Date: 10/09/2003

R7548: FINAL TECHNICAL REPORT

Development of clean gene technology for rice transformation and mapping of natural resistance to rice yellow mottle virus and nematodes in rice interspecific crosses.

R7548 is composed of two projects:
Project 1: Development of clean gene technology for rice transformation
Project 2: Mapping of natural resistance to rice yellow mottle virus and nematodes in rice interspecific crosses

R7548: TECHNICAL REPORT - Project 1 (A Afolabi, P. Vain)

Executive Summary

The purpose of this project is to provide enabling "clean-gene" technology for rice genetic engineering to related DFID programmes. An already developed *Agrobacterium*-mediated transformation system was used to develop new "clean-gene" technology aimed at producing rice plants free of undesirable selectable marker genes. New binary vectors able to transfer multiple T-DNAs into rice plants were constructed. Hundreds of independently transformed rice plant lines and thousands of progeny plants were produced and analysed providing proof of concept for "clean-gene" transformation technology in rice. The transgenic technologies developed in R7548-1 allowed DFID collaborative programmes (R8031) to introduce nematode resistance genes into rice without any selectable marker genes. The absence of selectable marker genes in transgenic crops is a key issue for biosafety and public acceptance of GMOs in developing countries.

Background

Half the world's population depends on rice as its major source of nutritional calories. Rice systems in W. Africa are intensifying to meet a demand for rice, which has, until now, been fed by imports. This process of intensification is taking place without a parallel adoption of practices to replace the restorative aspects of traditional fallowing.

Rice transformation technologies hold great promise for increasing rice productivity especially in areas where rice farmers have little means to counter damage caused by pests and disease. The absence of classic plant breeding solutions (limited genetic sources of resistance available) and the limitations of chemical treatments (not economically feasible under low input sustainable systems and extreme damage to the environment) presents an excellent opportunity for biotechnological solutions. Until recently, particle gun bombardment-mediated transformation is the most widely used technology to genetically engineer rice. Despite its efficiency, particle gun technology has several drawbacks due to the type of transgene integration patterns it generates in the plant genome. Uncontrolled transgene rearrangements and systematic transgene linkage results in transgenic loci of unpredictable structure and from which the different transgenes – including antibiotic resistance genes - cannot be separated by genetic recombination in segregating progeny. Transgenic plants containing such antibiotic resistance genes are a constraint to uptake (Select Committee / Royal Society recommendations). Therefore, improved transformation technologies need to be developed in order to generate transgenic loci of simple and predictable structure and plants free of undesirable marker genes. Such "clean-gene" technology could be achieved using *i*) *Agrobacterium*-mediated transformation systems for rice *ii*) alternative selectable marker genes. Once developed, these technologies can be used by other DFID projects and internationally.

Project Purpose

The purpose of this project is to provide enabling "clean-gene" technology for rice genetic engineering to related DFID programmes which concentrate on either improving rice resistance to nematodes (R8031) and viruses (R7415) or on studying transgene behaviour in rice (R6948). The absence of selectable marker genes in transgenic crops is a key issue for biosafety and public acceptance in developing countries.

Research Activities

1.1 Construction of 1st generation binary vectors for "clean-gene" technology

1.2 Identification of *Agrobacterium* strains competent to interact with DFID targeted rice varieties.

1.3 Production of transgenic rice tissues via *Agrobacterium* transformation.

1.4 Production of fertile transgenic rice plants containing 1st generation Clean Gene vectors.

1.5 Segregation analysis of transgenics to determine genetic linkage of different transgenes.

1.6 Molecular analysis to confirm genetic linkage of different transgenes.

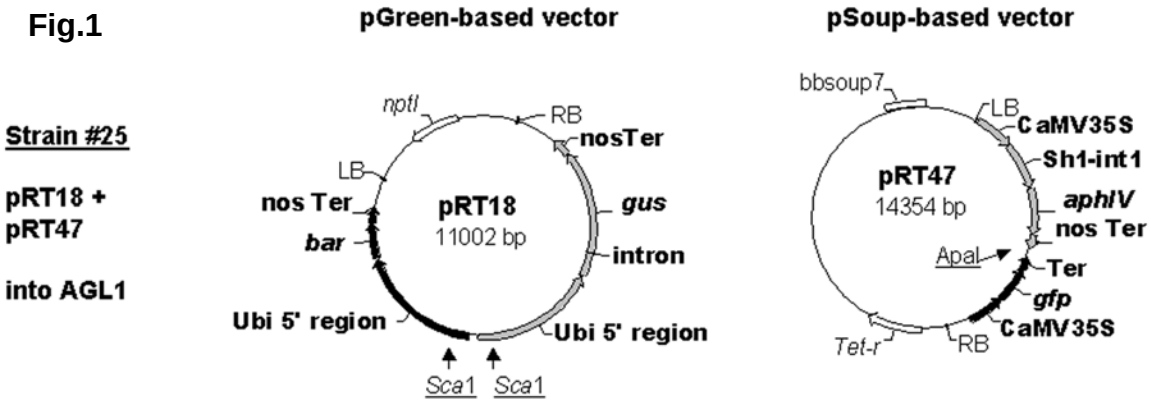
The activities relative to the "Production of transgenic plants containing alternative selectable marker genes" corresponding to Output 2 have been removed from the log frame in 2001 as "clean-gene" technology from Output 1 was available at this date. Transgenic rice plants without any marker gene could be produced. Alternative selectable marker genes were therefore not required.

Outputs

1.1 Construction of 1st generation binary vectors for "clean-gene" technology
- 6 binary vectors for rice "clean-gene" transformation were designed and constructed

1.2 Identification of *Agrobacterium* strains competent to interact with DFID targeted rice varieties
- *Agrobacterium* strain AGL1 harbouring the dual binary vectors pGreen/pSoup allowed rice transformation at high frequency
- pGreen-based binary vector pRT18 (containing one T-DNA with the *bar* and *gus* gene) and pSoup-based binary vector pRT47 (containing another T-DNA with the *aphIV* and *gfp* genes) were introduced into AGL1 (figure 1).

Fig.1



1.3 Production of transgenic rice tissues via *Agrobacterium* transformation
- Three selection regimes were applied during the transformation process with strain No. 25 *i*) dual selection of pRT18 and pRT47 T-DNAs *ii*) Selection of pRT18 T-DNA only *iii*) selection of pRT47 T-DNA only.
- The dual T-DNA selection regime was used to produce a large and random population of plants co-transformed and co-expressing all the transgenes present in the pRT18 and the pRT47 T-DNAs. This strategy was designed to by-pass the limitations of post-transformation screening for co-transformed and co-expressing lines in experiments designed to produce marker-free transgenic plants. Later on, the single T-DNA selection regimes were used to directly mimic experiments designed to produce marker-free transgenic plants.
- Hundreds of independently transformed callus lines were produced using single or dual T-DNA selection.

1.4 Production of fertile transgenic rice plants containing 1st generation Clean Gene vectors
- 403 independent plant lines were produced using single or dual T-DNA selection and were assessed for co-transformation and co-expression of transgenes (table 1).

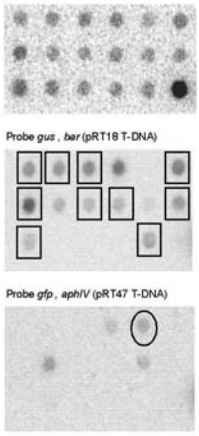
•

Table I. Summary of results of transformation experiments using different T-DNA selection regimes

	Selection regime		
pRT18 (pGreen-based) T-DNA	pRT18	pRT18	----
pRT47 (pSoup-based) T-DNA	----	pRT47	pRT47
No. of independent plant lines produced	10	208	185
No. of co-tranformed plant lines	80%(8/10)	100% (208/208)	131 (71%)
No. of plant lines co-expressing the transgenes	88% (7/8)	70% (146/208)	32 (24%)
No. of plant line studied in T_1	3	50	9
Unlinked lines	100% (3/3)	48% (24/50)	44 % (4/9)
Unlinked loci	63% (5/8)	44% (43/98)	33% (7/21)
"G-S locus" (linked pGreen and pSoup T-DNAs)	38% (3/8)	56% (55/98)	67% (14/21)
"G locus" (pGreen T-DNA alone)	50% (4/8)	31% (30/98)	14% (3/21)
"S locus" (pSoup T-DNA alone)	12% (1/8)	13% (13/98)	19% (4/21)

1.5 Segregation analysis of transgenics to determine genetic linkage of different transgenes

- Transgene inheritance and segregation of transgene phenotype were analysed in 62 independently transformed plant lines: 50 plant lines produced with dual T-DNA selelction **(**3109 T_1 plants studied) and 12 plant lines with single T-DNA selelction (727 T_1 plants studied).
- Transgene inheritance was studied using the genotyping data of the T_1 plants. The observed ratios for each line were compared statistically to those of 30 theoretical models representing all possible linkage configurations of pRT18 (pGreen-based) and pRT47 (pSoup-based) T-DNAs in up to four Mendelian loci. For each independent line, a three step analysis was undertaken to compare the observed ratios to the ratios from the 30 theoretical models 1) elimination of models predicting the absence of a given type of progeny when some where observed among the T_1 plants 2) ranking all possible models according to their probability (using Chi square analysis). 3) comparison of each probable model to the T_0 molecular data (*i.e. bar*, *gus*, *aphIV* and *gfp* gene copy numbers) and to the number of active loci determined by the segregation of transgene phenotype (see activity 1.6).
- In total, more than 4500 genotyping and 8000 phenotyping analyses were conducted as well as 7440 individual tests to identify all probable models for each of the 62 independently transformed plant lines.
- Unlinked T-DNA integrations were obtained in around half of the loci **providing proof of concept for clean gene transformation technology in rice**.



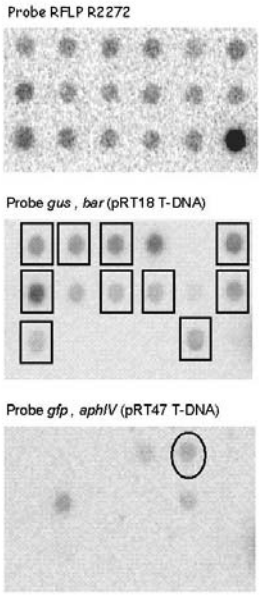
n genetic linkage of different transgenes

number, integration pattern, loci number) and expression were y transformed plant lines. Transgene presence and expression were

transgenes allowed clear identification of which T-DNA was present

es contained one locus or more harbouring a single-type T-DNA. t **for clean gene transformation technology in rice**.

Fig. 2 Dot blot analysis of T_1 rice plants produced by self pollination of a T_0 plant independently transformed with *Agrobactrium* strain No. 25 harbouring pRT18 (containing the *bar* and *gus* genes) and pRT47 (containing the *aphIV* and *gfp* genes) in AGL1. The membrane was probed with the R2272 rice genomic RFLP probe, then probes for the *bar, gus, aphIV* and *gfp* genes. Dot in right bottom corner contains wild-type rice DNA. Rectangle indicates T_1 plants containing pRT18 and not pRT47 T-DNAs, Oval indicates T_1 plants containing pRT47 and not pRT18 T-DNAs.



Contribution of Outputs

Project R4578-1 has sucessfully provided enabling technology for the production of transgenic rice plants free of selectable marker gene (*i.e.* containing only the genes of interest) to other DFID programmes which concentrate on improving rice resistance to nematodes (R8031). Hundreds of transgenic lines and thousands of progeny plants were produced and studied. In addition, R4578-1 contributed to an understanding of the factors controlling T-DNA integration in rice.

The transgenic technologies developed in R7548-1 allowed DFID collaborative programmes (R8031) to introduce nematode resistance genes into rice without any selectable marker genes. Upon confirmation of resistance under controlled conditions, transformed seeds will be made available to downstream users for testing at WARDA and IRRI. Germplasm would then be made available to NARS by the CGIAR centres, for the testing and release. The ultimate beneficiaries would be farmers in Africa and Asia

Publications:

- Vain P., **A.S. Afolabi**, B. Worland and J.W. Snape (2003) Transgene behaviour in populations of rice plants transformed using a new dual binary vector system : pGreen / pSoup. *Theor. Appl. Genet*. 107:210-217.
- **A.S. Afolabi** (2003) Development and understanding of a new "clean-gene" technology for rice (*Oryza sativa* L.) Ph.D. Thesis. University of East Anglia, Norwich, UK.

Internal Reports:

- Vain P., **A.S. Afolabi** and J.W. Snape (2002) Development of clean gene technology for rice transformation. DFID Annual Report.

- Vain P., **A.S. Afolabi** and J.W. Snape (2001) Development of clean gene technology for rice transformation. DFID Annual Report.
- **A. Afolabi**, JW Snape and P. Vain (2001) "Clean Gene" transformation technology for rice. Highlight 2001 DFID Annual report.
- Vain P., **A.S. Afolabi** and J.W. Snape (2000) Development of clean gene technology for rice transformation. DFID Annual Report.

Posters:

- **Afolabi AS**, B Worland, JW Snape and P Vain (2003) Development and understanding of new clean gene technology in rice. 7th International Congress of Plant Molecular Biology. June 23-28, Barcelona, Spain. S10-95.
- **Afolabi AS**, JW Snape and P Vain (2002) Development and understanding of new clean gene technology in rice. JIC Annual Science Meeting 2002. October 31st- November 1st. John Innes Centre, Norwich, UK.
- **Afolabi AS**, JW Snape and P Vain (2002) Development of *Agrobacterium*-mediated clean gene (marker free) technology for rice transformation using a novel dual binary plasmid Pgreen/pSoup. RF-NARO Conference on biotechnology, breeding and seed systems: research output that reaches farmers. Entebbe, November 4-7th, Uganda. p20.
- **Afolabi AS**, B Worland, JW Snape and P Vain (2002) Pgreen/pSoup dual binary vector in *Agrobacterium*-mediated co-transformation show good promise for generating marker free transgenic rice. 2nd Biennial Regional Rice Research Review (4Rs 2002) Meeting. April 9-12 2002. WARDA Headquaters, M'be/Bouake, Ivory Coast.
- **Afolabi AS**, JW Snape and P Vain (2001) Development of *Agrobacterium*-mediated clean gene technology for rice transformation. JIC Annual Science Meeting 2001. November 1-2. John Innes Centre, Norwich, UK.
- **Afolabi AS**, B Worland, P Mullineau, JW Snape and P Vain (2000) Development of clean gene technology for rice transformation. JIC Annual Science Meeting 2000. November. John Innes Centre, Norwich, UK.

Seminars:

- P. Vain (2000) WARDA, Bouake, Ivory Coast
- P. Vain (2000) DFID Plant Biotechnology Group Meeting, London, UK
- P. Vain (2000) Cereal Research Department seminar, Norwich, UK
- P. Vain (2000) DFID-GATSBY-WARDA Rice Biotechnology Meeting, Norwich, UK
- P. Vain (2000) DFID Plant Biotechnology Group Meeting, London, UK
- P. Vain (2001) DFID-PETRA Project Scientist Meeting, Norwich, UK
- P. Vain (2001) Crop Research Department seminar, Norwich, UK
- P. Vain (2001) DFID Plant Biotechnology Group Meeting, London, UK
- P. Vain (2002) Zhejiang University, Hangzhou, China
- **A. Afolabi** (2002) Crop Research Department seminar, Norwich, UK
- P. Vain (2002) Rice Research Institute of AAAS, Hefei, China
- **A. Afolabi** (2002) DFID Plant Biotechnology Group Meeting, London, UK
- **A. Afolabi** (2002) Conference on biotechnology, breeding and seed systems, Entebbe, Uganda
- **A. Afolabi** (2002) 2nd Regional Rice Research Review Meeting, WARDA, Bouake Ivory Coast.
- P. Vain (2003) DFID Plant Biotechnology Group Meeting, London, UK
- P. Vain (2003) DFID Plant Biotechnology Group Meeting, Norwich, UK

Other reports:

- **A Afolabi** (2002) UEA-JIC-Rockefeller Foundation. Annual report - Year 3 Ph.D.
- **A Afolabi** (2001) UEA-JIC-Rockefeller Foundation. Annual report - Year 2 Ph.D.
- **A Afolabi** (2000) UEA-JIC-Rockefeller Foundation. Annual report - Year 1 Ph.D.

R7548: TECHNICAL REPORT - Project 2 (J W Snape, M Koyama, P-L Ammousou)

Executive Summary

The purpose of this project was to map major genes/QTL for natural resistance to RYMV and nematodes in introgression lines derived from rice interspecific crosses (*Oryza sativa x O.glaberrima*), and to investigate QTL controlling components of yield. However, because of the political instabilities in the Ivory Coast and personnel changes at WARDA, it was not possible for WARDA to carry out weed competition experiments with the mapping populations, and hence they did not provide any data on yield components for QTL analysis. This part of the project was therefore not achieved. In addition, despite previous data given to us by WARDA indicating that the crosses examined were segregating for RYMV resistance, in containment experiments at JIC, no resistance was found in the parents of the two mapping populations, and, consequently, no genes for resistance could be mapped. Thus the main aspect of this project that was completed was the mapping of major genes /QTLs for nematode resistance.

Background

In 1991, in response to problems of rice cultivation in Sub-Saharan Africa, WARDA decided to create interspecific hybrids to generate a "low management rice plant type" for resource poor farmers in rainfed ecosystems to improve the welfare of rice producers and consumers. A wide hybridisation program was initiated in 1992, using as parents one inbred *O. sativa* japonica upland rice line (WAB 56-104) crossed with a cultivated *O. glaberrima* line (CG14) from Senegal. More than 300 backcross-introgressed lines (BC_2F_6) derived from these two parents have been generated through anther culture and double haploidization in the early generations. In general, these interspecific lines behave like *O.glaberrima* at the vegetative stage and like *O.sativa* at the reproductive stage. Those lines containing introgressions of *O.glaberrima* showed high yield potential and have been screened and characterized in the field for many traits affecting rice smallholder farmers throughout West Africa.

In general, hybridization between *O.sativa* and *O.glaberrima* produces a near sterile F_1s. However, by selecting certain varieties of *O.sativa* (e.g. WAB 56-104) and *O.glaberrima* (e.g. CG 14) for crossing, it was possible to reduce this level of sterility to 95% in F_1 generation. These F_1 plants showed a number of desired traits of the *O.glaberrima* parent including the lack of secondary branches on the panicles and early vigour. The F1 plants were then backcrossed twice with the *O.sativa* parent to increase fertility (5-95%) while at the same time helping to combine the *O.sativa* and *O.glaberrima* traits in the F_2 population. The majority of the population resembled either one or the other parent and with varying in levels of fertility. Up to one third of the F_2 population were intermediates showing combinations of traits from the two species.
Some of these have the following characteristics:

- Secondary panicle branching giving the high yield characteristic of *O.sativa* varieties
- Early maturity, with the plants ripening between 75 to 100 days after sowing
- Rapid vegetative growth with droopy leaves enabling the plants to compete successfully against weeds
- Production of high numbers of tall erected tillers
- Multiple disease and pest resistance traits

One *O.glaberrima* accession (TOG 5681) and one *O.sativa* indica (Gigante) have shown an immune reaction after being infected by different isolates of RYMV. Several *O.sativa* spp. japonica's upland varieties, like Moroberekan and OS6 have shown high levels of tolerance to RYMV (4% of yield losses). A tropical japonica variety Azucena has been used by breeders as sources of RYMV resistance in their breeding programs. A diallel analysis of resistance to rice yellow mottle virus in *O.glaberrima* suggested that this resistance was oligogenic. A mapping study on a double haploid population derived from a cross between IR64 and Azucena revealed a major QTL for RYMV resistance on rice chromosome 12. A study on of the inheritance of RYMV resistance demonstrated

that a single recessive gene, mapped on Chromosome 4, using two mapping populations respectively derived from TOG 5681 and Gigante, as donors of the resistance, was responsible for RYMV resistance and have been recently fine mapped.

In parallel, transgenic rice varieties resistant to RYMV have been produced using over expression by the plant of one part of the RNA polymerase of the virus. Moreover, through serial inoculations the emergence of breaking isolates without any link to either an initial high pathogenicity or an increase of virus content was observed.

Some of the WARDA BC_2F_6 interspecific hybrids were described as having levels of resistance towards RYMV (Monty Jones, personal communication). One of project R7548-2 objective was to investigate the genetics behind this resistance using four mapping populations derived from two BILs previously identified by WARDA as RYMV resistant.

O.glaberrima was found resistant to a number of nematodes species such as *Meloidogyne* spp and *Heterodera sacchari*. However, the resistance mechanisms involved have not yet been fully characterized.

Some of WARDA's interspecific (*O.sativa* x *O.glaberrima*) hybrids lines have raised the possibility of introgression nematode resistance. One interspecific WAB 450-IBP-160-HB has shown resistance for *Heterodera sacchari* isolates from Côte d'Ivoire and Ghana, and is reduced susceptibility compared to *O.sativa* lines towards *Meloidogyne graminicola*. The expression of resistance in the interspecific hybrids (*O. sativa* x *O. glaberrima*) suggested that resistance to *H sacchari* seems qualitative whilst that to *Meloidogyne* spp*.* looked rather quantitative. Another objective of project R7548-2 was to detect and map the nematode resistance originated from *O.glaberrima* using one mapping population derived from one BIL (WAB 450-IBP-160-HB).

Project Purpose

This project was set up to investigate, and when possible, characterize, the genetics behind the resistance of some rice interspecific material against three different species of plant parasitic nematodes (*Heterodera* spp., *Meloidogyne* spp. and *Pratylenchus* spp.) and RYMV.

Research Activities

1.1 SSR and anchor RFLP polymorphism levels between parental lines established

1.2 Four populations genotyped for polymorphic SSRs and RFLPs

2.1 Resistance to RYMV and nematodes phenotyped

2.2 QTL maps produced by combining resistance data and genotype characterisation

2.3 Diagnostic markers developed for marker-assisted selection

Outputs

1.1 SSR and anchor RFLP polymorphism levels between parental lines established
1.2 Four populations genotyped for polymorphic SSRs and RFLPs

Initially this study was focused on the following four mapping populations – See Figure 1
:

- WAB 450-IBP-160-HB x ITA 257 (105 F_4 families)
- WAB 450-IBP-160-HB x OS6 (19 F_5 families)
- WAB 450-IBP-160-HB x Moroberekan (48 F_5 families)
- WAB 450-11-2-BL1 DR1x Bouaké 189 (76 F_3 families)

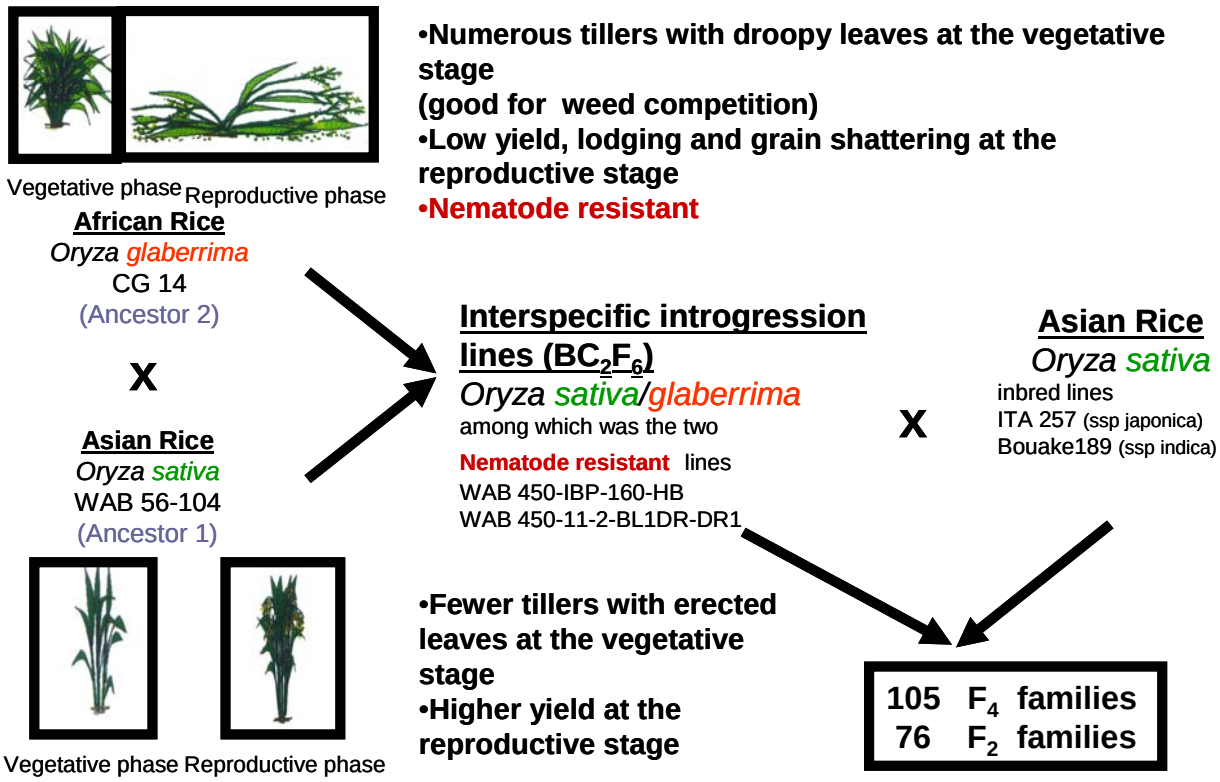


FIGURE 1 Description of the plant material used

The parental screens for markers included the following lines:

WAB 56-104 and CG14 (the two WARDA interspecifics ancestors), WAB 450-IBP-160-HB (WARDA interspecific BIL), ITA 257 (inbred line), OS6 (West African landrace) and Moroberekan (West African landrace), WAB 450-11-2-BL1-DR1 (WARDA interspecific BIL), Bouaké 189 (West African landrace). On top of those line a highly susceptible line to RYMV, ITA 212, was used as a positive control.

To perform the DNA extractions needed for this study, the protocols used were either a CTAB protocol or the DNeasy 96 Plant kit (Quiagen). Eluted DNA solutions are stored at 4ºC (for short term use) or at -20ºC (for long term use). SSR amplification was carried out using standard procedures developed at JIC and elsewhere. However, some recalcitrant markers were successfully amplified using this special PCR amplification profile (source: Plant Breeding Unit, Cornell University, USA). Denaturing acrylamide gels were used to analyse the SSR PCR products.
In addition to SSR markers, Sequence Tag Site (STS) procedure and the Cleaved Amplified Polymorphism (CAPS) procedure were used to generate further markers for specific regions of interest. The protocols were inspired from Konieczny and Ausubel, 1993 and from the Rice Genome Project of Japan website (http://www.rgp.dna.affrc.go.jp) from which the CAPS and STS primers sequences were found.

A set of 285 simple sequence repeat markers (SSR) and 3 sequence tag markers (STS), covering more than 300 loci were tested and used in the mapping populations and are shown in the Tables below :

1

RM323	1	
RM 1	19.3	
RM 84	26.2	
RM220	28.4	
RM 86	28.4	
RM283	31.4	
OSR 2	36.2	
RM272	37.3	
RM259	54.2	
RM243	57.3	
RM 35	57.3	
RM292	66.4	
RM 23	71.6	
RM312	71.6	
RM 81A	77.5	
RM238A	77.5	
RM140	78.4	
RM129	78.4	
RM294B	78.4	
RM 24	78.4	
RM157B	78.4	
RM329	78.4	
RM113	78.4	
RM150A	78.4	
RM158	78.4	
RM 9	92.4	
RM 5	94.9	
RM306	98.1	
RM 34	104.2	
RM237	115.2	
RM246	115.2	
OSR 27	134.8	
RM200	143.2	
RM302	147.8	
RM212	148.7	
RM319	150.2	
RM102	152.2	
OSR 3	154.8	
RM297	155.9	
RM265	155.9	
RM315	165.3	
OSR 23	186.6	
RM104	186.6	
RM165	186.6	
RM 14	194.1	

2

RM109	0
OSR 17	1.1
OSR 11	4.8
RM110	6.9
OSR 14	6.9
RM211	14.4
RM236	14.4
RM233A	16.3
RM279	17.3
RM 8	28.7
RM 53	32.7
RM174	47.5
OSR 9A	49.8
RM 71	49.8
RM322	49.8
RM191	51.9
RM327	51.9
RM324	66
RM 27	66
RM290	66
RM301	66
RM300	66
RM 29	68.9
RM262	70.2
RM341	82.7
RM106	123.2
RM263	127.5
RM221	143.7
RM318	150.8
RM 6	154.7
RM240	158
RM112	166
RM250	170.1
RM166	183
RM208	186.4
RM213	186.4
RM 48	190.2
RM207	191.2
RM266	192.2
OSR 26	196.8
RM183	2L
OSR 9B	
OSR 8	

3

RM 60	0
RM 81B	0
RM 36	4.5
RM 7	6.4
RM132	7
RM 22	11
RM231	15.7
RM175	23.9
OSR 16	42.9
OSR 13	53.1
RM218	67.8
RM232	76.7
RM157A	76.7
RM251	79.1
RM282	100.6
RM338	108.4
RM156	125.7
RM 16	131.5
RM347	131.5
OSR 31	157.3
RM 49	158.6
RM203	165.9
RM 55	168.2
RM186	168.2
RM168	171.2
RM293	193.4
RM143	207.3
RM130	208.2
RM114	208.2
RM148	224.2
RM 85	231
RM227	3L
OSR 5	

4

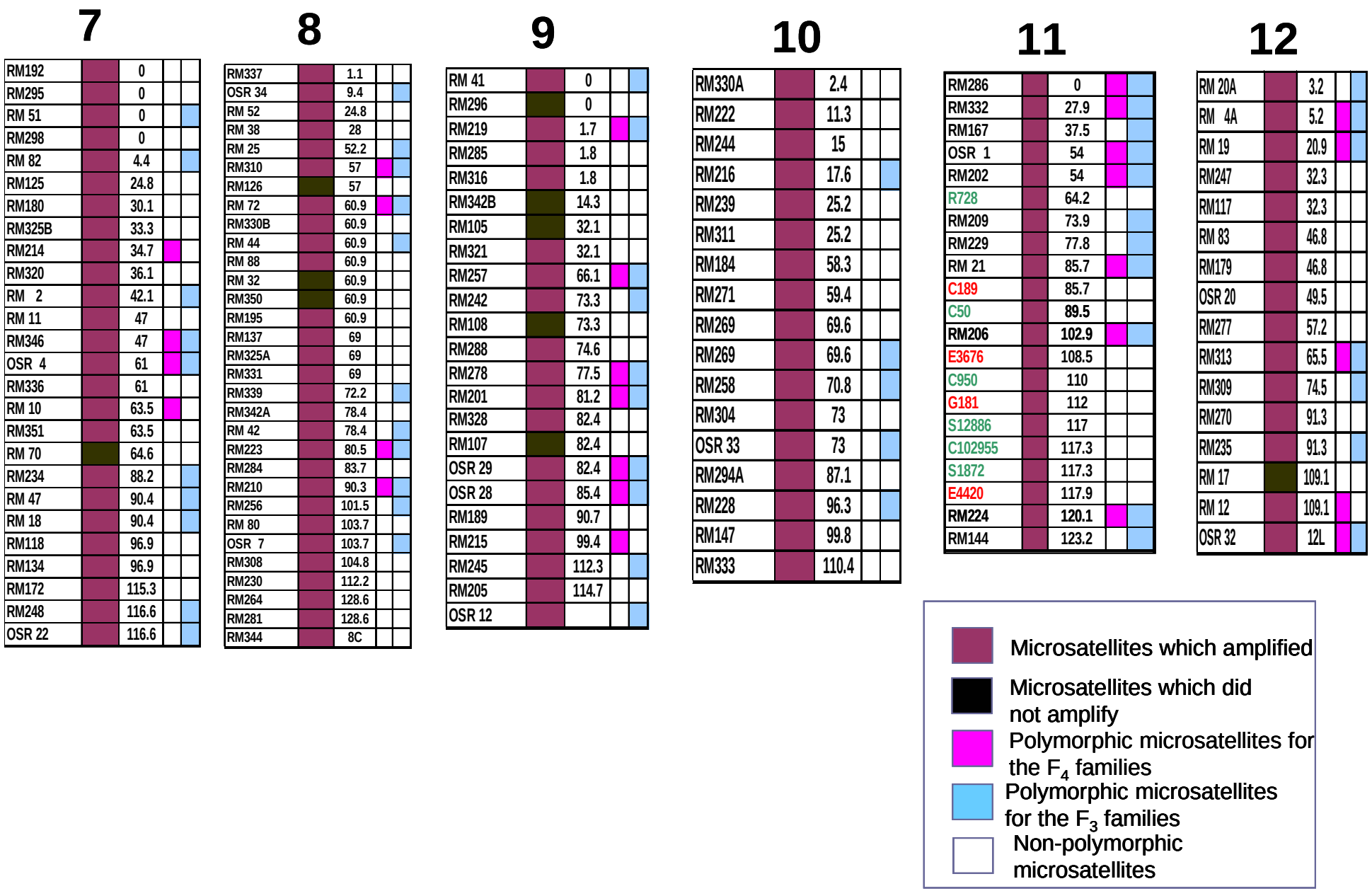
RM307	0
RM335	21.5
RM261	35.4
RM185	50.8
RM177	50.8
RM142	68.5
RM119	76.1
RM273	94.4
RM252	99
RM241	106.2
RM303	116.9
RM317	118.3
RM255	135.4
RM348	137.9
RM349	146.8
RM131	148.8
RM124	150.1
RM127	150.1
RM280	152.3
RM150B	4L
OSR 15	

5

OSR 35	0
RM159	0
RM122	0
RM 13	28.6
RM267	28.6
RM267	28.6
RM194	34.7
RM289	56.7
RM169	57.9
RM146	78.7
RM 39	78.7
RM291	78.7
RM163	78.7
RM164	78.7
RM161	96.9
RM305	96.9
RM173	99.8
RM188	100.6
RM233B	110
RM178	118.8
RM 26	118.8
RM 31	118.8
RM274	126.6
RM 87	129.2
RM334	141.8
RM249	5C
RM225	

6

RM133	0
RM170	2.2
RM197	2.2
OSR 19	7.4
RM204	25.1
RM225	26.2
RM115	26.2
RM217	26.2
RM314	33.6
RM111	35.3
RM253	37
RM 50	39.5
RM276	40.3
RM121	43.8
RM238B	46.1
RM136	51.2
RM 3	74.3
OSR 18	108.3
RM275	108.3
RM343	115.8
RM 30	125.4
RM340	133.5
RM141	143.7
RM103	143.7
OSR 21	143.7
RM176	145.2
RM345	145.2
RM150C	6C
RM193	6L
OSR 25	


7
RM192 0
RM295 0
RM 51 0
RM298 0
RM 82 4.4
RM125 24.8
RM180 30.1
RM325B 33.3
RM214 34.7
RM320 36.1
RM 2 42.1
RM 11 47
RM346 47
OSR 4 61
RM336 61
RM 10 63.5
RM351 63.5
RM 70 64.6
RM234 88.2
RM 47 90.4
RM 18 90.4
RM118 96.9
RM134 96.9
RM172 115.3
RM248 116.6
OSR 22 116.6
8
RM337 1.1
OSR 34 9.4
RM 52 24.8
RM 38 28
RM 25 52.2
RM310 57
RM126 57
RM 72 60.9
RM330B 60.9
RM 44 60.9
RM 88 60.9
RM 32 60.9
RM350 60.9
RM195 60.9
RM137 69
RM325A 69
RM331 69
RM339 72.2
RM342A 78.4
RM 42 78.4
RM223 80.5
RM284 83.7
RM210 90.3
RM256 101.5
RM 80 103.7
OSR 7 103.7
RM308 104.8
RM230 112.2
RM264 128.6
RM281 128.6
RM344 8C
9
RM 41 0
RM296 0
RM219 1.7
RM285 1.8
RM316 1.8
RM342B 14.3
RM105 32.1
RM321 32.1
RM257 66.1
RM242 73.3
RM108 73.3
RM288 74.6
RM278 77.5
RM201 81.2
RM328 82.4
RM107 82.4
OSR 29 82.4
OSR 28 85.4
RM189 90.7
RM215 99.4
RM245 112.3
RM205 114.7
OSR 12
10
RM330A 2.4
RM222 11.3
RM244 15
RM216 17.6
RM239 25.2
RM311 25.2
RM184 58.3
RM271 59.4
RM269 69.6
RM269 69.6
RM258 70.8
RM304 73
OSR 33 73
RM294A 87.1
RM228 96.3
RM147 99.8
RM333 110.4
11
RM286 0
RM332 27.9
RM167 37.5
OSR 1 54
RM202 54
R728 64.2
RM209 73.9
RM229 77.8
RM 21 85.7
C189 85.7
C50 89.5
RM206 102.9
E3676 108.5
C950 110
G181 112
S12886 117
C102955 117.3
S1872 117.3
E4420 117.9
RM224 120.1
RM144 123.2
12
RM 20A 3.2
RM 4A 5.2
RM 19 20.9
RM247 32.3
RM117 32.3
RM 83 46.8
RM179 46.8
OSR 20 49.5
RM277 57.2
RM313 65.5
RM309 74.5
RM270 91.3
RM235 91.3
RM 17 109.1
RM 12 109.1
OSR 32 12L
Microsatellites which amplified
Microsatellites which did not amplify
Polymorphic microsatellites for the F4 families
Polymorphic microsatellites for the F3 families
Non-polymorphic microsatellites

2.1 Resistance to RYMV and nematodes phenotyped

Virus work

Before being able to investigate the genetics behind the resistance towards RYMV in the mapping populations, a parental screen in JIC controlled environment rooms was conducted. The two experiments using an Ivorian Isolate were conducted using the following plant material:
WAB 56-104, CG14, WAB 450-IBP-160-HB, ITA 257, OS6 and Moroberekan.

Three virus purifications were carried out using a combination of the methods described by Fauquet and Thouvenel, 1977, Brisco *et al.* 1985 and Mansour (1990). Virus particles were chosen for the inoculation instead of RNA in order to be as close as possible to the field condition. The two experiments using a Nigerian isolate were conducted with the following plant material: WAB 450-11-2-BL1 DR1 and Bouaké 189 (6 plants for each concentration and 2 plants for negative control : one WAB 450-11-2 BL1-DR1 and one Bouaké 189). Observations of symptoms were carried out every 3 days (from 3 to 30 days). To standardize serological tests the antiserum was used as an already optimized 1:1000 dilution (recommended by WARDA)

No RYMV resistance was found to be segregating between the parents of the two mapping populations in controlled environment room conditions. Visual scoring were taken for the experiment with using the Ivorian isolates showing susceptibility to RYMV in all parental pairs (including the ancestors). In order to back those visual scoring with quantitative data, samples of 3 plants at each 4 concentrations was analysed by indirect ELISA for WAB 450 11-2-BL1-DR1 (*O.sativa* japonica *x O.glaberrima*) and Bouaké 189 (*O.sativa* indica) which form the most contrasting parental pair (in term of parental background) among the four crosses. For each concentration, equal amounts of leaves from each of the 3 sampled plants were mixed and titred together serologically. Global means for each variety (grouping the concentration means) were obtained and used to carry out a t-test of comparison of means to test the hypothesis of significance difference among the response towards RYMV for those lines. There was no significant difference among the mean of the two lines, therefore there was no evidence to conclude that those lines respond in a different way to RYMV infection. This implied no possibility of a mapping study using this material.

Nematode work

- *Response towards Heterodera sacchari.*

For each population, there were separate experiments conducted at CABI Bioscience by John Bridge and Judith Ineson. Two separate experiments conducted on the 105 F_4 families, their parents and the BIL ancestors using isolates RNC 71 and RNC 65, respectively, and one experiment for the 76 F_3 families, their parents and the BIL ancestors using isolate RNC 71. For each experiment, seedlings of each progeny family along with their parents and the BIL ancestors (WAB 56-104 and CG14), were grown. The rice seedlings were grown in 5 cm pots containing soil (Loam: Sand 3:1) in a completely randomised block design (see figure 2 step 0) of 5 replicates (30 randomly placed trays of 20 randomly placed pots each). In addition to those, 4 plants of IDSA6 (a susceptible cultivar from the Ivory Coast) were planted at one end of each tray and 7 were randomly placed within each replicate to act as susceptible checks to assess the most suitable harvesting time. However, depending of sampling on the IDSA6 plants two weeks before the theoretical harvesting dates, the effective harvesting date was calculated. Th4 testing protocols were as described by Bridge & Ineson. Counts were made of the numbers of cysts and white females under microscope.

The Table below shows the segregation patterns observed. Clearly, the parents showed clear discontinuous variation with the WAB 56-104 and CG lines resistant and the *O. Sativa* parents susceptible. This shows that the resistance of the Introgression line is derived from the *o. glaberimma* parent. Thus mapping of this resistance could proceed using the molecular markers.

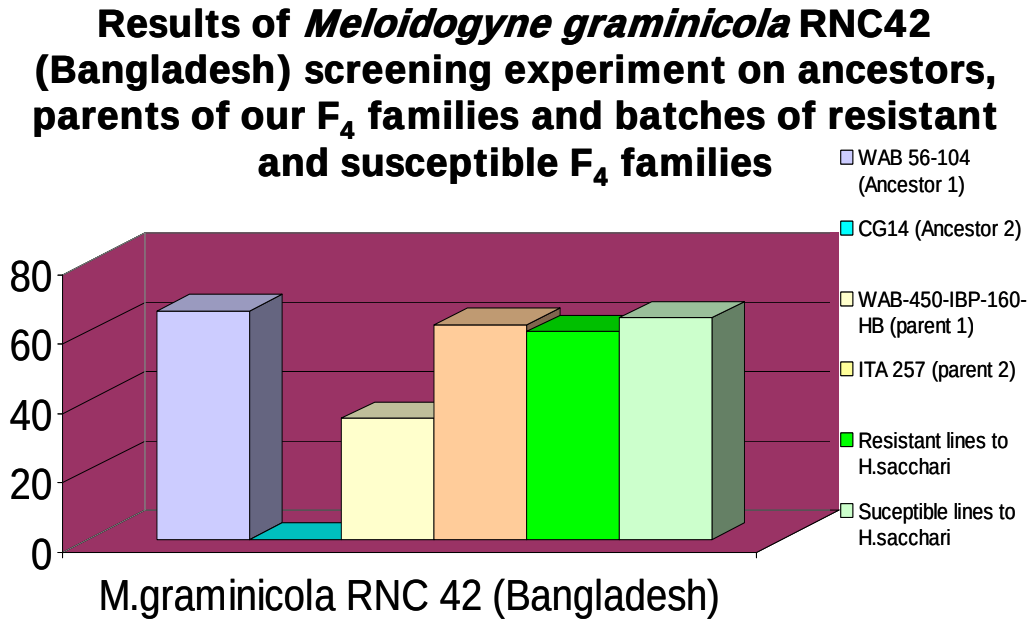
Results of *H.sacchari* RNC 71 (Ivory Coast) screening experiment on ancestors and parental lines

- *Response towards Meloidogyne graminicola.*

To extract *M.graminicola* J2's (RNC 42 isolate), galled roots from previously infected susceptible plants were washed free from soil in a bucket of water and finally washed over a 1mm sieve. Shoot heights before inoculation were recorded. About 100 J2's in 1 ml of water were inoculated, 15 days after planting, on the parents of the 105 F_4 families, the BIL ancestors and 8 selected highly susceptible and 8 selected highly resistant F_4 families towards *H.sacchari*. In this experiment, the plants were left infested for longer and a galling index used in place of nematode counts to determine rice plant susceptibility. At harvest, 23 DAI, shoot heights after inoculation were recorded. The soil was washed off the root by dipping in a beaker of water until all the soil is removed. Galling index was recorded according the percentage of galls on the roots. The root galling index was determined according to the following scale:0 = no galls; 1 = 10% galls; 2 = 20% galls; 3 = 30% galls, 4 = 40% galls; 5 = 50% galls; 6 = 60% galls; 7 = 70% galls; 8 = 80% galls; 9= 90% galls; 10 = 100% galls. Once scored, the roots were blotted to dry and weighted.

No resistance towards *Meloidogyne graminicola* was found to be segregating between the parents of the two mapping populations in glasshouse conditions – see below :

Results of *H.sacchari* RNC 71



- *Response towards Pratylenchus zeae.*

For the *P.zeae* testing, 100 J2's (of U23IRRI isolate), in 1ml of water were inoculated, 15 days after planting, on the parents of 105 F_4 families, the BIL ancestors, 2 selected highly susceptible and 2 selected highly resistant lines towards *H.sacchari* among the 105 F_4 families. At harvest, 50 DAI, roots were washed, weighted and immersed for 3 min in a boiling solution of equal parts lactic acid, glycerol and distilled water with 0.05% acid fuchsine. Roots were then cleared in a 50:50 mixture of glycerol and distilled water. Then, roots were cut into short lengths and placed in an electric mixer with 30 ml of water. Maceration of 10 sec is carried out to break open the plant material without damaging the nematodes. The macerated material was poured onto the tissue of a Baermann funnel and left for up to 144 hours. For each root sample, two subsamples of cleared maceration solution were taken within the first 48 hours and two others within 48 to 144 hours. The counts of J2's in 1 ml of cleared maceration solution were recorded and the total number of nematodes in the root system deduced as well as the density of nematodes per gram of root tissue.

No resistance was found toward *Pratylenchus zeae* neither in the ancestors nor in the interspecific parents or the batches of resistant and susceptible families towards *H.sacchari*.

The results of the phenotyping suggested that only resistance to *H.sacchari* exited in the mapping populations and all further mapping work was concentrated on this objective

2.2 QTL maps produced by combining resistance data and genotype characterisation

Two mapping populations were used for determining the inheritance of resistance to *H.sacchari*

- WAB 450-IBP-160-HB x ITA 257 (105 F_4 families)
- WAB 450-IBP-160-HB x OS6 (19 F_5 families)

The segregating families in these populations were populations were screened with polymorphic markers using a bulked segregant approach in the first instance to narrow down the area of the genome of interest. Genetic map distances was calculated using Joinmap 3.0 (Stam, 1993) .

To evaluate the segregation patterns in the populations the phenotypes of each of the segregating families was assessed at CABI for response to *H sacchaii* and the distributions were plotted – see below. Both populations behaved in a similar fashion and the discontinuous segregation observed indicated that resistance is controlled by a single recessive gene, see below.

Single recessive gene hypothesis tests

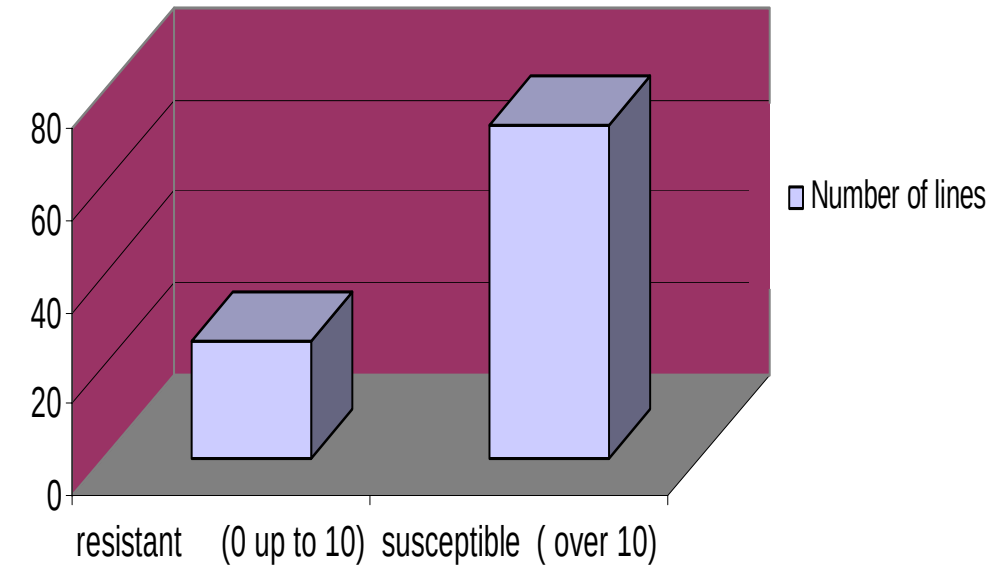
	Resistant		Susceptible	
	Observed	**Expected**	**Observed**	**Expected**
105 F_4 families	26	26.25	79	78.75

$\chi^2_{3:1}$=0.25, P>0.05

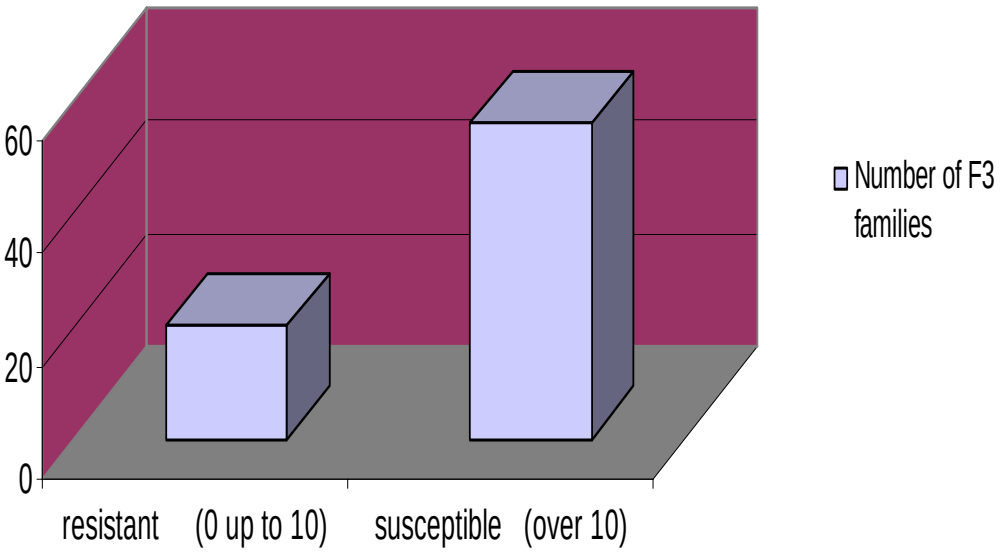
	Resistant		Susceptible	
	Observed	**Expected**	**Observed**	**Expected**
76 F_3 families	20	19	56	57

$\chi^2_{3:1}$=0.07, P>0.05

Division of 105 F_4 families tested with *H.sacchari* RNC71 into resistant and susceptible group

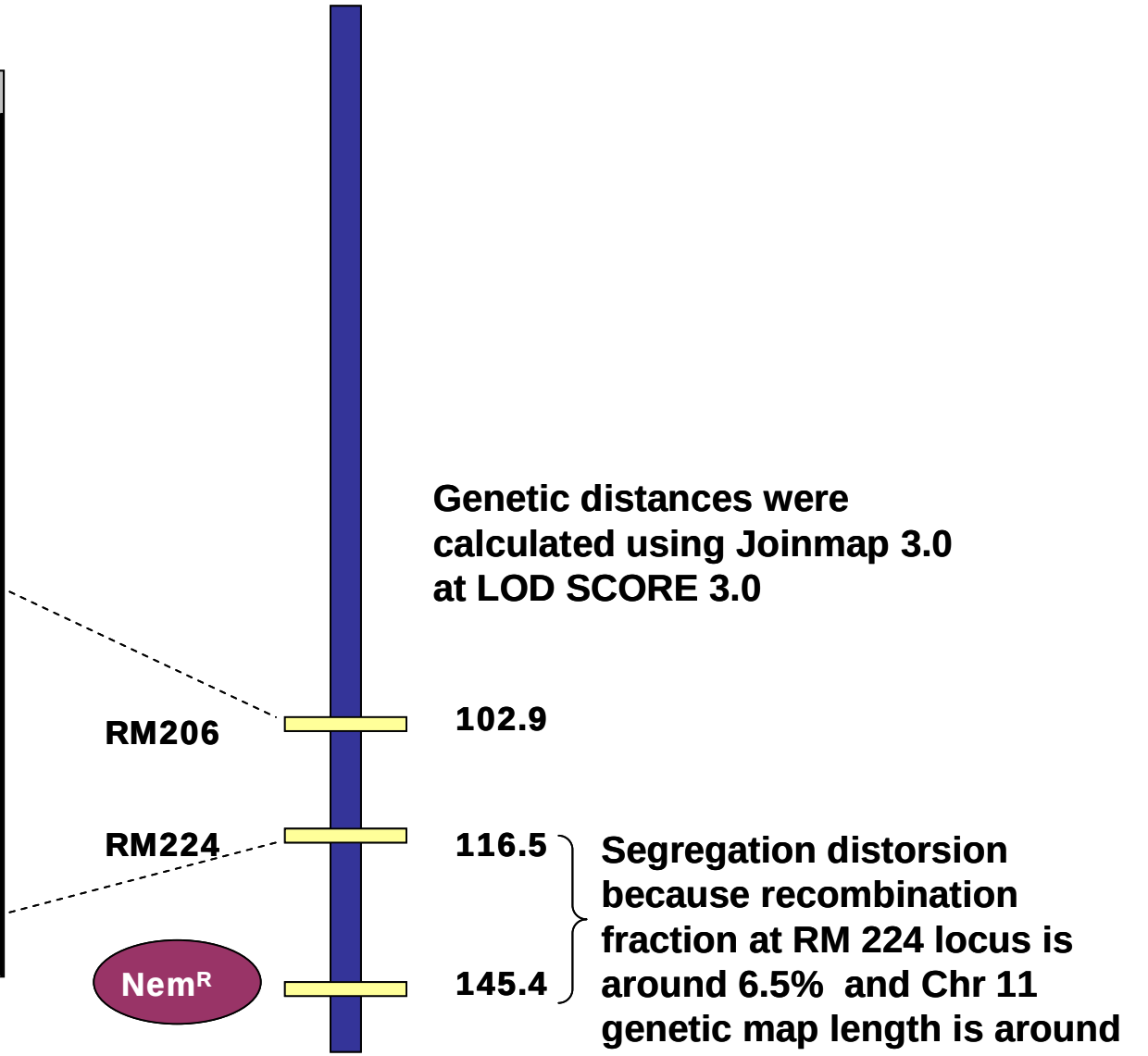


Division of 76 F_3 families tested with *H.sacchari* RNC71 into resistant and susceptible group



Microsatellites, CAPS, STS markers on Chromosome 11

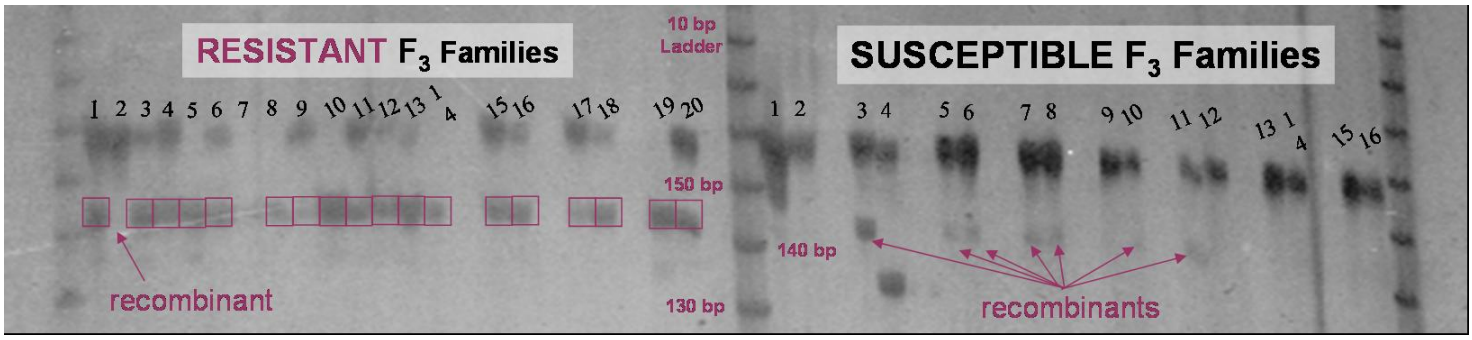
			F_4	F_3
RM 20B		0		
RM 4B		0		
RM286		0		
RM332		27.9		
RM167		37.5		
OSR 1		54		
RM202		54		
R728		64.2		
RM209		73.9		
RM229		77.8		
RM 21		85.7		
C189		85.7		
C50		89.5		
RM206		102.9		
E3676		108.5		
C950		110		
G181		112		
S12886		117		
C102955		117.3		
S1872		117.3		
E4420		117.9		
RM224		120.1		
RM144		123.2		



Genetic distances were calculated using Joinmap 3.0 at LOD SCORE 3.0

Segregation distorsion because recombination fraction at RM 224 locus is around 6.5% and Chr 11 genetic map length is around 123 cM on the consensus map

Using Bulk Segregant Analysis on the F_3 families (with at least 10 DNA samples per line), one microsatellite marker,RM 224 on Chromosome 11 appears to be reasonably linked to the resistance but in a dominant way. Also, the linked band (lower band on photo) segregated in a 3 : 1 ratio (51 : 21 with 4 amplifications failure) like our targeted gene and is present in most segregating and resistant F_3 families and absent or very weak in most of the susceptible F_3 families, confirming our single gene hypothesis.



In total, we found 10 recombinants (over 76 x 2 = 152 gametes in total) for that band indicating we are roughly at 6.5 cM distance from our gene. But this distance is likely to be over estimated by JOINMAP of the real distance from the resistant gene, as the length of chromosome 11 on the consensus map do not exceed 123 cM.

2.3 Diagnostic markers developed for marker-assisted selection

Since the mapping results indicates that there is a single recessive gene for resistance towards *H.sacchari*, located on chromosome 11. the one PCR-based marker linked to *H.sacchari* resistance is now available.

Contribution of Outputs

WARDA, which is the main conduit for promoting outputs and ensuring that technology reaches beneficiaries, have already the capacity to use marker-assisted selection (MAS) in conjunction with conventional breeding techniques. Once a more closely linked marker to this nematode resistance identified, this project will enable the Molecular Marker Group at WARDA to select efficiently rice plants carrying nematode resistance towards *H. sacchari*.

Project R7548-2 will provide enabling technology for marker-assisted selection (MAS) to be conducted in Africa and hopefully at WARDA. WARDA has already the capacity to use MAS in conjunction with conventional breeding techniques. Once a more closely linked marker to this nematode resistance identified, this project will enable the Molecular Marker Group at WARDA to select efficiently rice plants carrying nematode resistance towards *H. sacchari*. WARDA will be the main conduit for promoting outputs and ensuring that technology reaches beneficiaries in Africa.

Publications

None to date

Internal Reports

* M. Koyama, P-L Amoussou, J. Snape (2002): Mapping of natural resistance to rice yellow mottle virus and nematodes in rice interspecific crosses. DFID Annual report
* M. Koyama, P-L Amoussou, J. Snape (2001): Mapping of natural resistance to rice yellow mottle virus and nematodes in rice interspecific crosses. DFID Annual report

* M. Koyama, P-L Amoussou, J. Snape (2000): Mapping of natural resistance to rice yellow mottle virus and nematodes in rice interspecific crosses. DFID Annual report

Seminars

* P-L Amoussou (2003) Mapping of natural nematode resistance in rice for use by West African farmers. DFID Plant Biotechnology group meeting July 9th, John Innes Centre, Norwich, UK.
* P-L Amoussou (2003) Construction of an Oryza glaberrima Transformation Artificial Chromosome (TAC) library. February 13th. DFID Plant Biotechnology group meeting, London, UK.
* P-L Amoussou (2002) Mapping of natural nematode resistance in rice for use by West African farmers. Student Poster Interactive Talks (SPIT) site meeting. November 21st, Norwich, UK.
* P-L Amoussou (2002) Mapping of natural nematode resistance in rice for use by West African farmers. First General Meeting: Biotechnology, Breeding and Seeds Systems for African Crops. November 4-7th Entebbe, Uganda.
* P-L Amoussou (2002) Mapping of natural nematode resistance in rice for use by West African farmers. DFID Plant Biotechnology group meeting, July 25th, London, UK
* P-L Amoussou (2001) Transferable traits for rice in West Africa. Crop genetics research department seminar. June 25th , John Innes Centre, Norwich, UK.
* P-L Amoussou (2000) DFID-GATSBY-WARDA Rice Biotechnology Meeting, John Innes Centre, Norwich, UK.

Posters:

* P-L Amoussou, J. Ashurst, J. Bridge, M.P. Jones, J. Snape, M. Koyama. (2002) Mapping of natural nematode resistance in rice for use by West African farmers. First General Meeting: Biotechnology, Breeding and Seeds Systems for African Crops, November 4-7th Entebbe, Uganda.
  (This poster was voted "best poster" for the Crop Genetics Department at the John Innes Centre annual meeting, October 31st -November 1st, 2002)
* P-L Amoussou, J. Ashurst, J. Bridge, M.P. Jones, J. Snape, M. Koyama. (2002) Mapping of natural nematode resistance in rice for West African farmers. Plant, Animal and Microbes Genome conference X conference, January 12-16th San Diego, California, USA.
* P-L Amoussou, M. Koyama, J. Snape. (2000) Towards low-management rice for West African farmers. John Innes Centre annual meeting, November 2 -3rd Norwich, UK.

Other reports:

* P-L Amoussou (2003) UEA-JIC-Rockefeller Foundation. Annual reports -Year 3 Ph.D.
* P-L Amoussou (2001) UEA-JIC-Rockefeller Foundation. Annual reports -Year 2 Ph.D.
* P-L Amoussou (2000) UEA-JIC-Rockefeller Foundation. Annual reports -Year 1 Ph.D.

Follow-up activities

The Rockefeller Foundation, JIC and CIRAD are supporting a new follow-on project. This is a collaborative project between the John Innes Centre (JIC), the Centre de Cooperation Internationale en Recherche Agronomique pour le developpement (CIRAD) and the West African Rice Development Association (WARDA) funded by the Rockefeller Foundation (RF). Specifically, this Fellowship would enable the development of an *Oryza glaberrima* Transformation Artificial Chromosome (TAC) library. This library should contribute to finding additional markers which are more tightly linked to the *H.sacchari* nematode resistance gene and therefore more reliable to be used routinely for MAS by WARDA Plant breeding unit in the future.

Name and signature of authors of this report

R7548 project 1

Name: Philippe Vain

Signature:

Date:

R7548 project 2

Name: John W Snape

Signature:

Date: