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Fine mapping for blast resistance gene in rice (*Oryza sativa* L.) using bulked segregrant analysis

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ABSTRACT

TeTep and SocNau were used as the donors for blast resistance genes. Segregating populations were generated from the two crosses OM1308/TeTep, SocNau/OM997 for fine mapping. The phenotyping was carried out in the green house and in the CLRRI's field. Genetic analysis revealed that a single dominant gene controlling blast resistance in Tetep. DNA isolation from parents and two F_2 populations were amplified with STS marker. PCR-based DNA marker polymorphism (dominant and co-dominant) was recorded. One STS marker was detected to blast resistance at locus RG64, in chromosome 6. Two genetic fine maps containing Pi-2(t) and Pi-CI genes were established and all the selected DNA markers were constructed in the two mapping populations. The Pi-2(t) gene for blast resistance are linked with RG64 of chromosome 6 at a distance of 3.8cM and 1.0 cM in Socnau and Tetep, respectively. The Pi-CI gene for blast resistance are linked with RG64 of chromosome 6 at a distance of 4.8cM and 3.1 cM in SocNau and TeTep, respectively. RG64 could produce resistant and susceptible specific bands in bulk segregrant analysis. This indicated that RG64 might be a putative marker linked to the blast resistance gene. The fine mapping of blast using PCR-based marker will facilitate marker-assisted selection in rice breeding.

Key Words: PCR-based, Blast, Linkage map, BAC clone, Southern blot, Fine map

INTRODUCTION

Conventional genetic linkage maps of many crop species have been constructed by using morphological markers. But the morphological markers are usually difficult to apply in plant breeding program because they are few in number largely affected by environment. Isozyme marker is insufficient to saturate the genetic map for use in marker-assisted selection (MAS). Molecular markers are numerous in number and therefore ideal for gene tagging and use of marker-assisted selection (MAS) in plant breeding program. The MAS has been recently practised e.g. *tms-3* gene *(*Lang et al. 1998 a, 2000), *Rf-3 gene* (Lang et al. 1998b), *Bph-10* gene (Lang et al. 1999).

Major genes for blast resistance *Pi-1(t), Pi-2(t), Pi-4(t)* against *Magnaporthe grisea* (*Pyricularia oryzae*) have been mapped on chromosome 11, 6, and 12, respectively (Inukai et al. 1994). Both major and minor genes can contribute to durable resistance (Wang et al. 1994). At least 12 dominant genes conferring complete resistance to rice blast and 10 quantitatice trait loci associated with partial resistance have been loacated via linkage to genetic markers (McCouch et al. 1994). *Pi-1* was linked to RZ536 and RZ424 separated at a distance of 14.0 cM and 19.6 cM on chromosome 11. *Pi-2(t)* is closely linked to RG64 on chromosome 6 at 2.8cM. The *Pi-4(t)* gene was separated at a distance of 15.4cM at locus RG869 and 18.1 cM at locus RZ397 in chromosome 12 (Yu et al 1991). Another gene *Pi-6(t)* was located in chromosome 12 at a distance of 20cM from RG869. Two dominant loci associated with qualitative resistance to five isolates of blast fungus *Pi-5(t)* and *Pi-7(t)* were mapped in chromosome 4 and 11, respectively, in a 287-F_7 recombinant inbred lines (RILs) of Moroberekan/CO39. However, many genes have been mapped at a distance large than 5cM from the markers. This is difficult to be apply for MAS so that fine mapping is required.

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This study aims at identifying molecular marker linked to blast resistance gene and fine mapping facilitated with bulked segregant analysis.

MATERIALS AND METHODS

Plant material

Two populations were used for genetic fine mapping from F_2 of OM1308/Te Tep and Soc Nau/OM997. Populations consisted of 50 and 110 F_2' s individuals of OM1308/Te Tep and Soc Nau/OM997, respectively.

Phenotying

Blast nursery was carried out at OMon in a randomized complete block design with two replications and 10 plants per line for each replication. Nine leaves of three different plants (3 leaves per plant) in each of the tested lines were inoculated with blast isolated collected in Mekong Delta (resistance gene *Pi-Cl*) and in Philippines (resistance gene *Pi-2(t*)).

DNA isolation

DNA was extracted from rice leaves. Four to six gr of tissue were grinded in liquid nitrogen, then powder was transferred into 50 ml tube. The finer the grind, the higher the yield was taken care and the tissue did not thaw. 20 ml 2X CTAB extraction buffer (preheated to 65□ C) was added to the leaf powder, then mixed thouroughly and incubated at 65^0C for 30 min (up to 1 hour). Tubes were removed from water bath and let cool briefly. We added 20 ml 24:1 $CHCL_3$: isoamyl alcohol, then incubated at room temperature with moderate shaking for 20 min. Sample was centrifuged at 3000 rpm for 30 min at 4^0C.Supernatant (upper phase) was transferred to 50 ml tube with filtering through miracloth. We added 1 vol (20 ml) isopropyl alcohol, the mixed by inversion. Incubating at room temperature or COLDER (-20^0C) for 1 hour to overnight was done. Samlpe was centrifuged at 3000 rpm for 30 min at 4^0C. Supernatant was drained away from pellet. The pellet was washed with 70 % EtOH, air-dried, and dissolved nucleic acids in 5 ml TE.

Tubes were placed at 65^0C to help dissolution. We added 5µl RNAse (10 mg/ml) and incubate at 37^0C for 30 min. Then we added 1/10 vol 3 M NaAOc (500 µl) and 2 vols Absolute EtOH (10-11 ml). Incubate at -20^0C for one hour was done (or longer, at this step it is possible to leave the samples overnight at -20^0C). Two methods to collect DNA are introduced. First method was implemented:

- Use glass rod (sealed, bent Pasteur pipette) to hook pellet. Rinse in 70 % EtOH, drain dry.
- Transfer DNA to microfuge tube.
- Spin down for 30 sec. To 1 min. in microfuge. Drain any remaining alcohol and air dry. Dissolve pellet in 500 µl TE. Or centrifuge at 3000 rpm for 30 min, wash with 70% EtOH, air dry, dissolve in 500 µl TE, transfer to microfuge tube, rinse 50 ml tube with 200 µl TE, collect wash with rest of solution.
- Our yields obtained average 1-1.5 mg (or 700 µl of 1 - 2 µg/µl) starting from 4 g BC_1F_2 tissue which contains 75% *O. longistaminata* genome.

PCR analysis

The PCR reaction mixture contained 50ng template DNA, 50ng of each primer, 0,05mM dNTPs, 1X PCR buffer (10mMTRIS, pH 8.4, 50mM KCl, 1.8mM $MgCl_2$ and 0.01mg/ml gelatin) and 1 unit initially denatured at 94^0C for 5 min followed by 30 cycles of PCR amplification with the following parameters: 30 sec of denaturation at 94^oC, 30 sec of primer annealing at 60 ^{o}C and 1 min of primer extension at 72^oC. Final 5 min incubation at 72^oC was allowed for completion of primer extension. The amplified products were eletrophoretically resolved on a 1% agarose gel in 1X TAE buffer. We initially used 10µl of PCR product for gel electrophoresis to determine if PCR amplification was successful; the remaining 10µl of PCR products was used for restriction digestion. The total reaction volume was usually 15µl, the reaction mixture consisted of 3.2µl sterile distilled water, 1.5µl restriction buffer (10X), 0.3µl restriction enzyme (10U/µl) and 10µl of PCR products. The reaction mixture was incubated from 3 hrs to overnight at 37^oC. The DNA fragments produced by restriction digestion were separated through gel eletrophoresis (1.5% agarose) and visualized under UV light after staining with ethidium bromide.

Southern analysis

Restriction digestion, gel electrophoresis, and southern hybridization followed standard procedures (Sambrook et al 1989). DNA samples of bulks and parents were digested with three restriction enzymes*: Xba* I*, Bla* I *and Hae* III. Digested DNA samples were electrophoresed in 0.9% agarose gel and transferred to Hybond H^+ membranes (Amersham) by capillary action. PCR amplification products for which

polymorphisms had been confirmed on the individuals of the bulks, were isolated from the agarose gel and purified with Gene Clean Kit (Bio 101.Inc.). The isolated products were reamplified and checked by gel electrophoresis to ensure the purity of the isolated fragments. The purified fragments were used as probes in DNA-DNA hybridization analysis.

Fine mapping of blast resistance by chromosome flanking

According to the reported genomic location of *Pi-2(t)* (Yu et al 1991 and Hittalmani et al 1995), two mapping populations (F_2 and F_3) were developed from the crosses OM1308 (susceptible)/Tetep (resistant) and SocNau (resistant)/OM997 (susceptible). The polymorphisms of the two F_2 families from OM1308/Tetep and Socnau/OM997 were assessed. STS marker was determined by PCR amplification followed by *Xba* I *, Bgl* II *and Hae* III digestion.

Linkage analysis

Screened DNA markers from chromosome 6 where the *Pi-2* gene is know to be located and phenotypic scoring was resolved as co-dominant alleles. The values which were significant at 5% level in the Chi-square test for independence, were utilized for mapping. Recombination values were estimated using F_2 maximum likelihood algorithm with the linkage group. Three-point and multipoint analyses were done using the MAPMAKER computer program (Lander et al 1987). LOD score of 3.0 thresholds for linkage was used. Recombinant values were converted into the centi Morgan (cM), with genetic map distance using the Kosambi mapping function (Kosambi 1944).

Identification of overlapping clones

The positive bacterial artificial chromosome (BAC) clones were picked from the genomic library of IRRI and inoculated to 10ml of LB containing 12.5µg/ml of chloramphenicol and incubated at 37^{o}C overnight. DNAs of BAC individual clones were prepared by the standard alkaline-lysis method (Sambrook et al.1989).

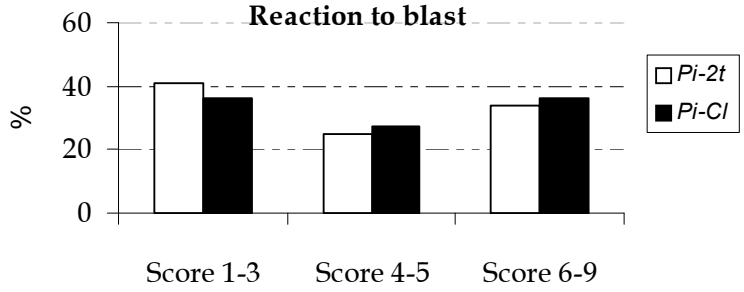


Figure 1: Reaction to blast of F2 in both two populations

RESULTS

STS markers' polymorphism in rice

A first step of exploiting the utility of STS marker in the rice genome-mapping program and eventually for marker-assisted breeding was implemented. Polymorphism and distribution of STS markers in rice genome were confirmed. Here, generation of an RFLP map with 8 markers was considered to convert into 16 primer pairs. Two F_2 populations of rice from 0M1308 / Tetep and Socnau / OM997 were used. 0M1308 / Tetep was considered as studying the level of polymorphism of in rice and their distribution along the rice genome in order to evaluate the efficiency of this marker technology in marker-assisted breeding. Among the STS markers, 16 primers were designed in table 1.

Table 1: Primer design from RFLP markers to detect *Pi-2t* and *Pi-CI* blast resistance genes

No.	Marker	Sequencing 5'----------3'	Product size (bp)
1	RG64	GTTGTTTGAGCTCTCCAATGCCTGTTC CTGCAGTGCAATGTACGGCCAGG	1400
2	G200	TTCCGTTATGCCCAGTGATG GGTATTATTCCCGACAAGTT	700
3	G122	CACCATGACAGACCAAGCCA CGGGGAGGAGTAACGAGAAG	1100
4	RZ19	AACAAGGACAATCCGCACATCCAC TTTGGCACCATCGCAGTGGCAG	1100
5	LP011 2	AGACCTTCAGACCGCTATGT CGGACGATGGTGGTTCTGAT	1100
6	G30	ATCCCTCACGCACTCCTTGT GCCGCCGCCTACCTCCTCAT	141
7	RG172	CTTGACGCCGTTCGAGACCA TAGACGGCATACAGTGAGTC	1800
8	RG123	TCAGGTGTGCTGTAGCAGTTGAG TCCTGCCAATTCCTCTTCCTT	600

Parental survey

Parental surveys were performed using 15 molecular markers from the entire length of chromosome 6. Polymorphism was observed among the parents: 0M1308 vs Tetep, and Socnau vs OM997. Eight STS markers can be used to detect resistance gene among the populations from two crosses 0M1308/Tetep and Socnau/OM997 (listed in table 1). No polymorphism was noticed with seven remaining markers.

Linkage confirmation between resistance gene and RG64 via Southern analysis

In the population developed from the cross OM1308/Tetep, DNA bands were shown resistant and susceptible alleles. Only one band was detected in susceptible parents. When the PCR products were digested with *Xba*I and RG64's probe (Figure 2A), the band in resistant and susceptible parents co-segregated with blast. We therefore concluded that at least one band detected by RG64 was linked to blast resistance gene and the band was in the nuclear genome as it segregated in the F_2 population.

Linkage of LPO111-LP0112 with target resistance gene

PCR primers were synthesized based on sequences of Genebank for both genes and sequence of LP0111-112. PCR products of the expected size (1.1kb) were obtained for LP0111 -112. The PCR analysis was first analysed in OM1308/TeTep population. A PCR band from TeTep is slighly larger than that OM1308, showing codominant polymorphism (Figure 2B). The progeny survey indicated that all lines carried the band from female parent (donor), were inherited resistance gene. In the Socnau/OM997 population, codominant polymorphism was observed with top band for Socnau and the lower for OM997) (Figure 2B).

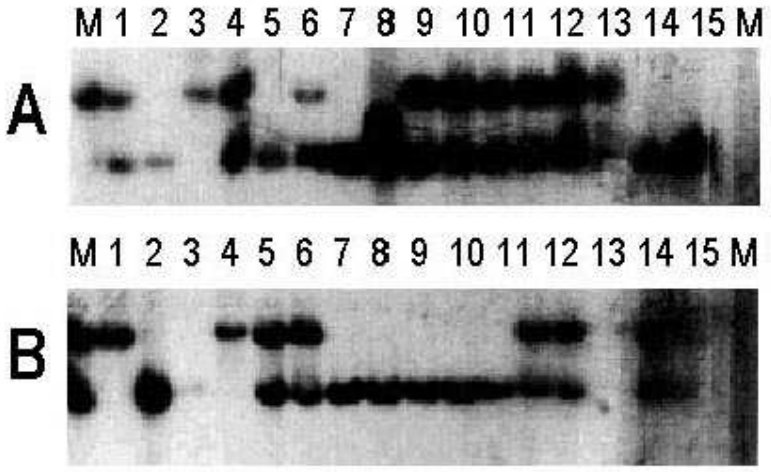


Figure 2: RFLP pattern in F2 population of a cross from OM1308/tetep. Total DNA was digested with Xbat and probed with RG64 on chromosome 6.
A: lane1: OM1308, lane2: Te tep, lane 3-15: F2 pupulation
B: lane1: Soc nau, lane 2: OM997: 1-15: F2 population

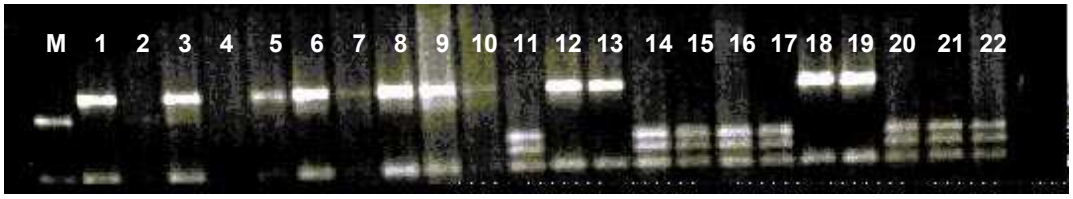


Figure 3: Marker banding pattern of selected F_2 population and their corresponding parent for STS marker linked to blast resistance. Lane 1-17: individuals of F_2 (OM1308 / Tetep). Lane 18: OM997, Lane 19: OM1308. Lane 20: Tetep. Lane 21: SocNau, Lane 22: Moroberekan

Fine mapping

An F_2 population (50 plants) from OM1308 / TeTep cross and 110 F_2 plants from Soc Nau / OM997 cross were chosen since it is segregating for blast resistance. By using 8-polymorphism DNA probes in 8 clones analysed in chromosome 6, blast resistance gene was linked with RG64 at a distance of 1.0cM. In this study, both STS markers are co-dominant and can be used to distinguish resistant individuals from susceptible ones, then they are representing either homozygous or heterozygous alleles. These primers have been efficiently used to select lines carrying blast resistance gene.

The parental lines and the F_2 plants of two mapping populations showed clear and distinct reactions to *Pi-2(t)* and *Pi-CI*. These genes were found to be inherited in Medelian fashion. Both populations fitted the expected ratio 3:1 (resistant : susceptible). Some STS markers in the region *Pi-2(t)* or *Pi-CI* also fitted the expected segregation ratio of 1:2:1 at the level 0.05

Figure 4 shows the two genetic maps containing *Pi-2(t)* and *Pi-CI* genes and all the selected DNA markers constructed in the two mapping populations. In Soc Nau / OM997, gene *Pi-2t was* linked with RG64 at a distance of 3.8cM, but 1.0 cM in OM1308 / Tetep (Figure 3). Gene *Pi-CI* was linked with RG64 at a distance of 4.8 cM in SocNau / OM997, but 3.1 cM in OM1308 / TeTep.

Gene *Pi-2t* was linked with LP0111-112 at a distance of 3.8 cM in OM1308/Tetep, but

18.6 cM in Soc Nau / OM997. Our results clearly indicated that RG64 marker was closest linked to two loci : *Pi-2t and Pi-Cl*

Identification and analysis of BAC clones near Pi-2t and Pi-Cl

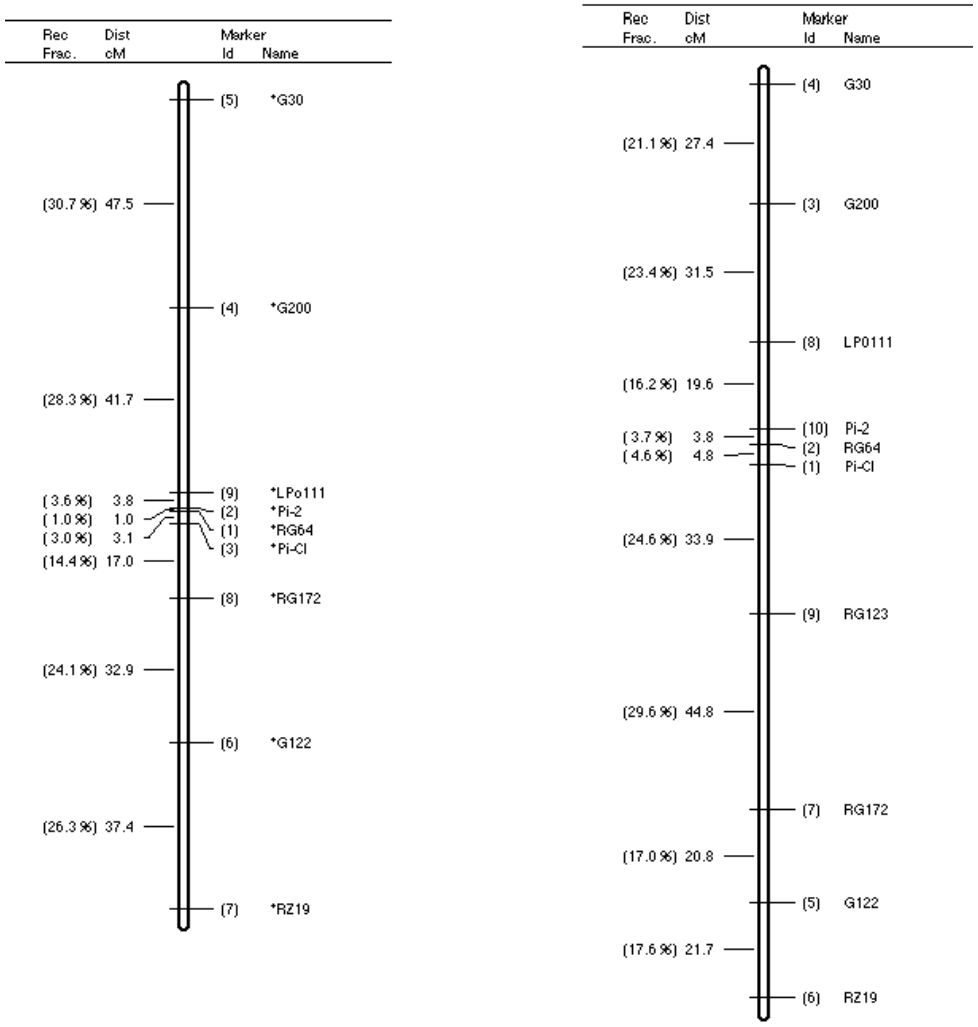
The two markers RG64 and LP0111 were used to screen the IR64 BAC library in order to identify overlapping clones near *Pi-2* . In addition, some markers were used to screen the library to determine the direction of chromosome walking. The RG64 (RFLP marker) did not detect any clone, but LP0111/LP0112 identified seven clones as 23D09, 23D06, 23H03, 23I13, 23 D05, 23J13 and 23 H09. Next orientation of the clones in contigs was individually determined based on the hybridization pattern of BAC end probe.

DISCUSSION

Transferring blast to different genetic backgrounds is very cumbersome and tedious. Identification of closely linked molecular markers is a first step towards expediting to transfer of the blast gene to different background through marker-assisted selection. Rice blast resistance which was controlled by single dominance gene, will be very easy to transfer. However, it would be difficult to identify under field conditions using conventional approaches so that marker-assisted selection will facilitate at early selection phase with greater accuracy. In this report, we identified one marker linked to blast resistance gene in rice using bulked segregant analysis.

According to the reported genomic location of RFLP (marker RG64) around the *Pi-2t* region in the population C101A51/CO39, it linked at the distance of 2.8cM, in chromosome 6. As part of our continuing effort to understand blast resistance, we evaluated the effectiveness of the resistance gene from OM1308/TeTep and SocNau/0M997 under field condition in Vietnam in both dry and wet seasons (1999-2000). Field performance of around 300 plants, their parents and local susceptible control (Ba Trang) were assessed at two blast hot spots with *Pi-Cl* in Mekong Delta (Vietnam) and with *Pi-2(t)* at greenhouse of IRRI. The phenotyping was carried out in a randomized complete block design with two replications. The resistance gene from Tetep included both *Pi-2t* and *Pi-Cl* . Gene *Pi-Cl* controled one specific isolate in Mekong delta under field blast nursery and screen house condition. Individuals from SocNau/OM997 have been continuously used to detected a genomic sequence of RG64. TeTep is considered as a good donor which controlled both blast and bacterial leaf blight (Okuno 1999). Fine mapping in chromosome 6 has been emphasized so far. From the analysis presented here, it indicated that the blast resistance gene *Pi-2t* is located in the same region as RG64 in chromosome 6. Another blast resistance gene *Pi-Cl* is also nearly located at locus RG64. It is possible that the two blast resistance genes *Pi-2t* and *Pi-Cl* reside at the same locus. Previously, Inukai et al (1992) concluded as the same way in case of *Pi-l* or *Pi-k* genes which reside at the same locus in chromosome 11. Based on this information, RG64 marker can be develop for marker-assisted breeding to facilitate the transfer of *Pi-2t* and *Pi-Cl* from one genetic background to another. Furtherstudy on the structure and function of the insertion will clarify the breeding strategy. The use of DNA markers permits the selection of plants with more than one gene without phenotyping (Hittalmani et al. 2000).

Bulk segregrant fine mapping approach can be compared to NIL or DH populations but it should be continuously done in RIL to offer the possibility of tagging several different characters simultaneously based on a single RFLP data set.



A **OM1308 / TeTep** **B** **SocNau / OM997**

Figure 4: Fine mapping *Pi-2t* and *Pi-CI* genes and all the selected DNA markers constructed in the two mapping populations

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SUMMARY IN VIETNAMESE

Lập bản đồ liên kết gen kháng bệnh đạo ôn với khoảng cách ngắn, trên quần thể con lai phân ly trồng dồn

Quần thể con lai F2 của hai tổ hợp lai OM1308/Tẻ Tép, và Sóc Nâu/OM997 được sử dụng cho kỹ thuật "fine mapping". Hai giống cho vật liệu gen kháng bệnh đạo ôn là Tẻ Tép và Sóc Nâu đã được công bố với hai gen kháng Pi-2t đối với nhiều nòi ở Philippines và Pi-CI đối với nòi phổ biến tại đồng bằng sông Cửu Long. Sử dụng 8 cặp primer được thiết kế từ RG64, định vị trên nhiễm thể số 6 để tìm gen kháng trên từng cá thể con lai thông qua đánh giá kiểu gen. Hai bản đồ liên kết gen và marker được ghi nhận với kết qủa như sau:

- *Gen Pi-2t liên kết với RG64 với khoảng cách di truyền là 3,8cM trong Sóc Nâu và 1,0cM trong tẻ Tép*
- *Gen Pi-CI liên kết với RG64 với khoảng cách di truyền là 4,8cM trong Sóc Nâu và 3,1cM trong Tẻ Tép*

Phân tích đa hình cho thấy kết qủa có tính chất co-dominant rất rõ trong quần thể con lai với các băng thể hiện thể đồng hợp tử của tính kháng và tính nhiễm, bên cạnh các băng thể hiện thể dị hợp tử bao gồm cả alen kháng và alen nhiễm.
