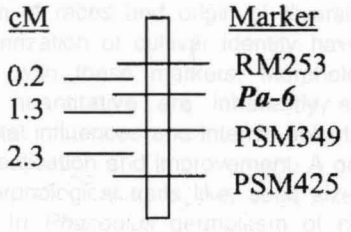

Fig. 2. Linakge map showing gene for purple apiculus, *Pa*-6 with linked microsatellite markers on chromosome 6 in rice

Table 3. Genotypic segregation ratio of different markers linked to gene for purple apiculus

Marker	A/A*	A/B	B/B	χ^2 (1:2:1)
PSM 349	44	76	31	2.52
PSM 425	43	77	31	2.25
RM253	43	78	31	2.29

*A/A: Homozygous for the HJX 74 allele; A/B: Heterozygous; B/B: Homozygous for the Lian Jian 33 allele

6. Thus C and *Pa*-6 appears to occupy the same region on short arm of rice chromosome 6, however, mapping approach was different. It thus proves that the approach of using 3-S Lines for gene mapping is as efficient as others. Taking advantage of the current mapping of the gene (*Pa*-6) with closely linked SSR markers, cloning can be afforded through map-based approach. For that matter, however, fine mapping of the gene with more numbers of segregating plants and markers is highly suggested.

In this study, besides apiculus, purple color was found in leaf sheath, leaf margin including leaf tip, and stigma. Similar observation was also made by Jena and Khush [3]. Through application of MAAL, Jena and Khush [3] estimated the gene for purple stigma to be monogenic dominant and mapped it to chromosome 3 of *O. officinalis*. It thus can be inferred that complementary loci or multiple allele are involved for the development of the purple color. Therefore, further investigation at this locus is required to elucidate the involvement of allelic differentiation.

The introgression lines (ILs) or near-isogenic lines (NILs) are useful tools for precise mapping of gene/QTL, clarifying the specific gene effect and evaluating the genotype/environment interaction of individual QTL [10, 11]. The unique difference between such population and the 3-S Lines is that while the ILs may contain more than one substitution segments, the 3-S lines contain only one substitution segment from the donor. Hence, estimate of gene effect and mapping through 3-S lines is more precise and efficient. Use of simple, cost effective and highly polymorphic SSR markers have made the gene mapping process more powerful and efficient. The 3-S Lines were effectively used to map the QTL affecting heading date in rice [12]. The greatest advantage of this approach is that the 3-S Lines identified with the desirable gene(s) can be directly subjected to the trial for development of new variety, because such lines are developed on the genetic background of elite cultivars [13]. Lines identified in the current study have already been advanced to develop some black rice varieties with improved quality and yield enhancement. Successful mapping of purple apiculus gene in rice established validity of 3-S Line approach for transfer of useful gene(s) to elite varieties.

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