R7415: FINAL TECHNICAL REPORT Date: 30/4/05

TITLE OF PROJECT: Containment testing of transgenic rice lines with resistance to RYMV, the development of markers to assist selection of nematode and RYMV resistance in WARDA introgression lines from *O. glaberrima* and *O. sativa* crosses

Executive Summary

The project set out to provide virological and molecular analysis of West African transgenic rice lines of varieties ITA212 and BG90-2 from the previous DFID PSP project R6355. In bioassays, resistance to a Nigerian strain of RYMV was found to be unstable with loss of resistance over generations. Molecular analysis showed that there was selection against resistant homozygotes.

It was also planned that these lines, and the previously characterised Bouake 189 resistant transgenic line10/12 with complete RYMV resistance (from project R6355), be evaluated for agronomic performance under African conditions. However, due to initial problems at WARDA with setting up their containment testing facilities and the subsequent government coups and war, WARDA was evacuated and abandoned and this part of the project was never realised.

T2 seed from resistant transgenic Bouake 189 line 10/12 has been sent to Bangor University, Wales for crossing experiments to check for transferability of the resistance in future breeding programmes. Before it was sent, the T3 generation of line 10/12 was bioassayed for complete RYMV resistance but was found to display only partial resistance consistent with natural multigenic partial resistance found in some japonica subspecies. This result is supported by a set of parallel experiments carried out at IRD, Montpelier, France (pers. comm. D. Fargette). If the current crossing experiments show that this resistance can be transferred, it could then be combined with other forms of RYMV resistance in rice gene pyramiding experiments in the continuing effort to overcome RYMV as a constraint to rice production.

The project also aimed to produce and characterise new lines with improved constructs against RYMV, free from undesirable selectable marker genes. Using the Positech^{TM} mannose plasmid construct from Syngenta, the construct PMI:RYMV3.4:AMP was made among others. Many transformation experiments using bombardment technology were carried out, including using just the PMI:RYMV fragment of the construct to avoid the ampicillin selectable marker (AMP) from the plasmid backbone and address biosafety concerns. However, although transformed rice plants were produced using PMI selection, none of the lines showed good resistance to RYMV in subsequent bioassays.

Background

Rice yellow mottle virus (RYMV) disease is endemic to Africa where it was first reported in Kenya in 1966. It has only become a serious disease of epidemic proportions since the introduction of highly susceptible exotic Asian varieties of rice.

RYMV is a very serious disease in most rice growing regions of West Africa, East Africa and Madagascar. These environments have been targeted as regions for the greatest future rice expansion with greatest yield potential. Yield losses have been more than 60 % in some irrigation schemes. No other disease is rated as highly in terms of importance as RYMV in the lowlands and irrigated rice ecosystems (WARDA Medium Term Report, 2000).

The need for this project was identified and driven by WARDA and carried out in collaboration with, and funded by, DFID. At that time, only partial quantitative resistance to RYMV had been identified in some *japonica* subspecies of *Oryza sativa*. More recently varieties / lines such as Gigante and TOG 5681 have been shown to have complete resistance to RYMV via a single gene. Transformation technologies have great potential to increase rice productivity with disease resistance. Thus, this project R7415, and its predecessor R6355, set out to develop transgenic resistance that could be

combined with existing alternative sources of resistance, in an effort to provide durable resistance to RYMV and more sustainable yields for smallholder farmers in Africa.

The previous project, R6355, produced transgenic African rice varieties (Bouaké 189, BG90-2 and ITA 212) using bombardment-mediated transformation with RYMV transgenes from a Nigerian isolate (Pinto *et al.*1999). Transgenic Bouaké 189 exhibited complete resistance to RYMV (virus particles or RNA inoculum) at a range of concentrations (Pinto et al, 1999). Therefore, continued use of this method to produce potentially resistant rice lines with improved transgenic constructs was thought prudent. In addition, the resistance of the transgenic Bouaké 189 lines was consistent with a homology dependent mechanism, which has been demonstrated for several plant viruses to date, (Sanford & Johnson, 1985). A pathogen gene, here a highly conserved region of the RYMV genome, expressed in the host, stops the pathogenic process. The strategy relies on a high level of homology between the expressed transgene and the incoming virus.

Successful genetic transformation requires not only efficient gene delivery, but also an efficient selection system to distinguish transgenic from non-transgenic events (Potrykus & Spangenberg, 1995). Several different selection strategies have been used in cereals, mainly using antibiotic or herbicide resistance genes.

However, there have been concerns over the use of these selective markers and although risk assessment reports have suggested that there are no scientific, health or safety reasons to restrict the use of some of these selectable markers, this may not apply to all of them. Concerns have been raised that the selectable marker genes could be transferred into microbes and increase the number of resistant pathogens, or that horizontal transmission of the marker gene into wild relatives may result in weedy pests (Dale, 1992; Nap et al, 1992). The use of some selective agents may have negative effects not only on non-transformed cells, but could also limit the regeneration of transgenic tissue. Elimination of marker genes is therefore a desired aim.

Several strategies to eliminate marker genes have been employed. Co-transformation using binary vectors carrying two separate T-DNAs were used for rice transformation and the segregation of the transgenes in the progeny allowed the retrieval of marker-free plants (Komari et al, 1996). Transient expression of the *cre*/*lox*P site-specific recombination system (Dale & Ow, 1990; Gleave et al, 1999) together with a conditional dominant lethal gene *cod*A (Perera et al, 1993) resulted in the elimination of marker genes from transgenic plants without sexual crossing.

Systems allowing the selection of transgenic tissue with the help of sugars that cannot be metabolised by untransformed cells, have also been used. These include the xylose isomerase gene and the phosphomannose isomerase (PMI) gene. This selection strategy using selectable marker genes based on a physiological advantage rather than detoxification is called positive selection. This is because the selection gives a clear advantage to the transformed cells rather than killing non-transformed cells. Mannose has been reported as a novel selectable agent and the phosphomannose isomerase gene (*pmi*) as its suitable selectable marker (Joersbo and Okkels, 1996; Lucca et al, 2001). Recently, transgene removal systems denoted as Multi-Auto-Transformation (MAT) vectors have been developed to generate marker-free transgenic plants through a single step transformation (Ebinuma & Komanine, 2001; Ebinuma et al, 2001).

Most plants except legumes are unable to utilize mannose-6-phosphate as a carbon source. The introduction of the phosphomannose isomerase (*pmi*) gene during transformation makes this an ideal system for positive selection whereby plants containing the gene are able to survive on a medium containing mannose. Plant cells transformed with this gene can convert mannose-6-phosphate to frustose-6-phosphate that is easily metabolised. Mannose selection therefore provides a metabolic advantage to transformed cells while the untransformed cells are starved and lose their viability (Joersbo et al, 1998).

This marker has been found to be very effective in the selection of wheat and maize transgenics, although not much information is available on the selection of rice (Lucca et al. 2001). Use of the PMI selection system resulted in higher transformation frequencies in maize and wheat possibly because toxic compounds are not released on the death of cells as with other selection systems using herbicides or antibiotics (Wright et al, 2001). Marginally expressing transgenes may also be recovered. Extensive safety tests have also been done (Privalle et al, 1999). Several different explants, for example, callus, embryos, protoplasts, cotyledons, floral buds and microspore embryos have been used for transformation procedures using biolistic or *Agrobacterium*-mediated techniques followed by selection using mannose (Table 1). Transformation frequencies have varied from 0.94% sugarbeet cotyledons (Joersbo et al, 1998) to 50% for maize callus using biolistics (Wright et al, 2000).

Table 1: Plant systems for which PMI and mannose selection have been used (Privalle et al, 2001).

Plant	Transform. method	Target tissue	Transf. freq. (%)	Reference
Maize	Biolistic	Callus	50%	Wright et al, 2000
	Agrobacterium	Embryos	30%	Negrotto et al 2000
	Protoplasts	Protoplasts	-	Evans et al 1996
Wheat	Biolistic	Callus	25%	Wright et al, 2000
Sugar beet	Agrobacterium	Cotyledons	0.94%	Joersbo et al, 1998
Arabidopsis	Agrobacterium	Floral buds	-	Melanson et al, 1999
Rice	Agrobacterium	Embryos	10-20%	Unpublished
	Agrobacterium	Embryos	41%	Lucca et al, 2001
Canola	Agrobacterium	Microspore embryos	1%	Unpublished
Barley	Biolistic	Embryos	3%	Unpublished
Tomato	Agrobacterium	Cotyledonary petioles	-	Unpublished

Project Purpose

Project R7415 set out to characterise any resistance found in the previously produced transgenic BG90-2 and ITA212 varieties (project R6355), and to develop new transgenic lines with improved bombardment-mediated transformation constructs that addressed current biosafety concerns (use of the PMI gene as the selectable marker) and using locally adapted West African rice varieties. Sequence analysis of the RYMV replicase gene of different RYMV isolates was also carried out to ensure sufficient homology with that part of the Nigerian RYMV sequence used in the transgenic constructs. This was done in order to ensure a broad-spectrum resistance. This project also extends work that was previously carried out, Plant Sciences Programme Project - R6355; Genetically engineered resistance to rice yellow mottle virus (RYMV).

Research Activities

1.1 Bouake 189 transgenic lines tested against RYMV isolates from Ivory Coast in containment and risk analysis assessments made.
1.2 Mock trials set up in containment.

2.1 Resistance of transgenic BG90-2 and ITA212 tested with different inoculum types / concentrations.
2.2 Molecular analysis of transgenes.
2.3 Resistance phenotype tested for stability over generations (T_1-T_4)
2.4 Resistance tested with different RYMV strains including new ones identified in 3.

3.1 Collection and purification of different isolates and characterisation by RT-PCR.
3.2 Calculation of phylogenetic distances between isolates initiated.

3.3 Design of new transgenes to any isolates that overcome resistance at WARDA or JIC.

4.1 Transformation of 3 varieties with improved transgenes and regeneration from tissue culture attempted.
4.2 Transgene expression in transformed lines ascertained.
4.3 Optimize transformation with new particle gun and mature seeds protocol.
4.4 Crosses set up at Bangor to confirm transferability of transgenic resistance to susceptible material.

The activities 1.1 and 1.2 were not achieved due to the war that broke out in the Ivory Coast in 2002 resulting in WARDA closing down. There was also considerable civil unrest and military coups that had hindered the project in this respect previous to the war breaking out. Research activity 2.4 was not carried out because no stable resistance could be found in the ITA212 or BG90-2 transformed lines from project R6355 and so it was not relevant to bioassay with different RYMV isolates. Activities 3.1 and 3.2 were carried out, however, all sequence data is stored on a designated server (jii04) at the John Innes Centre and, at the time of writing the report, the author could not access the server or the programs to display similarity indices or phylogenetic distances. However, the results of these experiments are summarised here in this report. Similarly activity 3.3 was not necessary to do since resistance to RYMV was never tested for at WARDA in the transgenic lines and the partial breakdown of resistance in the Bouake189 10/12 transgenic line was only confirmed at the end of the project.

Outputs

1. Containment testing of transgenic rice lines at WARDA

As a result of the war that broke out in the Ivory Coast in 2002, WARDA closed down. Previous to this, problems WARDA experienced with the building contractors in the construction of the containment facilities together with general political unrest and military coups hindered greatly this aspect of the project.

2. Transgenic rice lines from varieties ITA212 and BG90-2 molecularly characterised and bioassayed.

Fifty-seven progenies derived from transgenic rice varieties ITA212 and BG90-2 resistant at the T1 generation, were characterised for transgene integration pattern and stability over generations (T0 to T3) and bioassayed against the Nigerian isolate of RYMV. These lines derived initially from eight bombardment-mediated transformation events (from DFID project R6355), six from ITA212 and two from BG 90-2 (Table 2). Although good resistance to RYMV was seen at T1 in the bioassays (Figure 1), the resistance deteriorated over subsequent generations. From the molecular analysis, no homozygous T2 or T3 RYMV resistant lines were found in the 57 progenies. Thus, there appeared to be some early selection against resistant homozygotes and subsequent loss of resistance over generations in this material. As the resistance was unstable no further work was carried out with this material and it is now in storage (JIC seed store).

Table 2: Molecular, genetic and phenotypic analysis of progenies derived from transgenic ITA212 and BG90-2.

RYMV-Resistant Clone (T1)*	T1 +ve PCR for transgene R = resistant	No. of bands in Southern analysis	No. T2 families	T2 +ve PCR for transgene	Resistance phenotype S=susceptible R=resistant
ITA212 3.4-3	Yes R	7	1	0	S
ITA212 3.4-11	Yes R	2	7	0	S
ITA212 3.4-14	Yes R	2	10	1	1R + 9S
ITA212 2.7-4	Yes R	5	9	2	2R + 7S
ITA212 2.7-6	Yes R	3	4	0	S
ITA212 2.7-14	Yes R	3	7	0	S
BG90-2 2.7-7	Yes R	2	6	4	4R + 2S
BG90-2 2.7-10	Yes R	2	13	9	9R + 4S

*Plants transformed with HYG:RYMV:GUS:AMP (DFID project R6355)

Figure 1: Comparison of phenotype between resistant transgenic and susceptible non-transgenic ITA212.

3. Sequencing of RYMV isolates

Thirty-one RYMV isolates were collected (Table 3). Purification and sequencing of the majority of isolates (at least 20) were carried out, however, all sequence data is stored on a designated server (jii04) at the John Innes Centre and, at the time of writing the report, the server or the programs to display similarity indices or phylogenetic distances could not be accessed. For DFID PSP progress

meetings phylogenetic trees were produced using the program PHYLLP from the sequence data available at that particular time. Virus particles of 31 RYMV isolates are stored at the JIC. Much more detailed phylogenetic sequence analysis of RYMV has been carried out at IRD, Montpelier (D. Fargette research group). For the purposes of this project, sequence analysis was used purely to ensure the necessary level of homology between the RYMV transgene sequence and the corresponding sequence of the attacking virus isolate to effect the pathogen-derived homology-dependent resistance mechanism.

Table 3: The RYMV isolates sent mostly from WARDA to JIC for sequence homology analysis.

Mali	Kleila
	Niono 4
	Kayo
	Longorola
Nigeria	Edozigi
Niger	Bonfeba
	Kollo
	Kirkissaye
	Say 1
Burkina Faso	Banzon 3 & 4
	Banfora
Ivory Coast	Mbe 1&2
	Sassandra 1&2
	Gagnoa P, 00 & 90
	Guehiebli 1 & 2
	Odienne 1 & 2
	Sakassou 1 & 2
	Tapeiguia 1 & 2
	Danane 1 & 2
	Korhogo
Uganda	Uganda 1

4. New Rice Transformation.

Optimization of new particle gun and development of the mature seeds protocol for African varieties Bouake 189, ITA212 and BG90-2.

The particle gun used for the previous project (R6355) was replaced with the BIORAD particle gun at the start of this project. This necessitated an initial period of optimisation of the protocols used for the transformation experiments where different bombardment conditions and target explants were tried using mainly the GUS:HYG:AMP transformation construct. Observations were made for selection on media, regeneration and a series of GUS assays to study transient expression after the transformation experiments were carried out. As a result of the optimisation, embryogenic calli derived from proliferating scutella of mature zygotic embryos of *indica* rice varieties, Bouaké 189, BG90-2 and ITA 212; and from small callus pieces from mature zygotic embryos of *japonica* variety Nipponbare (Vain et al., 2003), were used as target tissue for gene delivery. The latter target tissue from the Nipponbare variety was optimised for bombardment in order to provide a high throughput system in which novel constructs could be tested quickly. This high throughput system was not suitable for the African rice varieties. The details of these experiments including the culture of mature zygotic embryos can be found in Appendix 1.

Improved Trangenes and Rice Transformation

The widely-used Positech mannose construct was obtained from Syngenta in June 2001. Subsequent cloning gave rise to 2 constructs: PMI:GUS:AMP and PMI-RYMV3.4:AMP. Another alternative marker gene, tryptophan decarboxylase (TDC), had also been obtained for which a construct TDC:GUS:AMP was made to test its selective effectiveness. However, as the focus was on the PMI constructs which has a proven selective capacity in rice, all time and effort was placed in the PMI constructs and the TDC construct was stored at JIC for potential future use.

All transformation experiments are detailed in Table 4. Before using the PMI-RYMV3.4:AMP construct the PMI selection system was optimised using the GUS-PMI:AMP construct in order to see how many transformants could be recovered. Eight experiments using 60 immature embryos of Bouaké 189, 85 callus pieces of BG90-2 and 886 callus pieces from Nipponbare were bombarded with GUS-PMI:AMP. Of these the calli of the African variety Bouaké 189 necrotized one week after bombardment. For BG90-2 the calli went through a series of subculturing events but when a GUS assay was performed on the calli none were found to be GUS positive and no regeneration took place. However, for Nipponbare, of the 886 bombarded callus pieces, 243 were sampled for the GUS assay and 159 of them were GUS positive (between two and ten weeks after bombardment). Many plant lines were then regenerated and 73 of them, not previously assayed at the calli stage, were GUS assayed 4 months after the initial bombardment with no positive results. This showed that African varieties were much more difficult to transform and regenerate and that although Nipponbare seemed initially to be easy to transform (as shown with the positive GUS assay results on calli lines) and regenerate, there was a risk of loss of plasmid/gene function during the regeneration process.

Following these experiments with the GUS:PMI:AMP, a series of transformation experiments using the construct PMI:RYMV3.4:AMP took place. This construct was similar to the construct used in the previous project R6355 in that it contained the full-length replicase gene of RYMV (3.4 Kb), but we had greatly improved the construct by removing the hygromycin selective marker and the GUS reporter gene, and replacing these with the new PMI selectable marker from Syngenta. However, the plasmid still had the ampicillin resistance gene (AMP) present in the backbone. As it was necessary to devise a strategy to remove this and it was going to take some time, it was thought prudent to go ahead with this set of transformation experiments using the whole PMI:RYMV3.4:AMP plasmid.

Table 4: Details of all bombardment transformation experiments carried out.

Cultivar	Explant	n	Construct	Result
Bouké189	Immature embryo	60	GUS:PMI:AMP	No plants
BG90-2	Callus pieces	85	GUS:PMI:AMP	No plants
Nipponbare	Callus pieces	886	GUS :PMI:AMP	159 callus lines GUS +ve, but no GUS +ve plant lines
Nipponbare	Callus pieces	1008	PMI:RYMV3.4:AMP	118 plantlets from mannose^R calli 4 transgenic plant lines (RYMV +ve and fertile)
Bouaké189	Mature embryo	160	PMI:RYMV3.4:AMP	No plants
ITA212	Mature embryo	65	PMI:RYMV3.4:AMP	No plants
Nipponbare	Callus pieces	1300	PMI:RYMV3.4(-AMP)	138 mannose^R calli →plants
Bouaké189	Mature embryo	560	PMI:RYMV3.4(-AMP)	7 plants from mannose^R calli
ITA212	Mature embryo	330	PMI:RYMV3.4(-AMP)	2 plants from mannose^R calli
BG90-2	Mature embryo	445	PMI:RYMV3.4(-AMP)	No plants
BG90-2	Callus pieces	230	PMI:RYMV3.4(-AMP)	5 mannose^R calli → plants

Of the 1008 Nipponbare callus pieces bombarded with the PMI:RYMV3.4:AMP plasmid, 118 plant lines were regenerated that were mannose resistant. Dot blots and PCR assays were performed on all these lines to check for the presence of all three genes together with controls which were the genomic probe R2272 for the Dot blots and the *odh* gene for the PCR assays. Of the 118 plant lines, 4 lines were found to be positive for all three genes and were fertile. Seed was collected from these lines and bioassays carried out on the T1 generation with whole RYMV virus particles (from infected leaf sap) and with infective RYMV RNA (80 ng/plant). All four lines showed RYMV symptoms, chlorosis in youngest leaves, necrotic tips and brown streaking, 2 of the lines showed less severe symptoms (C12 and C3) but all lines had a good seed set except one line (C4). The T0 and T1 seed of these lines have been stored.

For the African lines, 160 Bouake 189 and 65 ITA212 callus pieces were bombarded with PMI:RYMV3.4:AMP. However, no plantlets were regenerated from the bombarded calli.The final set of experiments involved a series of bombardments with a fragment PMI:RYMV3.4 (-AMP). The fragment was produced through complete restriction enzyme digestion of the PMI:RYMV3.4:AMP plasmid using SacII. The digest was then run at 4C, 70 V for 24 hours. This gave rise to two well-separated bands on the gel, one 8.75 Kb PMI:RYMV fragment and one 1.8 Kb fragment of the ampicillin gene. The fragments was extracted, purified and quantified for use in bombardment experiments.

For Nipponbare, 1,300 callus pieces were bombarded with the PMI:RYMV3.4-AMP fragment from which 138 independent plant lines were regenerated on mannose selection media. At the callus stage, a102 of these (available at that time) were DNA extracted for dot blot and PCR analysis and five calli were found to be positive for RYMV and PMI (5/8:C108, C109, C112, C126, C127). However, DNA samples from the regenerated plantlets of these positive calli were obtained in due course and unfortunately, at that later stage, none were found to be PMI positive or RYMV positive. These plants were not bioassayed.

For the regenerated African lines, 6 independent Bouake 189 plant lines and 2 ITA212 plant lines resulting from bombardment experiments with the PMI:RYMV3.4:-AMP fragment also had DNA extracted from them for dot blot and PCR analysis but they were found to be negative for the presence of both genes.

However, due to time constraints (Miki Koyama going on maternity leave in June and Barbara Woodward leaving in July 2003) there were some mannose^{R} regenerated plant lines that were not molecularly analysed. These included 1 Bouake 189 (27/11) plant line,36 of the 138 Nippobare plant lines (25/2 and 11/3) and 5 BG90-2 plant lines (25/2 and 11/3). These should be in storage at –70 °C freezer at the John Innes Centre for future analysis.

Breakdown of complete transgenic resistance in Bouake 189 (R6355)

In preparation for sending the resistant Bouake 189 T2 seed (Pinto Y et.al. 1999) to the University of Wales, Bangor for crossing experiments, we first bioassayed the T3 generation with both whole virus particle and viral RNA inoculations. We found that the whole T3 generation had lost its complete resistance and was now partially resistant. Plants would show full symptoms initially and then appear to recover and set seed, whilst the control plants remained very sick. IRD, Montpelier, France have also carried out a comparative experiment with this seed against other lines / varieties with RYMV resistance and reported that these transgenic lines now displayed resistance consistent with the natural multigenic partial resistance found in some japonica subspecies.
Both the T0 and T2 seed of this resistant Bouake 189 line have now been sent to Bangor for crossing experiments to demonstrate the transferability of this partial transgenic resistance. If this resistance can be transferred it can then be combined with other natural multigenic partial and monogenic complete resistances to RYMV. Aliquots of RYMV virus for bioassays and non-transgenic seed of the

African varieties of Bouake189, BG90-2 and ITA212 for use in the crossing experiments have been sent to Bangor.

Contribution of Outputs

This project has shown that the Positech mannose-based selection system can be used for rice transformation but can lead to a large number of escapes. Transgenic resistance based upon the introduction of the RYMV replicase gene in the plant genome of African (R6355) or Japonica (R7415) rice varieties via particle gun bombardment does not lead to high and stable levels of resistance against RYMV. Partial resistance observed in some japonica transgenic lines could be due to natural multigenic partial resistance found in some japonica subspecies, however, in bioassays, transformed lines had generally less symptoms than the controls and set seed. Putative transgenic plants developed during R7415 will be molecularly characterised further by DFID project R8031 and bioassayed against RYMV if transformed. Positech Mannose-based selection could provide, if developed further, "clean" transformation technology for plant species where no other alternative currently exists for producing marker-free transgenics. T2 seeds from the partially resistant transgenic Bouake 189 line 10/12 (DFID project R6355) have been sent to Bangor University, Wales for crossing experiments to check for transferability of the resistance in future breeding programmes. If transgenic partial resistance is confirmed in this material under controlled conditions and is found to be transferable, it could benefit rice farmers in Africa.

Posters

Koyama ML, Woodward B, Amoussou P-L, Snape JW (2002) "GM rice in Africa? Complementing biotechnology with conventional solutions against the devastating rice yellow mottle virus" Presentation in the House of Commons, London.

Woodward B, Snape JW, Koyama ML (2002) "Resistance to Rice Yellow Mottle Virus disease in African rice" Poster presentation at the 10th IAPTC & B congress (International association of Plant Tissue culture and biotechnology).

Proceedings

Koyama, ML. 2000. "Resistance to rice yellow mottle virus disease in African rice – complementing biotechnology with conventional solutions". Proceedings of the International Conference on Sustainable Agriculture in the Next Millennium - the Impact of Modern Biotechnology on Developing Countries. Brussels, Belgium.

Seminars

2000-2003 Biennial DFID Biotechnology Group progress meetings, London, UK (7 seminars).
April 2002: Visit to WARDA, Bouake, Cote d'Ivoire, to attend and contribute to the Rice Regional Research Review meeting and discuss projects with collaborative scientists.
July 2001: CIRAD meeting. John Innes Centre, Norwich, UK
June 2000 "Introduction to Biosafety / Risk Assessment Issues for Transgenic RYMV Resistance." John Innes Centre, Norwich.
2000. DFID-Gatsby-WARDA Rice Biotechnology Meeting, John, Innes Centre, Norwich, UK.
June 2000 "Transgenic resistance to RYMV / Mapping RYMV resistance in interspecific crosses." IRD / CIRAD, Montpelier, France.
May 2000 "Resistance to RYMV in West African Rice –Complementing Biotechnology with Conventional Solutions." Transgenic Crops for Sustainable Agriculture Conference, Brussels, Belgium
May 2000 "Transgenic RYMV resistance and mapping QTLs for natural resistance." West African Rice Development Association WARDA, Ivory Coast

Appendix 1

Materials and Methods

Plant material

Embryogenic calli, derived from proliferating scutella of mature zygotic embryos of *indica* rice varieties, Bouaké 189, BG90-2 and ITA 212, and from small callus pieces from mature zygotic embryos of *japonica* variety Nipponbare, were used as target tissue for gene delivery. For the *indica* varieties mature zygotic embryos were cultured for 5-7 days on a proliferation medium (MSM or N6) containing 2,4-D. Small callus pieces were derived from three week-old cultures of proliferating scutella, according to Vain et al, 2003. Optimization experiments for culture of mature zygotic embryos were carried out as follows:

Optimization of mature embryo culture

The culture of mature embryos of the African varieties, Bouaké 189, BG90-2 and ITA212 was investigated to obtain an optimal method for later transformation with the PMI construct. Seeds of the cultivars were dehusked and sterilized in 79% ethanol (3min) followed by rinsing in sterile distilled water. This was followed by a sterilization step in 6.5% sodium hypochlorite (10min), followed by three rinses in sterile distilled water. The seeds were cultured on proliferation medium (MSM, N6 or MS4), for 5-7 days in order to allow the scutellum to proliferate. Following this step the proliferated scutella were excised and subcultured through a series of proliferation, regeneration and germination steps, to produce plants. This is outlined in the diagram below.

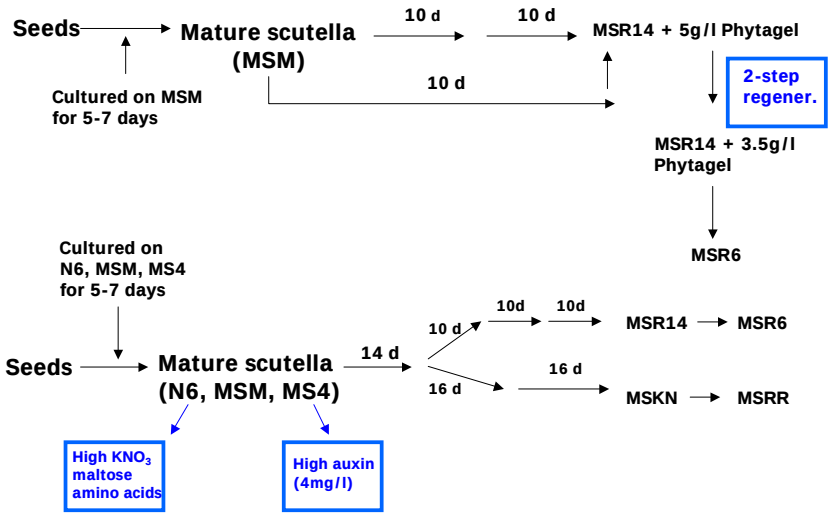

Figure 1. Diagram to show scheme of proliferation, regeneration and germination in the optimisation of mature embryo culture of the African lines Bouaké 189, BG90-2 and ITA212.

Mature proliferated scutella were bombarded with the HYG:GUS construct in order to examine transient expression levels to determine the optimal length of initial time on proliferation medium prior

to bombardment and regeneration. Mature embryos of the three cultivars were bombarded at 5, 6 and 7 days on proliferation medium according the method below. Scutella were incubated overnight at 26^0C and then stained for GUS expression by counting the number of blue spots. Histochemical staining of tissue was done using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson (1987). Scutella were vaccum infiltrated with a solution containing 0.5mg/ml X-gluc, 50mM sodium phosphate, pH 7.0, 1mM EDTA, and 0.5% Triton X-100 and incubated overnight at 37^0C. Tissue was examined for blue spots using a binocular dissecting microscope.

Transformation

Transformation frequency is defined as the number of independent events recovered per explant bombarded. Each rice target plate was composed of between 16-25 proliferating mature embryos with the scutella uppermost (about one week old), or about 50 small callus pieces (1-2mm) arranged in a $1cm^2$ area in the centre of a 50mm petri dish. Tissues were exposed to a high (0.4M) osmotic treatment (plasmolysis) for 4 hours prior to bombardment. For both explant types the proliferation medium (MSM, N6 or NB) supplemented with 36.4g/l mannitol and 36.4g/l sorbitol served as the osmotic medium.

Fragment DNA was precipitated onto gold microcarriers (0.9 or 0.7 µm) as described in the DuPont Biolistics™ Manual. Genes were delivered to the target tissue cells using the PDS-1000He Biolistics™ device. The settings were as follows: 20mm between the rupture disc and the macrocarrier, 10mm between the macrocarrier and the stopping screen and 5cm between the stopping screen and the target. The rice target plates were shot with 0.3µg DNA using 1100psi rupture discs. Following gene delivery the tissues were incubated overnight in the dark at 26^0C. Following this, callus was subcultured onto fresh proliferation medium and incubated in the dark for 2-3 weeks.