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Linkage mapping of Hsa-1^{Og}, a resistance gene of African rice to the cyst nematode, *Heterodera sacchari*

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Abstract Inheritance of resistance to cyst nematode (*Heterodera sacchari*) in *Oryza sativa* was investigated by inoculation tests with isolate 244 from Congo in segregating populations derived from hybridisation between *O. sativa* and its African sister cultivated species, *O. glaberrima*. We found that the resistance was controlled by one major gene, *Hsa-*1^{Og}, with codominance of susceptible and resistant alleles. To map *Hsa-*1^{Og} on the rice genome, we pooled the data obtained from segregation of the resistance trait and microsatellite markers in three kinds of progeny: BC_1F_3, BC_1F_4, and pseudo-F_2 populations. *Hsa-*1^{Og} was unambiguously located between Cornell University's RM206 and RM254 markers on chromosome 11. Two additional microsatellite markers derived from Monsanto publicly available sequences were found to be tightly linked to the *Hsa-*1^{Og} gene. It is possible that numerous plant resistances to a pathogen in fact exhibit a codominant inheritance, possibly explaining misleading conclusions in several reports on resistance segregation.

Keywords Rice (*Oryza sativa* L., *Oryza glaberrima* Steud) · Cyst nematode (*Heterodera sacchari*) · Resistance gene · Molecular mapping

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Introduction

Four species of cyst nematodes, *Heterodera elachista* Ohshima, *H. oryzicola* Rao and Jayaprakash, *H. oryzae* Luc and Berdon and *H. sacchari* (Luc and Merny 1963), are known to attack both upland and irrigated rice (Barker et al. 1998). As its name signifies, *H. sacchari* was first described as a parasite of sugarcane in Congo-Brazzaville (Luc and Merny 1963). It was later found on rice in West Africa (Babatola 1983; Merny 1970), India, Trinidad, Pakistan, and Thailand (Reversat and Destombes 1998). Like other *Heterodera* species, *H. sacchari* is a root endoparasite. To date, only two crops are known to be host plants – rice and sugarcane – but it has quite a wide host range among the African weeds. It is triploid and reproduces by mitotic parthenogenesis as do few other *Heterodera* species, while most remaining species of this genus are amphimictic. Its complete life cycle is about 4–5 weeks, meaning that at least three generations may succeed during a rice growing season (Bridge et al. 1990; Evans 1998).

H. sacchari has been shown to be highly pathogenic on susceptible upland rice in sandy soil (Coyne 2000). Production losses from *H. sacchari* infection on rice can be high under upland conditions, but are less severe under flooded conditions (Babatola 1983). By causing root necrosis, chlorosis, and reduced vigor and tillering, early *H. sacchari* infection even with very low initial population densities can result in severe grain losses (Coyne 2000). Moreover, *H. sacchari*-infected plants are likely to increase their susceptibility to various biotic and abiotic stresses, especially drought (Audebert et al. 2000).

With increasing restrictions on the use of chemical pesticides, the role of natural host resistance in plant breeding for nematode control has grown in importance. However, for rice, no source of high resistance to *Heterodera* spp. or *Meloidogyne* spp. nematodes has been reported in the *Oryza sativa* L. germplasm. Only quantitative differences in the final population sizes of *H. sacchari* were found between the accession tested (Reversat and Destombes 1995). Recently, Coyne et al.

(1999) and Reversat and Destombes (1998) have shown that some accessions of the African rice cultivated species, *O. glaberrima* Steud, can be partially to extremely resistant to this parasite.

Many plant major genes or quantitative trait loci (QTLs) conferring partial or high resistance to cyst or root-knot nematodes have been mapped or even characterised by map-based cloning (Brown et al. 1996; Cai et al. 1997; Concibido et al. 1996; Cregan et al. 1999; Djian-Caporalino et al. 2001; Heller et al. 1996; Kreike et al. 1996; Kretshmer et al. 1997; Lecouls et al. 1999; Leister et al. 1997; Lu et al. 1999; Milligan et al. 1998; Ogbonnaya et al. 2001; Schuster et al. 2001; Tamulonis et al. 1997; Taylor et al. 1998; van der Voort et al. 1999; Veremis et al. 1999; Wang et al. 2000; Williamson 1999). However, to our knowledge, no similar investigation has so far been reported on rice. With the recent discovery of a natural source of medium- to high-level resistance in the African *Oryza* spp. germplasm, we initiated mapping studies to locate genes or QTLs for resistance to nematodes in rice. We present here the analysis of the inheritance mode of resistance to *H. sacchari* in rice and the molecular tagging of a major gene for this trait.

Materials and methods

Genetic material

Three kinds of populations segregating for the resistance to *Heterodera sacchari* were obtained from the cross IR64 × TOG5681. IR64 is an *Oryza sativa* L. ssp. *indica* high-yielding variety (Luc and Merny 1963) which is very susceptible to *H. sacchari*. TOG5681 is an *O. glaberrima* steud landrace showing high-level resistance to *H. sacchari*. As the interspecific F_1 hybrid was totally male-sterile, a BC_1F_1 population was developed using IR64 as the recurrent parent. A semi-fertile BC_1F_1 plant was chosen to develop segregating BC_1F_3 populations. After having identified markers boarding the major resistance gene, hereafter named *Hsa-1^{Og}*, using two BC_1F_3 families (n = 83 individuals), we derived two types of segregating populations from BC_1F_3 genotypes in order to increase the number of informative meioses. First, a BC_1F_4 population consisting of 147 individuals was derived from a BC_1F_3 plant with a heterozygote genotype for the *Hsa-1^{Og}* region. Second, ten pseudo-F_2 populations (subsequently called pseudo-F_2-A population) were also derived from the selfing of ten pseudo-F_1s of the cross between IR64 and a BC_1F_3 genotype homozygote for the TOG5681 microsatellites alleles around *Hsa-1^{Og}*. In total, the pseudo-F_2-A population consisted of 233 individuals. All 463 genotypes were first inoculated with *H. sacchari* and then analysed for molecular markers following the protocols described below. Moreover, 251 additional pseudo-F_2 individuals (subsequently called pseudo-F_2-B population) derived from three pseudo-F_1s were first analysed for molecular markers around the *Hsa-1^{Og}* gene, and the individuals showing a recombinant genotype between the boarding markers were then inoculated with *H. sacchari*. Figure 1 summarises production of the genetic material. A total of 714 informative selfed genotypes were thus available, which enabled precise estimation of recombination fractions and accurate determination of loci orders.

Nematode culture

H. sacchari isolate no. 244 was collected in 1986 from the sugarcane plantation of SUCO at Nkayi in Congo-Brazzaville. It was multiplied on rice (cv. UPLRi5 or IR64) grown on sandy substrate in 100-ml PVC tubes (Reversat et al. 1999). Ten weeks after inoculation, infested rice root systems were recovered by gentle washing of the tube content in a water bucket. These were highly infested with protruding brown cysts and white females. The roots systems were then kept for at least 5 weeks in Dropkin solution (0.3 *M* NaCl), which allowed all of the white females to mature into brown cysts. Cysts needed for the making of inoculum for the screening tests were isolated from these root systems under the binocular in batches of 60. The batch of cysts was crushed on a sieve made of stainless steel gauze (0.25-mm mesh) using the rounded end of a polystyrene haemolysis test tube. The whole content of crushed cysts was vigorously shaken in a closed jar with 60 ml of distilled water in order to separate eggs and juveniles from cyst debris. The suspension obtained was then filtered through the 0.25-mm mesh sieve to remove cyst debris. The suspension was ready to be used for inoculation purposes, and the content of eggs and juveniles was estimated by counting a sample of the aliquot under the binocular (about 150–200 individuals/ml). The process was repeated until the required amount of inoculum was achieved.

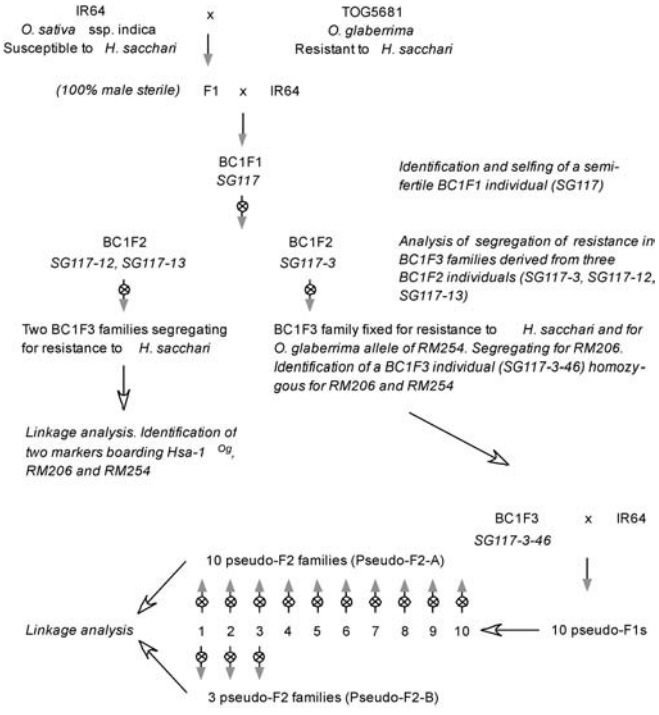

Fig. 1 Scheme showing production of genetic material in segregation for resistance to *Heterodera sacchari*, starting from a cross between the two cultivated species of rice

Resistance screening tests

Culture tubes containing 100 ml of sandy substrate (Reversat et al. 1999) were prepared. A rice seed of the series to be tested was inserted into the wet substrate at a depth of 10–15 mm. The culture tube was covered with a transparent polystyrene centrifuge tube turned upside down. This cover maintained high moisture content of air and improved the germination of seeds and the release of coleoptiles from the substrate. After 1 week, the transparent covers were removed, and the length of the coleoptiles was about 20–50 mm. Each culture tube was provided with an inoculation shaft of a 1-ml Expender tip vertically inserted 40 mm in the substrate and an irrigation black PVC tubing through which 15 ml of one-tenth Hoagland's solution was provided each night. Culture tubes were kept in a room under fluorescent light [14/24-h (day/night)

photoperiod] and a controlled temperature (25–30 °C). Two weeks after sowing, the first inoculum of about 200–250 individuals was introduced into the inoculation shaft; this was followed by two equivalent inoculations, the second 4 days and the last one 7–8 days after the first one. For every screening test (100–240 entries), two series of six control tubes were inoculated in the same way (one series of susceptible controls, with one seed of IR64, and one series of resistant controls, with one seed of *O. glaberrima* TOG5681). Ten to twelve weeks after the final inoculation, root systems of the tested rice plants were scrutinised for their susceptibility or resistance.

Molecular markers

Plant DNAs were isolated from fresh leaves using a simplified version of the CTAB method (Murray and Thompson 1980).

To find markers linked to *Hsa-1^{Og}*, we scanned the rice genome using the two BC_1F_3 families with microsatellite markers from Cornell University (Chen et al. 1997; Panaud et al. 1996; Temnykh et al. 2001). These markers were chosen for their even dispersion on the genetic map. Complete information about these markers is available from the Gramene database Web site (http://www.gramene.org). Polymerase chain reactions (PCR) reactions were carried out under the same conditions as described in Temnykh et al. (2001). Primers were labelled with IRD-700 or IRD-800 dyes, and PCR products were run on 6% acrylamide gels using a LiCor DNA sequencer (Lincoln, Neb.).

After having identified the chromosomal region containing the *Hsa-1^{Og}* gene, we tried to refine the genetic map in generating simple-sequence length polymorphism (SSLP) using rice sequence data containing short tandem repeats made publicly available by the Monsanto Company (http://www.rice-research.com/rice_ssr.html). Primers were designed from candidate sequences supposed to be located in the vicinity of the Cornell microsatellite markers of the *Hsa-1^{Og}* gene. When needed, PCR conditions were optimised for each couple of markers in modifying $MgCl_2$ concentration, annealing temperature or in using touchdown-PCR. Labelling and migration conditions were identical to those for the Cornell markers.

Data analyses

Estimations of recombination fractions were obtained using a special version of MAPMAKER v. 3.0b software compiled for Macintosh PowerPC-based machines (Lander et al. 1987) (http://abraxa.snv.jussieu.fr). As the different segregating populations were obtained from selfed heterozygous genotypes, we could analyse the data as a single F_2 population of 714 individuals. Likelihood ratios for the different possible multipoint orders were computed with the multipoint COMPARE function. Recombination fractions were converted to centiMorgans (cM) with the Kosambi's mapping function (Kosambi 1944). The map was drawn using MAPDISTO v. 1.2 software (http://mapdisto.free.fr).

Results

Segregation of *H. sacchari* resistance

In the early observations on the two BC_1F_3 populations, two levels of infestation were observed: (1) a high number of dark-brown cysts, most of them protruding outside the roots; close to the phenotype observed on IR64 controls; scored as susceptible (S); (2) no visible cyst or only one or two small light-brown cysts embedded in roots; close to the phenotype observed on TOG5681 controls; scored as resistant (R). The observed segregation of R:S phenotypes fitted well to a 3:1 segregation (Table 1).

Table 1 Fit of observed segregation for resistance to *Heterodera sacchari* in selfed interspecific rice families

Segregation[a] in BC_1F_3 population				
Population name	R	S	Total	χ^2 3:1
BC_1F_3	60	23	83	0.98 NS
Segregation[a] in BC_1F_4 and pseudo-F_2-A[b] populations				
Population name	R	MS	S	Total	χ^2 1:2:1
BC_1F_4	43	69	35	147	1.42 NS
Pseudo-F_2-A	50	127	56	233	2.20 NS

[a] S, susceptible; MS, medium-susceptible; R, resistant; NS, non-significant

[b] Data for the pseudo-F_2-B population were not shown as resistance screening was only performed on recombinant individuals, and the segregation test was non-applicable (see text)

In the remaining BC_1F_4 and pseudo-F_2 populations, more attention was given to the resistant class, and three clear levels of infestation could be observed: (1) a high number of big and dark-brown cysts, most of them protruding outside the roots; close to the phenotype observed on IR64 controls; scored as susceptible (S); (2) only few to moderately numbered, small light-brown cysts embedded in roots; scored as medium-susceptible (MS); (3) no visible cyst; close to the phenotype observed on TOG5681 controls; scored as resistant (R). The observed segregation of S:MS:R phenotypes in the BC_1F_4 and pseudo-F_2-A population fitted well to a 1:2:1 segregation (Table 1). For the pseudo-F_2-B population, we could not test for fit to a segregation pattern, as resistance screenings were performed only on recombinant individuals. Consequently, the segregation test was non-applicable since the expected frequencies of the three phenotypic classes were unknown.

These segregation data clearly indicate a monogenic control of the resistance. Moreover, the data collected on the BC_1F_4 and pseudo-F_2 populations provide evidence for a codominant or partially dominant inheritance of the resistance. However, due to difficulties encountered in scoring some of the genotypes showing intermediate phenotypes between R and MS, the trait was scored as a dominant one, i.e. R and MS phenotypes were scored as being resistant for the mapping computations.

Identification of molecular markers linked to *Hsa-1^{Og}*

The genome scanning using the Cornell microsatellites allowed us to identify four markers linked to *Hsa-1^{Og}*. These markers are RM 229, RM 206, RM 254 and RM 224, located on the long arm of rice chromosome 11. *Hsa-1^{Og}* was located between RM206 and RM254.

From the Monsanto public sequences, primers were successfully designed for only nine of the 53 (16.9%) of the candidate chromosome 11 sequences. Four primer combinations gave polymorphic electrophoretic patterns. Segregation analysis using the BC_1F_3 populations showed

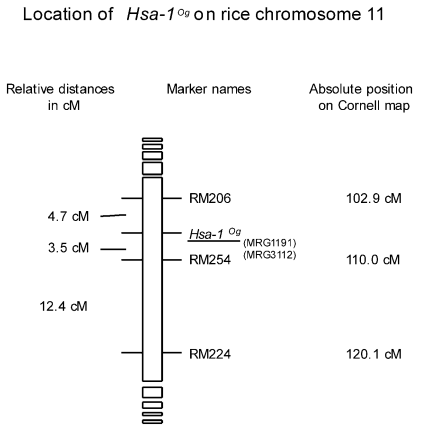

Fig. 2 Genetic location of the *Hsa-1*^{Og} gene on the long arm of chromosome 11 of rice. The markers MRG1191 and MRG3112 were not placed on the framework map as their positions were inferred from only 83 BC_1F_3 individuals. Mapping function: Kosambi

that three of them (MRG1191, MRG3112 and MRG6443) were located on chromosome 11. However, for an unknown reason, we only succeeded in obtaining distinct electrophoretic patterns for the BC_1F_3 populations. In the other populations, the patterns did not clear identification of the amplicon corresponding to the TOG5681 allele. The remaining marker, MRG6170, was not linked to chromosome 11. This could be due to several causes, including incomplete sequencing data leading bioinformatic tools to give false chromosome assignment. All of the microsatellite markers were scored as codominant loci.

Genetic mapping of *Hsa-1*^{Og}

The integration of all available segregation data for the markers found in the vicinity of *Hsa-1*^{Og} lead to the map shown in Fig. 2. The two closest markers from *Hsa-1*^{Og} were RM254 and RM206, respectively at 3.5 cM and 4.7 cM. Nevertheless, on the basis of the small set of segregation data available (n = 83), the two markers MRG1191 and MRG3112 were found to be closer to *Hsa-1*^{Og}, respectively at 1.5 cM and 3.0 cM. However, they were not placed in the framework map, as their positions were inferred from a too small data set. The markers RM229 and MRG6443 were not represented on the map as they showed only loose linkage with *Hsa-1*^{Og}.

As for the resistance trait, no distortion was observed in the segregation of the genetic markers, indicating that the presence of genes causing interspecific *O. sativa* × *O. glaberrima* sterility barrier in this region is improbable. More, the recombination fractions observed were comparable to those of the other intraspecific rice genetic map. This is an important point for generating a high-resolution map around the *Hsa-1*^{Og} gene without being limited by repression of recombination.

Discussion

We successfully mapped the major *Hsa-1*^{Og} gene on rice chromosome 11 between two microsatellite markers, RM206 and RM254. To our knowledge, this is the first localisation of a resistance gene acting against a nematode species on the rice genome. Moreover, as the resistance source came from the *O. glaberrima* species, we think that this original result shows the importance of investigating the possible input of this species for the breeding and improvement of rice varieties.

In this study, we observed that homozygous genotypes for the resistant parent TOG5681 alleles at the markers linked *Hsa-1*^{Og} showed a clear resistant behaviour, while heterozygous genotypes showed a medium-susceptible behaviour. An allelic dosage effect can be invoked to explain this effect. Indeed, other authors have already reported such segregation patterns. For example, Tzortzakakis et al. (1998) showed a dosage effect of the *Mi* gene on partially virulent isolates of *Meloidogyne javanica* attacking tomato. Also, resistance to *M. javanica* in carrot exhibited some allelic dosage response (Simon et al. 2000). The evidence for codominant inheritance is important information, which could explain the differences in segregation patterns that have been observed by various authors in genetic studies involving the same host-pathogen model. In fact, it is possible that numerous plant resistances to a pathogen exhibit a codominant inheritance. However, due to difficulties in identifying the intermediate phenotypes, we propose that many authors have been led to classify these phenotypes either as resistant or as susceptible. This could be the case for a study carried out by Amoussou et al. (2002), who analysed the inheritance of resistance to *H. sacchari* in an interspecific rice pseudo-F_2 population where the resistance donor was CG14, an *O. glaberrima* accession. The authors observed two phenotypic classes, resistant and susceptible, and concluded that there was a monogenic and recessive control of the resistance with respect to the observed frequencies in the two classes. This conclusion, which is not in accordance with our results, could be explained by the classification of MS phenotypes as susceptible. However, the authors did not find markers linked to the involved resistance gene. Thus, although highly probable, we cannot be certain that this gene is identical to *Hsa-1*^{Og}. For example, a source of incongruity between outcomes of the two studies could be the fact that, to overcome the interspecific sterility barrier, advanced backcross lines were used as donor parents in both studies instead of an *O. glaberrima* accession. Thus, it is possible that different portions of the *O. glaberrima* genome, bearing different resistance genes, were segregating in the two studies, leading to apparently contradictory results.

The microsatellite markers MRG1191 and MRG3112 are good candidates for starting a fine-mapping project of *Hsa-1*^{Og}. However, they did not give clear patterns in pseudo-F_2 populations. We believe that it should be possible to address this problem in using primers defined

from larger genomic sequences. Indeed, the Monsanto sequences only contained 100 bases at "left" and "right" around the microsatellite sequence itself, which may be insufficient. We thus expect that we will be able to confirm these marker positions in using larger data sets. We are currently in the process of defining new PCR-based markers from the different rice sources of genomic sequences publicly available.

Classical selection for nematode resistance is difficult and time-consuming. It involves labour-intensive protocols and manipulations and is not really feasible on large populations. We hope that the markers that we have identified around the *Hsa-1^{Og}* gene will help in breeding tropical rice for resistance to *H. sacchari*.

The main result of the present study, genetic mapping of the *Hsa-1^{Og}* gene, is obviously the first step toward its fine mapping and positional cloning. In order to improve the precision of the genetic map, we are currently performing resistance screenings using pseudo-F_3 families issued from the two pseudo-F_2 populations used in the present work. This will enable us to classify the F_2 individuals showing the intermediate MS phenotype with better accuracy, allowing us to use the full information given by codominant segregation of the resistance. The acquisition of more closely linked markers will be greatly facilitated by the increasing availability of whole genomic sequence data, new sets of microsatellite markers (http://www.gramene.org/microsat/IRMI.html), and bioinfomatic tools for rice.

A knowledge of the structure of the *Hsa-1^{Og}* gene should lead to a better understanding of its function and role in the resistance process. Moreover, cloning of this gene of interest could provide a resource for introducing resistance to *H. sacchari* by genetic transformation in its other host, such as sugarcane, for which no source of natural resistance has yet been found.

In addition to resistance to *H. sacchari*, it has been shown that some *O. glaberrima* accessions are also highly resistant to different *Meloidogyne* species, including *M. graminicola, M. incognita* and *M. javanica* (Coyne et al. 1994; Plowright et al. 1999; Soriano et al. 1999; P. Quénehervé, personal communication; our unpublished data). These root-knot nematodes have a large host spectrum and cause severe loss in yield for many crops, including rice. As for *H. sacchari*, no source of resistance has been found in *O. sativa* accessions. We thus expect that interspecific crosses between the Asian and African rice cultivated species will enable the tagging of resistance alleles to *Meloidogyne* spp. with the help of molecular markers. Such markers would be helpful for breeding for resistance to these nematodes and to initiate map-based cloning of the corresponding resistance genes.

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