J. Plant Biol. (2012) 55:218-225
DOI 10.1007/s12374-011-0276-z

ORIGINAL ARTICLE

Fine Mapping of *Pa-6* Gene for Purple Apiculus in Rice

Xu Liu[1,†], Xu Sun[1,†], Wenying Wang[1], Hanfeng Ding[1], Wei Liu[1], Guangxian Li[2], Mingsong Jiang[2], Changxiang Zhu[3] and Fangyin Yao[1,*]

[1]*High-Tech Research Center, Shandong Academy of Agricultural Science, Jinan 250100, P.R. China*
[2]*Shandong Rice Research Institute, Jinan 250100, P.R.China*
[3]*State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, Shandong 271018, P.R. China*

Received: September 23, 2011 / Accepted: January 10, 2012

Abstract Purple apiculus is one of the important agronomic traits of rice. Single-segment substitution line (SSSL) W23-07-6-02-14 in the genetic background of an elite rice variety Huajingxian74 (HJX74) with the substituted interval of RM225-RM217-RM253 on the chromosome 6 was found to have purple apiculus (Pa). To map the gene governing Pa, W23-07-6-02-14 was crossed with the recipient HJX74 to develop an F_2 secondary segregation population. The ratio of purple apiculus to green apiculus showed a good fit to 3:1 ratio, indicating that Pa was controlled by a major dominant gene. The gene locus for Pa was tentatively designated as *Pa-6*. Using 430 individuals from the F_2 segregation population, the *Pa-6* locus was mapped between two SSR markers RM19556 and RM19561 with genetic distances of 0.2 and 0.3 cM, respectively. For fine mapping of the *Pa-6* gene, a large $F_{2:3}$ segregation population of 3890 individuals was developed from F_2 heterzygous plants in the RM19556-RM19561 region. Recombinant analyses further mapped the *Pa-6* gene locus to an interval of 41.7-kb bounded L02 and RM19561. Sequence analysis of this 41.7-kb region revealed that it contains eleven open reading frames (ORFs), of which, ORF5 is classified as the one that is associated with the *C* (chromogen for anthocyanin) gene, it was presumed to be the candidate gene for Pa. This result provided a foundation of map-based cloning and function analysis of the *Pa-6* gene.

Keywords *Pa-6*, Physical mapping, Purple apiculus, Rice, Single-segment substitution line (SSSL)

Introduction

Anthocyanin pigmentation in rice is not only an important morphological marker, but also an important trait for studying rice domestication (Saitoh et al. 2004). Anthocyanin pigmentation in rice was extensively studied by earlier workers (Nagao and Takahashi 1963; Takahashi 1982; Maekawa and Kita 1987; Reddy et al. 1996). These studies showed that 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 mainly on leaf sheath, leaf blade, leaf margin, stigma, apiculus, internode, and pericarp. The distribution of red/purple colors in different plant parts among indica lines produces considerable variation (Reddy et al. 1995). The tissue-specific pigmentation, especially the apiculus coloration, has long been used as a morphological marker for the variety identification and linkage analyses in rice.

A powerful tool in rice genetics and molecular biology would be developed if the anthocyanin pathway and the related genes were characterized in detail (Reddy et al. 1995). For this purpose, mapping the gene with tightly linked molecular marker is essential.

So far a great deal of study has been made on the anthocyanin pigmentation of rice. Kinoshita (1984) showed that the *C* gene was located on the group 1 of the Japanese classical linkage map of rice. After the publication of the molecular linkage map of rice by the Tanksley's group of the Cornell University, USA (McCouch et al. 1988), the Japanese group constructed another molecular map and aligned it with the classical map (Kishimoto et al. 1992). The *C* locus was mapped between XNpb165-1 (G165) and XNpb200 (G200) on rice chromosome 6. In another development, through synteny of maps between rice and maize, a rice homologue (*OsC1*) of the maize *C1* anthocyanin regulatory gene was cloned from cDNA library (Reddy et al. 1995). Through

†These authors contributed equally to this work
*Corresponding author; Fangyin Yao
E-mail : yaofy@163.com

comparative sequence study between tawny-colored and colorless, Mikami et al. (2000) showed that this *OsC1* might be responsible for colored apiculus. In addition, through comparison of colored and colorless NILs, the *OsC1* locus was located on chromosome 6 at a distance of 6.2 cM from the linked RFLP marker RZ588. More studies have located *C* locus in the same region with a little variation (Li et al. 1995; Wang et al. 1998; Li et al. 2001). Recently, the *C* locus was delimitated to a 59.3kb region in which *OsC1* was located (Fan et al. 2008). However, the nature of the gene is yet to be ascertained.

For map-based cloning of the *Pa* gene, the first step is to locate the gene locus on a delineated DNA fragment. With the completion of the sequence of the rice genome, a nearly unlimited number of molecular markers can be developed using known sequence information obtained from public databases. In the present study, the SSSL W23-07-6-02-14 in the genetic background of an elite rice variety Huajingxian74 with the substituted interval of RM225-RM217-RM253 on the chromosome 6 was found having a gene for purple coloration. Since the apiculus coloration matched the leaf sheath, leaf blade and stigma coloration, for ease of description, only the apiculus coloration was used. To map the gene, we developed a secondary population from a cross between SSSL and a recurrent parent HJX74. In the process of mapping, we first constructed the genetic linkage map of the *Pa* locus by using SSR markers. The analysis of recombination events in the *Pa* region with newly developed SSR markers further delimitated the *Pa* locus to a 41.7kb region. The sequence analysis of this fragment using RICE GAAS (http://ricegaas.dna.affrc.go.jp/) predicted 11 Open Reading Frames (ORFs). The ORF5 encodes a gene classified as association with the Anthocyanin regulatory C1 protein. The gene is probably the candidate gene for purple coloration. This result will be very useful in molecular cloning of the *Pa* gene.

Materials and Methods

Identification of QTLs for Purple Apiculus Using SSSLs

To evaluate the potential advantages of the SSSLs constructed by our group (Zhang et al. 2004), phenotype variation of purple color was investigated under natural conditions on the farm of High-Tech Research Center, Shandong Academy of Agricultural Science, Shandong in May-October 2008. The field experiment was designed in randomized plots without replications. For each SSSL and parent, 60 plants were planted in six rows, 20 plants in the middle of each plot were selected to collect the data. Each entry was planted at a spacing of 16.6 cm from plant to plant, and 25 cm from row to row. The purple apiculus was recorded before maturity. At heading, the tip of Lemont apiculus is purple, but the color fades and is hardly distinguishable at maturity.

Mapping Population

For fine mapping the gene, the target SSSL was crossed with the recipient HJX74 to develop an F_2 segregating population. The resultant three F_1 plants were self-pollinated to produce F_2 plants. Four hundred and thirty individuals from F_2 population were used for molecular tagging and chromosome mapping of the purple apiculus gene. Five F_2 plants, in which the region around the target gene locus was heterozygous, were used to develop an F_3 segregating population containing 3890 individuals for high-resolution linkage mapping of the target gene.

Field Experiments and Purple Apiculus Investigation

The segregating population and the corresponding parents were planted in the normal rice growing season on the farm of the High-Tech Research Center in April-October 2009 and 2010, Shandong Academy of Agriculture Science, Jinan, P.R.China. Each entry was planted at a spacing of 16.6 cm from plant to plant, and 25 cm from row to row, purple and green apiculus of each plant was scored before maturity. In the same time, the apiculus coloration matched to leaf sheath, leaf blade and stigma coloration.

DNA Extraction and PCR Amplification

Rice genomic DNA was extracted from fresh leaves harvested from each plant using a microisolation method described by Zheng with minor modifications (Zheng et al. 1995). The leaf samples were cut into small pieces of 1 cm long and placed into 1.5 mL tubes, some nitrogen was added, and samples were ground with a small plastic pestle. Then, 1 mL of isolation buffer [100 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA (pH 8.0), and 1 mol/L KCl] was added to the powdered tissue, incubated at 75°C for 30 min, centrifuged at 12,000 rpm for 10 min. The supernatant was collected and DNA precipitated with cold absolute ethanol.

PCR amplification was conducted as described by Panaud et al. (1996) with slight modifications. The PCR was performed in 20 μl reaction volume containing 30 ng of template DNA, 0.15 μl of 10 mmol/L dNTPs, 1.5 units of *Taq* DNA polymerase, 2 μl of 10 × PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, and 0.01% gelatin], and 1.5 μl of 2 μmol/L forward and reverse primers. Cycling conditions were 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were subjected to electrophoresis on 6% polyacrylamide gel. After

completion of the electrophoresis, the gels were silver stained by Li et al. (2002).

Marker Selection and Primer Design

SSR markers were used to determine the genotypes of marker loci for each plant. Known SSR markers were adopted from the public database released by the International Rice Microsatellite Initiative (www.gramene.org), other newly developed SSR and InDel markers were from the Plant Molecular Breeding Research Center, South China Agricultural University, Guangzhou, China. The sequence between two flanking markers, downloaded from a publicly rice genome sequence (www.ncbi.nlm.nih.gov) was exploited to design new SSR markers by using SSRIT procedures (www.gramene.org/db/searches/ssrtool) and the primer premier version 5.0. Sequence diversities between *indica* '93-11' and *Japonica* 'Nipponbare' were used to develop InDel markers. All primer pairs flanking SSRs or InDels were designed in light of the following parameters: 18-25 nucleotides in length, devoid of secondary structure, a GC content around 50%, and a melting temperature around 55°C.

Map Construction

The genetic map of the gene locus was developed using MAPMAKER/EXP version 3.0 based on genotypic and phenotypic data for 430 segregating individuals in F_2 population (Lander et al. 1987). The genetic distance (centimorgans) was calculated using the Kosambi function (Kosambi 1944).

Fine Physical Mapping of the Gene for Purple Apiculus

An F_3 segregating population comprising 3890 individuals was used to fine map the gene locus. SSR markers, which flanked the target gene, were first used to detect the recombinants. Additional PCR-based markers were subsequently developed according to the sequence information of the reference Nipponbare and 93-11. Physical map of the target gene was constructed by using bioinformatics analysis. Molecular markers linked with the target gene were landed on the BAC or PAC clones of the reference Nipponbare and released by IRGSP using the sequence homology search tool BLASTN (www.blast.ncbi.nlm.nih.gov/BLAST.cgi). Sequences of these clones were downloaded and aligned using the sequence alignment tool pairwise BLAST (boast.ncbi.nlm.nih.gov/blast.cgi/b12seq/b12.html) for constructing the BAC/PAC contigs spanning the target gene locus. At the same time, the genotype and phenotype of the recombinants and their progenies were analyzed in the present study.

Results

Identification of QTLs for Purple Color

The apiculus coloration matched the leaf sheath, leaf blade and stigma coloration. For ease of description, only the apiculus coloration was used hereafter. The phenotypes of apiculus in SSSL W23-07-6-02-14 and HJX74 were showed in Fig. 1. SSSL W23-07-6-02-14 had a substituted segment from Lemont on chromosome 6 with an estimated length of 5.6 cM from RM225 to RM253. Purple color between W23-07-6-02-14 and control HJX74 showed a significant difference, indicating that there was a QTL for purple color at the interval of RM225-RM217-RM253, and the QTL was tentatively named as *Pa-6*.

Phenotypic Segregation

The segregating plants were recorded to produce purple apiculus. Meanwhile, purple color in leaf sheath, leaf margin, and stigma was also observed. Segregating plants were therefore recorded as either purple or green apiculus. The segregation of purple to green apiculus based on 430 F_2

Fig. 1. Phenotypes of the apiculus in SSSL W23-07-6-02-14 and HJX74. Left: SSSL W23-07-6-02-14 with purple apiculus; right: HJX74 with green apiculus.

Table 1. Chi-square test for plants segregating for purple apiculus

Phenotypic classes	Observed value	Expected value	χ^2 value (3:1)
Green apiculus	100	107.5	0.79
Purple apiculus	330	322.5	
Total	430	430	

plants fitted a ratio of 3:1 (χ^2=0.79<$\chi^2_{0.05,1}$=3.84) (Table 1), indicating the gene for purple color is dominant over green color and is controlled by a major gene. The QTL was tentatively named as *Pa-6*.

Construction of the Genetic Map for the *Pa-6* Locus

Fifteen pairs of SSR markers located at the substituted interval were used to screen parents, HJX74 and W23-07-6-02-14, of which six pairs exhibited polymorphism. The randomly selected 430 F_2 individuals was used as the initial mapping population, the individuals were genotyped using the six positive markers. Based on the genotype of marker loci, a linkage map was constructed using MAPMAKER analysis and the target gene locus for purple apiculus was mapped between two SSR markers RM19556 and RM19561, with genetic distances of 0.2 and 0.3 cM, respectively. The physical distance between RM19556 and RM19561 is 106 kb (Fig. 2a).

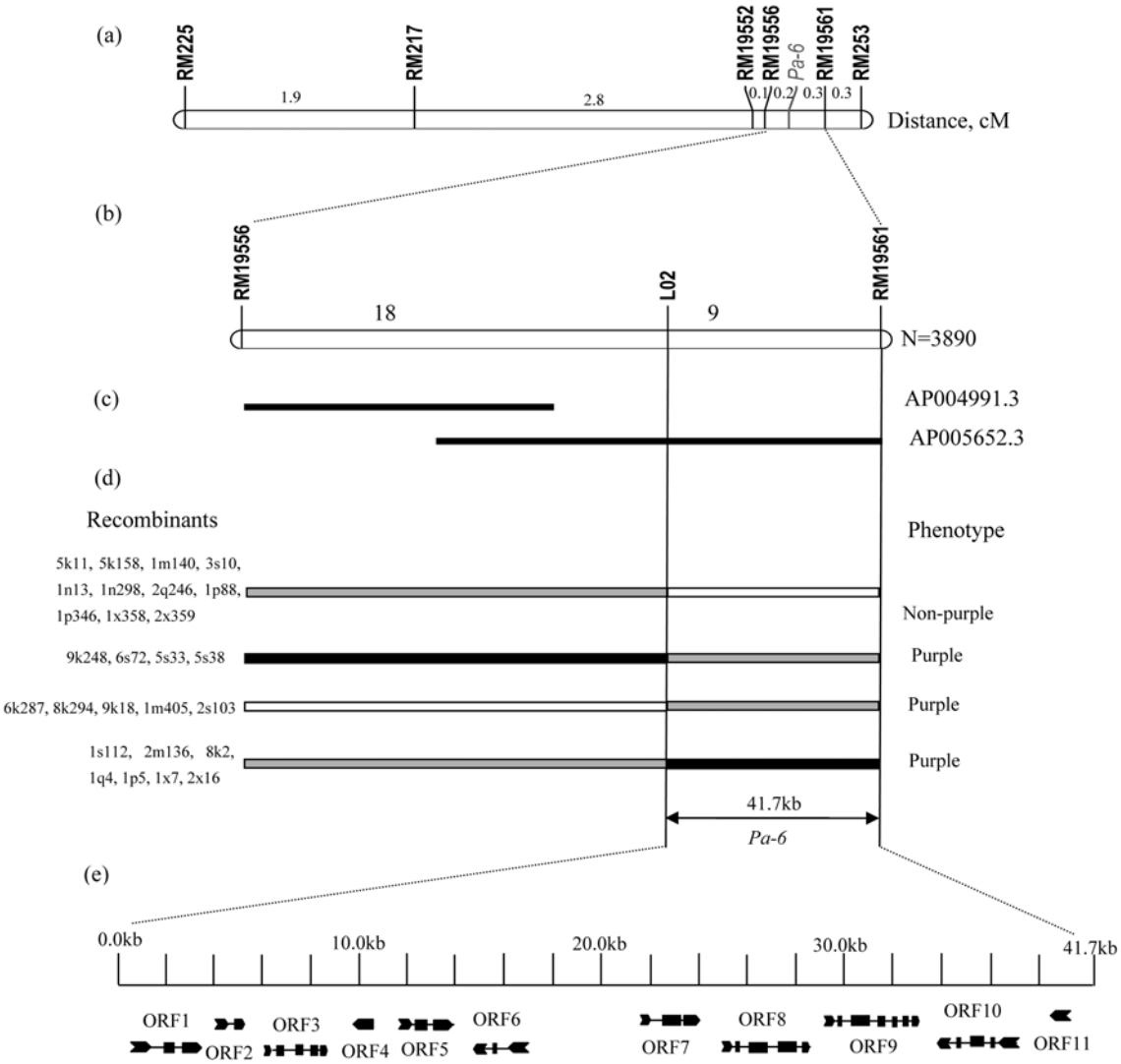

Fig. 2. A genetic and physical map covering the *Pa-6* locus. (a) Location of the *Pa-6* locus on rice chromosome 6. (b) To further reduce the region of the *Pa-6* locus, one polymorphic marker developed between RM19556 and RM19561 was used to identify recombinants. The number of recombinants between the adjacent markers is shown above the linkage map. (c) The horizontal line represent BAC clones. (d) Analysis of overlap-mapping of the *Pa-6* locus with nine key recombinants indicated the *Pa-6* locus was finally delimitated to a 41.7-kb DNA fragment. Open, filled and grey bars represent homozygous fragments from HJX74, heterozygous chromosome segments, and possible interval of crossover, respectively. (e) The horizontal arrows represent predicted ORFs using RiceGAAS.

Fine Genetic and Physical Mapping of the *Pa-6* Locus

For fine genetic and physical mapping of the *Pa-6* locus, a large F_3 segregating population containing 3890 individuals developed from F_2 plants with the heterozygous region around the *Pa-6* locus was used to identify recombinant events between the *Pa-6* locus and tightly linked markers. The two flanking SSR markers RM19556 and RM19561 were used to identify recombinants, and twenty-seven recombinants were screened out (Table 2). Based on mapping results, it was clear that three types of segregation pattern represented three genotype classes: HJX74 homozygous (1/1), W23-07-6-02-14 homozygous (2/2), and heterozygous (1/2).

In an attempt to pinpoint the *Pa-6* locus, the sequence between RM19556 and RM19561 was downloaded from publicly available rice genome sequence (http://www.ncbi.nlm.nih.gov) and new SSR markers were designed by using the primer premier V. 5.0 and SSRIT procedures (http://www.gramene.org/db/searches/ssrtool). Meanwhile, sequence diversities between the *indica* CV. 93-11 and the *japonica* CV. Nipponbare were used to develop InDel markers. Nine new markers were developed (Table 3), among which, only one SSR marker showed polymorphism between the parents. The polymorphic marker L02 was used to screen the 27 recombinants between M19556 and RM19561 to identify the crossover point. Genotypes of recombinants labeled with three polymorphic SSR markers were showed in Table 2.

Among these 27 recombinants, four purple recombinants (9k248, 6s72, 5s33 and 5s38) showed heterozygous alleles (1/2) at the left-border marker L02, and homozygous allele (1/1) at the right-border marker RM19561, indicating that the *Pa-6* locus was located downstream of RM19561. Five other purple plants (6k287, 8k294, 9k18, 1m405 and 2s103) showed homozygous alleles at the left-border marker L02, and heterozygous alleles at the right-border marker RM19561, indicating that the *Pa-6* locus was located upstream of L02. It can be deduced that the *Pa-6* locus is located between L02 and RM19561. Sequence analysis indicated that the physical

Table 2. The genotype of 27 recombinants at the polymorphic SSR markers in the RM19556/RM19561 region harboring the *Pa-6* locus

No.	Recombinants	SSR markers			Character
		RM19556	L02	RM19561	
11	5k11, 5k158, 1m140, 3s10, 1n13, 1n298, 2q246, 1p88, 1p346, 1x358, 2x359	1/2	1/1	1/1	Non-purple
4	9k248, 6s72, 5s33, 5s38	1/2	1/2	1/1	Purple
5	6k287, 8k294, 9k18, 1m405, 2s103	1/1	1/1	1/2	Purple
7	1s112, 2m136, 8k2, 1q4, 1p5, 1x7, 2x16	1/1	1/2	1/2	Purple

Table 3. Sequences of newly developed polymorphic primers from BAC sequence data of Nipponbare on chromosome 6 at the RM19556-RM19561region

Primer	Primer sequence (5'-3')	Motif	Polymorphism
L01	F: TGAGAAGTGGAGAAGAGGGAG R: GGCTTTAAGTCCTAGCCCGTT	(at)27	–
L02	F: GTGATAACTACTACTAGATGGG R: CAGTAGAATAATGCCTCACCTG	(ga)13	+
L03	F: ACAGAGACAGAGAAGAGCTCT R: CTTCATCCCTTCCTTTGCGC	(ag)9	–
L04	F: TCGTCGTCATCGCCACCGC R: TCGGCGTCTGAAACTCGGC	(tc)11	–
L05	F: ACCTCGCACCCACATCGCC R: GGGAAGCTTTATTGGGGCTC	(cg)5 and (ct)7	–
L06	F: ATGCATGTACATATGTATTGAAAC R: TACATGGCCCTGTTGCTGC	(at)9	–
L07	F: CTCATCTACAAGTACATGGCC R: ACGAGAGGACAATGTACGTAC	(gcc)7	–
L08	F: GGTGGTCCCTAAAATTAAACTTCT R: GTACTTGTATACTCCCTCGCTCCC	(taa)5	–
L09	F: ACAGCAGCGGAGCGTGTGG R: CGCGGTGGTGGATCGAAAA	(tcg)5	–

distance between L02 and RM19561 was approximately 41.7-kb in length (Fig. 2b, 2c, and 2d).

To further confirm our conclusion, the nine recombinants (9k248, 6s72, 5s33, 5s38, 6k287, 8k294, 9k18, 1m405, 2s103) at the loci L02 and RM19561 (the respective genotypes: 1/1 and 1/2 or 1/2 and 1/1) and their progenies were identified for their genotypes and apiculus coloration. Progenies from the nine purple recombinants with heterozygous alleles (1/2) at the locus RM19561 or L02 produced different phenotypes, indicating the segregation occurred in offspring. This consistency between genotypes of the *Pa-6* locus and phenotypes for the key recombinants and their offsping definitely indicated the accurate mapping of the *Pa-6* locus.

Candidate Genes in the 41.7- kb Region

Gene prediction analysis of the 41.7-kb DNA fragment using the Rice Genome Automated Annotation System (ricegaas.dna.affrc.go.jp/) identified eleven putative open reading frames (ORFs) (Fig. 2e). Among eleven ORFs, three ORFs (ORF4, ORF6 and ORF11) encode unknown proteins, and the functional annotations of the remaining eight ORFs are as follows: ORF1 produces a transcript of 1203 bp containing three exons, which is predicted to a encode putative cell wall lytic protein, ORF2 encodes a gene with a transcript length of 281 bp, and is classified as a putative MYB protein, ORF3 is classified as putative ATG12A (AUTOPHAGY 12 A) protein, ORF5 encodes Anthocyanin regulatory C1 protein, ORF7 is a putative transposase family protein encoding gene, ORF8 and ORF9 are putative reverse transcriptase genes, ORF10 is a putative RNA-directed DNA polymerase gene.

Discussion

A Novel QTL Controlling Purple Apiculus

Mapping of genes with tightly linked marker facilitates cloning, and cloning facilitates understanding the function of a gene. Well-characterized anthocyanin pathway in maize, barley, *Petunia* and *Antirrhinum* has been extensively utilized for gene isolation, expression, regulation, and transformation (Peterson 1986; Dooener et al. 1991; Liyod et al. 1992), similar utilization can be achieved in rice too. Genetic control of apiculus color is a very fascinating research topic, and has attracted many researchers to work on it.

Most researchers believed that the anthocyanin trait was controlled by one or two pairs of dominant genes. Japanese researchers found that pigmentation was controlled by the *C_A_P* system, including the basic gene *C* (generating pigment), the activating gene A and the distributing gene *P* (Mori and Takahashi 1981). The basic *C* gene producing chromogen was tagged to DNA markers on the short arm of chromosome 6 (Mikami et al. 2000). Yue et al. (2006) reported that three main-effect QTLs for purple color were detected on chromosomes 1, 6, 10, and they designated the locus governing the purple color on chromosome 1 as *lsc1* flanked by two SSR markers RM129 and RM5. Wang et al. (2009) has mapped the *PSH1*(t) gene for purple leaf sheath to chromosome 1 between two SSR markers L03 and L05. Fan et al. (2008) delimited the *C* (Chromogen for anthocyanin) gene to a 59.3-kb region bounded by RM19559 and RM19565. However, the product and function of the gene are yet to be ascertained. In present study, the gene for purple apiculus was finely mapped to an interval of 41.7 kb located on a BAC clone AP005652.3.

With high level of uniformity of the genetic background excepting for a single substituted segment, all the phenotypic variations were associated with the substituted segments in those lines (Eshed and Zamir 1995; He et al. 2005a; Xi et al. 2006; Zhao et al. 2008; Liu et al. 2008). Thus the secondary mapping populations are more efficient than the primary populations in fine mapping of individual QTLs, even minor QTLs. In this study, SSSLs developed by marker-assisted selection were utilized to map the gene for purple color. SSSLs enable us to precisely locate QTLs, because such lines are isogenic to the recurrent parent, and any differences between substitution lines and recurrent parent must be due to QTL located in the substituted segment. These lines are suitable material for verification and characterization of the QTLs detected. Once a SSSL with desirable QTL was detected, this SSSL can be used as a parent material to cross with recurrent parent for developing secondary segregating population, which can be used to identify recombinants within the introgression segment using flanking marker. It is very useful to map the QTL more precisely. The results will make QTL analysis and backcross breeding more effective, and provide a good basis for the fine mapping of QTLs.

The *Pa-6* locus in this work was mapped to the chromosome 6 between L02 and RM19561, a overlapping interval was detected between RM19559 and RM19565, indicating the *Pa-6* locus in this study was consistent with the *C* locus (Fan et al. 2008). So the *Pa-6* and *C* loci were mapped in similar chromosomal location. Therefore further analysis will be necessary to clarify the relationship between *Pa-6* and *C*. The *Pa-6* gene is a major dominant gene, and can be used directly in the breeding of male sterile lines and restoring lines. Breeders will produce hybrids with *Pa-6*, thus it is very helpful to identify other seedling mixed in rice hybrid seedlings based on the purple color of *Pa-6*. So the *Pa-6* not only can be used as a morphological marker directly in the genetic research and breeding activity, it is

also useful for identifying the purity of variety. At the same time, this result should be very useful for cloning of the *Pa-6* gene.

Possible Candidate Gene

In this study, the *Pa-6* locus was finely mapped to a BAC clone. Although molecular tools and sequence information have been rapidly accumulated, the phenotypes of plants are still the most crucial factor in the genetic analysis of target genes. Purple apiculus is a visible morphological trait that can be easily identified. At the same time, the phenotypes and genotypes of the recombinant individuals identified for fine genetic and physical mapping of the *Pa-6* locus (Table 3) have been further confirmed by examining the presence of purple color and marker genotypes of the respective F_3 progenies. In this way, the phenotype of a recombinant could be accurately evaluated by identifying the *Pa-6* gene performance of a recombinant and its offspring. Analysis of genotypes for the key recombinants and their offspring further confirmed that the results for fine genetic and physical mapping of the *Pa-6* gene were reliable.

Although QTLs for anthocyanin has been finely mapped (Fan et al. 2008), little is known about the isolation of genes for purple color. Here, we have reported the mapping of a major dominant gene, *Pa-6*, controlling the trait of purple apiculus. Rice Genome Automated Annotation System analysis of this 41.7 kb DNA fragment predicted eleven candidate genes. It is possible to infer the candidate gene with the help of bioinformatic analysis at this time. Gene prediction analysis of the 41.7-kb DNA fragment using the Rice Genome Automated Annotation System (ricegaas.dna.affrc.go.jp/) identified eleven putative open reading frames (ORFs) (Fig. 2e). Of them, three ORFs (ORF4, ORF6 and ORF11) encode unknown proteins. Among the eight putative known function genes, ORF1 is predicted to encode putative cell wall lytic protein, and its function possible take part in the development of cell wall growth and division. ORF2 is a putative MYB protein encoding gene, the function of putative MYB proteins is still unknown. ORF3 encodes a putative ATG12A (AUTOPHAGY12A) protein, which is involved in autophagic vacuole formation. ORF7 is a putative transposase family protein encoding gene, and its function possible participate in transposition which is a method of recombination in which an element of DNA is excised and inserted into a new location in the genome. Both ORF8 and ORF9 are putative reverse transcriptase genes, and their biological function is photosynthesis. ORF10 is a putative RNA-directed DNA polymerase gene, and its biological function is RNA-dependent DNA replication. Therefore, these genes seem to have nothing to do with purple apiculus development and cannot be a candidate for *Pa-6*.

It is well known that anthocyanin regulatory gene may play an important role in regulating reproductive growth and the development of color (Cone et al. 1993). Genetic studies in maize have identified several regulatory genes that control the tissue-specific synthesis of purple anthocyanin pigments in the plant. *c1* regulates pigmentation in the aleurone layer of the kernel, whereas pigmentation in the vegetative and floral tissues of the plant body depends on *pl*. The *c1*gene encodes a protein with the structural features of eukaryotic transcription factors and functions to control the accumulation of transcripts for the anthocyanin biosynthetic genes. Previous genetic and molecular observations have prompted the hypothesis that *c1* and *pl* are functionally duplicate, in that they control the same set of anthocyanin structural genes but in distinct parts of the plant. Hence, ORF5, which codes anthocyanin regulatory C1 protein, is considered the most interesting candidate gene. Paying more attention to confirming the candidate gene function by transformation or by checking its mutation will be worthwhile. Using RT-PCR, we are attempting to find differences of these two gene's transcripts between the SSSL W23-07-6-02-14 and HJX74. Functional analysis of the candidate genes by transformation and other strategies is on going.

Acknowledgements

We are grateful to professor Zhang Guiquan, South China Agricultural University, Guangzhou for kindly providing us with the SSSL populations. This work was financially supported by the China National Natural Science Foundation (No. 31171529, No. 31000696), the National Program on Research and the Development of Transgenic Plants (No. 2011ZX08001-001, 2009ZX08001-010B), the Open Foundation of the State Key Laboratory of Crop Biology, China (No. 2010KF07).

References

Cone K C, Cocciolone SM, Burr FA, Burr B (1993) Maize anthocyanin regulatory gene *p1* is a duplicate of *c1* that functions in the plant. The Plant Cell 5:1795–1805

Eshed Y, Zamir D (1995) An introgression line population of *Lycopersicon pennellii* in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. Genetics 141:1147–1162

Fan FJ, Fan YY, Du JH, Zhuang JY (2008) Fine mapping of *C* (*Chromogen for Anthocyanin*) gene in rice. Rice Science 15:1–6

He FH, Xi ZY, Zeng RZ, Tulukdar A, Zhang GQ (2005a) Mapping of heading date QTLs in rice (*Oryza sativa* L.) using single segment substitution lines, Scientia Agricultura Sinica 38:1505–1513 (In Chinese)

Kinoshita T (1984) Current linkage maps. Rice Genet Newslett 1:16–

27
Kishimoto N, Shimosaka E, Matsuura S, Saito A (1992) A current RFLP linkage map of rice: Alignment of the molecular map with the classical map. Rice Genet Newslett 9:118–123
Kosambi, DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175
Lander ES, Gree P (1987) Construction of multilocus genetic linkage maps in human. Proc Natl Acad Sci USA 84:2363–2367
Li WT, Zeng RZ, Zhang ZM, Zhang GQ (2002) Mapping of *S-b* locus for F_1 pollen sterility in cultivated rice using PCR based markers. Acta Bot Sin 44:463–467
Li Z, Pinson SRM, Stansel JW, Park WD (1995) Identification of quantitative trait loci (QTLs) for heading date and plant height in cultivated rice (*Oryza sativa* L). Theor Appl Genet 91:374–381
Li ZK, Luo LJ, Mei HW, Wang DL, Shu QY, Tabien R, Zhong DB, Ying CS, Stansel JW, Khush GS, Paterson AH (2001) Overdominant epistatic loci are the primary genetic basis of inbreeding depression and heterosis in rice: I. Biomass and grain yield. Genetics 158:1737-1753
Liu GF, Zhang ZM, Zhu HT, Zhao FM, Ding XH, Zeng RZ, Li WT, Zhang GQ (2008) Detection of QTLs with additive effects and additive-by-environment interaction effects on panicle number in rice (*Oryza sativa* L.) with single segment substitution lines. Theor Appl Genet 116:923–931
Maekawa M, Kita F (1987) Genic analysis for weakness induced with gamma-ray treatment and mechanisms for the occurrence of triploid plants of rice. Jpn J Breed 37(Suppl. 2):308–309 (In Japanese)
McCouch SR, Kochert G, Yu ZH, Wang ZY, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829
Mikami I, Takahashi A, Khin-thidar A, Sano Y (2000) A candidate for *C* (*Chromogen for anthocyanin*) gene. Rice Genet Newslett 17:54–56
Mori KI, Takahashi ME (1981) Differentiation of multiple alleles for anthocyanin color character of apiculus in indica rice varieties-genetical studies on rice plants, LXXXI. Japan J Breed 31: 226–238
Nagao S, Takahashi T (1963) Trial construction of twelve linkage groups in Japanese rice. Genetical studies of rice plant, XXVII, J. Fac Agric Hokkaido Univ 53:72–230
Reddy AR (1996) Genetic and molecular analysis of the anthocyanin pigmentation pathway in rice. In: Khush G S. Rice Genetics III. Manila, Philippines: IRRI:341–352
Reddy AR, Scheffler B, Srivastava MN, Kumar A, Sathyanarayan P V, Nair S, Mohan M (1995) Chalcone synthase (CHS) gene in rice (*Oryza sativa* L): expression and molecular mapping. Plant Genome III, San Diego, California, USA. P121
Reddy VS, Dash S, Reddy AR (1995) Anthocyanin pathway in rice (*Oryza sativa* L): identification of a mutant showing dominant inhibition of anthocyanins in leaf and accumulation of proanthocyanidins in pericarp. Theor Appl Genet 91:301–312
Saitoh, K, Onishi K. Mikami I, Thidar K, Sano Y (2004) Alleic diversification at the *C* (*OsC1*) locus of wild and cultivated rice: nucleotide changes associated with phenotypes. Genetics 168:997–1007
Takahashi ME (1982) Gene analysis and its related problems. J. Fac. Agr. Hokkaido Univ 61:91–142
Wang J, Liu KD, Xu CG, Li XH, Zhang QF (1998) The high level of wide-compatibility of variety "Dular" has a complex genetic basis. Theor Appl Genet 97:407–412
Wang WY, Ding HF, Li GX, Jiang MS, Li RF, Liu X, Zhang Y, Yao FY (2009) Delimitation of the *PSH1*(t) gene for rice purple leaf sheath to a 23.5-kb DNA fragment. Genome 52:268–274
Xi ZY, He FH, Zeng RZ, Zhang ZM, Ding XH, Li WT, Zhang GQ (2006) Development of a wide population of chromosome single-segment substitution lines in the genetic background of an elite cultivar of rice (*Oryza sativa* L.) Genome 49:476–484
Yue B, Cui KH, Yu SB, Xue WY, Luo LJ, Xing YZ (2006) Molecular marker-assisted dissection of quantitative trait loci for seven morphological traits in rice (*Oryza sativa* L.). Euphytica 150: 131–139
Zhang GQ, Zeng RZ, Zhang ZM, Ding XH, Li WT, Liu GM, He FH, Tulukdar A, Huang CF, Xi ZY, Qin LJ, Shi JQ, Zhao FM, Feng MJ, Shan ZL, Chen L, Guo XQ, Zhu HT, Lu YG (2004) The construction of a library of single segment substitution lines in rice (*Oryza sativa* L.) Rice Genetics Newsletter 21:85–87
Zhao FM, Liu GF, Zhu HT, Ding XH, Zeng RZ, Zhang ZM, Li WT, Zhang GQ (2008) Unconditional and conditional QTL mapping for tiller number at various stages by using single segment substitution lines in rice (*Oryza sativa* L.). Agricultural Sciences in China 7:257–265
Zheng KL, Huang N, Bennett J, Khush GS (1995) PCR-based marker-assisted selection in rice breeding. In IRRI Discussion Paper Series. No. 12. International Rice Research Institute, Manila, Philippines