Appendix 1C: FINAL TECHNICAL REPORT
R6453: Transgenic Crop Resistance in Upland and Lowland Rice to Nematodes

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1: EXECUTIVE SUMMARY

Nematodes have been identified as a major pest problem in DFID reports on many crops including upland and lowland rice. The objective of this work is to develop a general defence against a wide range of nematodes that infect rice. Once this has been achieved, the defence would be suitable for the wide range of transformable crops in subsistence agriculture that suffer problems with *Meloidogyne* spp (root knot nematode) or other nematodes.

There were three phases in the generation of transgenic rice in this project. Constructs were designed and made in *Centre for Plant Sciences* (CPS). Biolistic rice transformation was carried out at *John Innes Centre* before protein expression and assessments of efficacy against nematodes was evaluated in Leeds. New containment glasshouses provided by University of Leeds (55%), BBSRC (45%) and DFID (5%) funds enhanced our capacity for such trials.

A promoter from a root-specific tubulin has been shown to provide root-specific expression in rice. It is active at the majority of infection sites of *Meloidogyne* but it is possible that *Pratylenchus* can develop in older root tissues where the promoter is less active. The extent that this may limit future efficacy of the defence against this nematode has yet to be evaluated. Root-specific expression is important in ensuring no new protein is expressed in foods made from transgenic rice plants.

The first demonstration of transgenic resistance in any monocot to nematodes was achieved using a constitutive promoter (CaMV35S) and protein engineered rice cystatin (Oc-IΔD86). The level of resistance was c65% over a single generation. It was not sufficiently close to our target of >80% / generation to justify evaluation in field trials. A constitutive promoter should provide high levels of expression which are important in work to select the cystatin of greatest interest. The constitutive promoter ubiquitin was used to replace CaMV35S using both Oc-IΔD86 and chicken egg-white cystatin (CEWc). The latter was used as a standard for comparison with plant cystatins as it has the highest known affinity for any protein inhibitor of the cysteine proteinase, papain. The constructs did not provide sufficient expression to justify challenge of plants with nematodes. Constructs driven by the CaMV35S promoter did express CEWc. These plants did not show a higher level of resistance than achieved in a similar construct with Oc-IΔD86 as the cystatin. However they were used to show expression of a cystatin was not detrimental to the vegetative growth of the rice plants.

A construct was made using the root-specific promoter from a tubulin gene to control expression of CEWc. This provided only low levels of expression of the cystatin (<0.1% total soluble protein) but the promoter is known to be more active in feeding sites of *M. incognita* than generally in the root system. This effect may ensure measurement of cystatin expression for uninfected roots is not a reliable basis for separating expressing from non-expressing transgenic plants. Comparison with wildtype of the same cultivar ITA212, established that transgenic plants showed a statistically significant level of resistance of c 91% and 88% in two assays. This method of expressing the results does not summarise the effect accurately. Many transgenic plants provided no eggs after 63 days of infection with *M. incognita*. In total 38 of 56 transgenic plants provided no eggs whereas the corresponding value for untransformed rice of the same cultivar was only 4 of 18 plants. This effect is very highly statistically significant and exceeds our target level of resistance set for the project.

Further constructs are being inserted into rice and will be bioassayed later to obtain lines expressing a cystatin naturally expressed in maize or rice seed. The main future priority for the approach is to demonstrate stable expression and high resistance levels over many generations. Establishment of homozygous lines, consideration of biosafety issues plus extensive containment and field trials must be completed before this royalty-free technology can be distributed through WARDA, IRRI and LARS to poor rice farmers. The approach has received much publicity as a distinctive and important contribution of UK science to plant biotechnology.

2: BACKGROUND

Work at IRRI has suggested that *Meloidogyne* spp and *Pratylenchus zeae* occur in 50% and 100% of upland rice fields causing losses of 0-75% and 0-50% respectively (see Chapter 3 in Luc. M, Sikora, R. A. & Bridge, J. 1990, In *Plant Parasitic Nematodes in Sub-Tropical and Tropical Agriculture, CABI, Wallingford, 629pp ISBN 0-85198-630-7* for a summary of the pest status of nematodes on rice).

Resistance to nematodes is measured as a reduction in egg numbers relative to a susceptible cultivar. Effort has concentrated on *Meloidogyne* (root knot nematodes) because RNRRS reports have previously identified this as an important crop pest problem on wide range of crops (e.g. see RNR Annual Report for 1991, p 13). Once efficacy has been achieved in rice the technology can be readily adapted to other crops. Our expectation is that transgenic rice with a high level of resistance to *Meloidogyne* would be also prove resistant to *P. zeae* and *Hirschmanniella* spp. Unfortunately the loss of the post-doctoral worker to a permanent post meant that the project had a staffed duration of only 2.5 rather than 3 years. Therefore the final phase of testing with these two nematodes was not carried out. This work will be completed in the near future within other research projects (R7294).

We set the research objective of achieving >80% resistance for a transgenic cultivar over a single generation of *Meloidogyne*. This would provide a higher level of resistance than this value at harvest providing each generation of the nematode was curtailed to a similar extent. Protection is particularly important in the early growth of the crops.

The work programme centred on construct design. Constructs were optimised to establish effective levels of resistance and to develop a promoter for root-specific expression. Constructs were then transferred to Dr P. Vain at John Innes Centre, Norwich (JIC) where all rice transformation was carried out. After a minimum of about 9 months plant material was returned to Centre for Plant Sciences, University of Leeds for evaluation of expression at the protein level and subsequent challenge by nematodes in a containment glasshouse.

3: PROJECT PURPOSE AS DEFINED IN PROJECT MEMORANDUM

This project will demonstrate that *Meloidogyne*, *Pratylenchus* and *Hirschmanniella* can be controlled on upland and lowland rice. Optimisation of the defence will involve selection of one or more effector genes plus promoters that provide either root-specific or expression at the locale of the parasite only. The resistance will be demonstrated in field trials at IRRI and WARDA. The project will provide an important contribution for DfID to rice improvements in the developing world.

4: RESEARCH ACTIVITIES

4.1: Promoter activity

Two promoters were considered of interest for the programme. The constitutive promoter from a ubiquitin gene was selected to provide a higher level of expression in the monocot rice than occurs when CaMV35S from cauliflower mosaic virus (CaMV35S) is used. High levels of expression were considered of value for comparing the efficacy of different cystatins. The second promoter that was selected was from a root specific tubulin gene. This is the preferred promoter for field use as it ensures no new expression of the cystatin in either aerial tissue or food. Other root-specific promoters are available for the programme but effort was not dissipated in comparing them at this stage in the work. Constructs were made with both promoters using *uidA* (GUS). These constructs allow promoter activity to be reported by a blue coloration of plant tissue where and when a promoter is active on addition of an appropriate substrate.

Meloidogyne invades near root tips where both promoters are highly active (Fig 1a) and may complete development in a region of high promoter activity (Fig 1b). The root often continues to grow and so occasionally mature females may occur at sites at which the promoter is no longer active (Fig 1c). *Pratylenchus zeae* was found in roots with different levels of promoter activity (Fig 2). The distribution of the two nematode species in regions of differing promoter activity is summarised in Fig. 3. These studies have not yet been completed because molecular work was prioritised when the duration of the project was shortened (see earlier). The data presented in Fig 3 suggest that once a high level of resistance to *Meloidogyne* is achieved, it will be necessary to determine if the level of resistance is also sufficient to suppress multiplication of *P. zeae*. We do not know as yet if locality on the root influences the feeding success of *P. zeae* . More active roots with higher levels of promoter activity may also be more suitable sites for parasitism. Such information is necessary to ensure the defence works well in rice against both *Meloidogyne* spp and *P. zeae*.

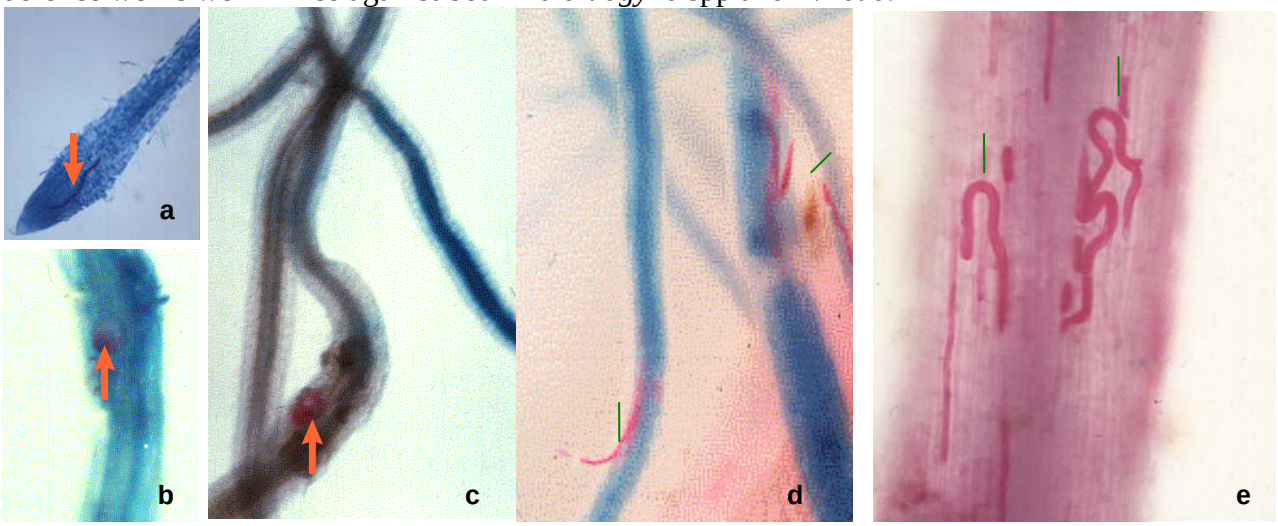


Fig 1a -c *Meloidogyne incognita* and rice roots showing variation UBI promoter activity (GUS reporter). at sites of invasion and establishment . a) Juvenile *M. incognita* close to a root tip and inducing a gall. b) An enlarged saccate female within an area of promoter activity c) a second adult female in an area of very low promoter activity at its feeding site. Arrows indicate nematode parasites. The juvenile and female are c400µm and c700µm in length respectively.

Fig 1 d-e *Pratylenchus zeae* and rice roots showing TUB promoter activity (GUS reporter).d) *P. zeae* penetrating within a root region where the promoter is active and e) a region of another root system where no promoter activity is reported. *P. zeae* is c 350-600µm in length

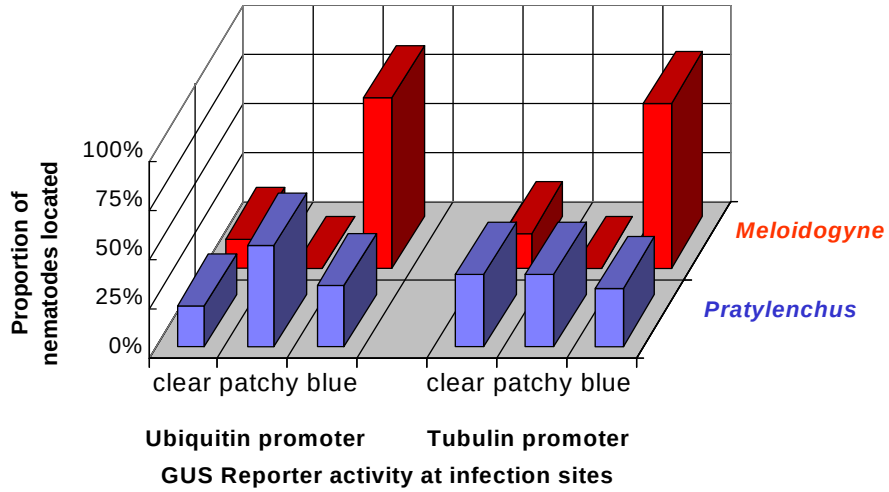


Fig 2: Tubulin and Ubiquitin promoter activity reported by GUS at 7,14 and 28 days (combined data) at sites of *Meloidogyne* and *Pratylenchus* infection in roots.

4.2: Development of conditions for challenging rice plants with *Meloidogyne* spp

Fig 4: Rice plants ready for expression level determination prior to re-planting associated with start of a nematode challenge.

The basis for challenging rice plants with nematodes were developed during the work programme of this project following the commissioning of new containment glasshouses. They were built with University, BBSRC JOI and DFID funds have been in use since in December 1997. Initial effort centred on establishing reliable protocols for containment trial of rice lines to quantify resistance levels. The current protocol is summarised briefly in Table 1. Procedures have also been developed for migratory endoparasites. The variation in data obtained establish that resistance of 50% or more can be detected with statistical certainty ($P<0.05$) with 8 plants per treatment. We have also maintained colonies of *M. incognita* (ex-Ivory Coast), mixed *Meloidogyne* spp (ex-Bangladesh), *M. graminicola*, *P. zeae* and *Hirschmanniella* spp (all ex-The Philippines) for use in bioassays. To-date we have exclusively used the *M. incognita* population.

1. The glasshouse was set at 28 ± 2C day and 26 ± 2C night, relative humidity 80%, day length (13h light :11h dark).
2. *M. incognita* or *M. graminicola* infected plants harvested, roots and soil mixed were diluted in a 3x series. Most of the soil is stored at 15C for 10 days while aliquots used in bioassay using aduki bean over 7 days to estimate invasion in a 3 fold dilution of the infested soil. Acid fuschin staining was used to estimate nematode establishment. Information gained provided a basis for determining the soil dilution chosen that provided c100 females/ rice plant in first generation. Assays with constructs involving the tubulin promoter used a lower challenge based on adding eggs to soil (see later).
3. Rice plants were grown for 4 weeks before test and control plants were paired for equal tiller numbers at infection. Plants were arranged in a randomised block design with c 10 plants per replicate. Vegetatively propagated plants are older but of a similar size to those grown from seed at infection.
4. Plants were grown in infected soil for at least 42 days. At harvest, total fresh wt, root weight and tiller number are measured for each plant. Egg numbers are determined per plant and per g root.
5. Watering systems have been developed during the course of the work. They involve automatic trickle watering system. Soil tensiometers have been obtained and in future the soil matric potential (pF) will be maintained at cpf1.4-1.6 during the initial 10 days when *M. incognita* is invading the plants. We also intend adding a water retaining gel in future work to buffer changes in pF during this critical phase. This is needed to ensure reliable infection particularly at the lower densities that simulate conditions in the field. For an example, see section on challenge of plants using the tubulin promoter (see later).

Table 1: Summary of protocol for challenging rice plants with *Meloidogyne* spp.

4.3: Constructs

A wide range of constructs have been made during the course of the work. They take about 3 months of post-doctoral worker time each to produce. Each therefore involves a considerable amount of effort to produce and collectively they form an important resource. They are summarised in Fig. 6. They can be sub-divided into a) those that provided of insufficient interest to justify bioassay b) those that were bioassayed c) those for which the transgenic plants for bioassay are not yet available.

4.4: Transgenic Rice Plants and Bioassays

Preliminary demonstration of transgenic rice achieved with *CaMV35S/OciΔD86/NOS*

The first proof of principle demonstration of transgenic nematode resistance in any monocot was achieved. This work was been published (Vain *et al* 1998) and a poster of the work presented at an international conference in plant molecular biology (Richard *et al* 1997). The data is presented in Fig.5. The level of resistance was very encouraging but the level of expression of the cystatin was less than the level of c0.5% tsp we have shown in our other work is required for high efficacy.

These plants were not advanced to containment or field trial in The Ivory Coast. The level of resistance was not considered to be sufficient and The Ivory Coast still lacks the national regulations and legislation to accept transgenic rice plants. Further work has centred around the design of further constructs to raise the level of resistance obtained to an agronomically effective level. We have set the objective of achieving >80% resistance over a single generation of *Meloidogyne*. Resistance to nematodes is measured as a reduction in egg numbers relative to a susceptible cultivar. Our effort can be sub-divided into effort centred on the promoter and a parallel effort to optimise a cystatin choice.

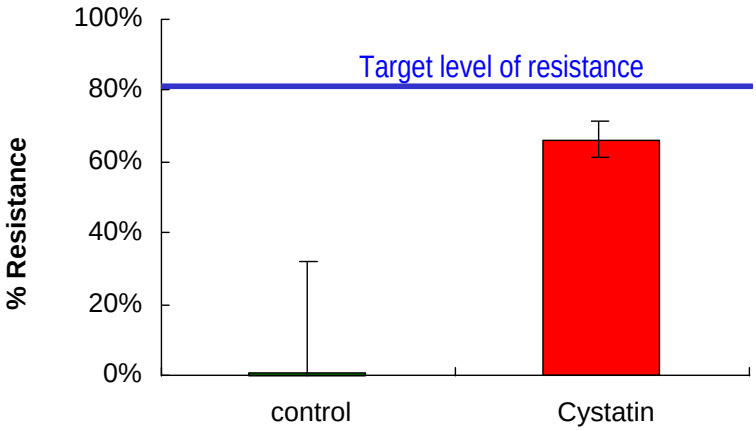
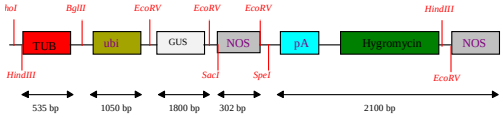


Fig 5: The level of resistance obtained in plants expressing low levels of OcIΔD86 under control of the CaMV35S promoter. The target level of resistance set for the project is shown (>80%).

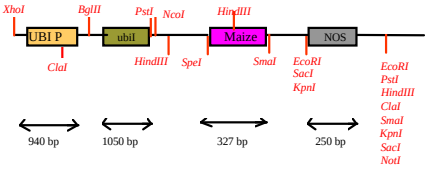
Rice transformed with *UBI/ubi/Oc-IΔD86/NOS*:

The CaMV35S promoter was replaced by a ubiquitin promoter that provides higher expression in rice. The ubiquitin intron (ubi) was also added as the intron favours expression in monocots. 42 lines of this construct *UBI/ubi/Oc-IΔD86/NOS* were generated at JIC. 9 lines were western positive but no improvement in expression was obtained. All lines expressed Oc-IΔD86 at <0.05% tsp.. This suggest that the 50% level of resistance was not limited by use of CaMV35S as the promoter. These plants were not prioritised for bioassay. The low expression may arise because of homology dependent silencing. This effect may occur because rice expresses OcI and the addition of OcIΔD86 may trigger the homology dependent gene silencing in which neither the natural nor the introduced gene are expressed.

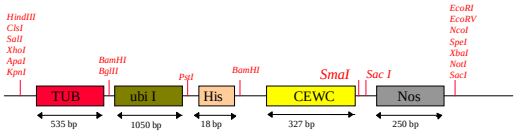
TUB/UBI/Gus



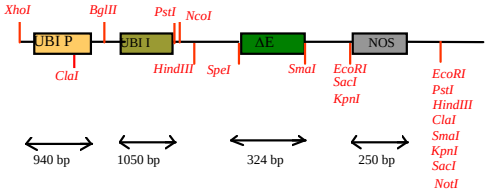
Ubi/Ubi/ Maize



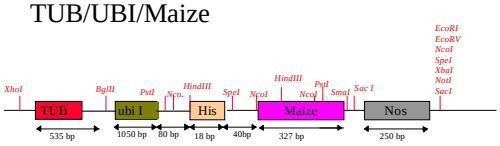
TUB/UBI/CEWc



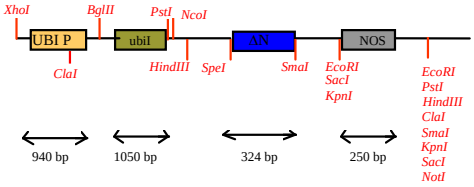
Ubi/Ubi/ ΔE



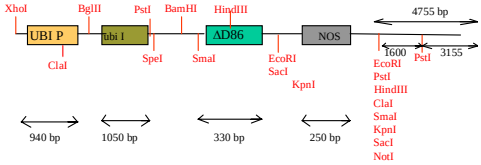
TUB/UBI/Maize



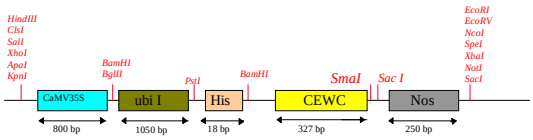
Ubi/Ubi/ΔN



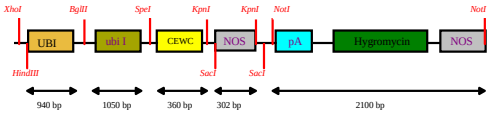
Ubi/ubi/ Δ D86



CaMV35S/Ubi/CEWc



Ubi/Ubi/ CEWc



CaMV35s/Ubi/ Δ 86

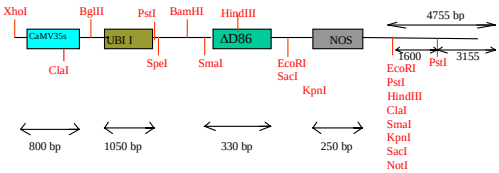


Fig 6. Maps for the constructs made during tenure of the grant.

TUB= Root specific promoter of a Tubulin gene

CaMV35S = A constitutive promoter from cauliflower mosaic virus used in dicots but known to have activity in rice. **UBI** = Constitutive promoter from the ubiquitin gene. A constitutive promoter with high activity in monocots.

ubi = an intron from the ubiquitin gene that may enhance expression in monocots. **NOS** = is a standard terminator sequence used in most plant gene constructs.

CEWc = Chicken egg-white cystatin; **OcI** = rice cystatin; **Δ86** = OcIΔD86,a rice cystatin lacking its 86th amino acid (Aspartic acid; D); **maize** = maize cystatin; **ΔN** = maize cystatin missing an amino acid in a similar position to D86 in OcIΔD86; **ΔE** = maize cystatin missing an amino acid in a similar position to D86 in OcI.

Restriction enzyme sites and length of nucleotide sequences in base pairs are also shown.

Rice transformed with *UBI/ubi/CEWc/NOS*: Constructs were generated with a constitutive promoter and Chicken egg-white cystatin (CEWc) to overcome possible problems with homology dependent silencing of Oc-IΔD86. CEWc is known to be an effective cystatin and has worked well against Potato cyst-nematode in our 1998 field trial (SOAEFD funding). These plants are experimental and not intended for uptake. 22 transformation lines of *UBI/ubi/CEWc/NOS* were generated at JIC but no plants were western positive.

Resistance Levels achieved with *CaMV35S-UBI-CEWc* plants: In parallel, to the above constructs, 155 transformation lines carrying the construct *CaMV35S/ubi/CEWc/NOS* were generated. Many were tested as positive in western blots (duplicates) and a sub-set of the plants were used for challenge with nematodes in two trials.

First Trial: Western blots demonstrated that these plants provided much lower levels of expression than expected or required for effective nematode resistance. Their level of expression of T_1 generation of transgenic plants was about 0.01% CEWc tsp. They were therefore not expected to be nematode resistant. They were used in an initial trial which established some effects but also involved a very high level of challenge. The western positive plants did show reduction on rate of growth of *M. incognita*. The experiments allowed the bioassay protocol in Table 1 to be developed. The plants were then used in a second trial.

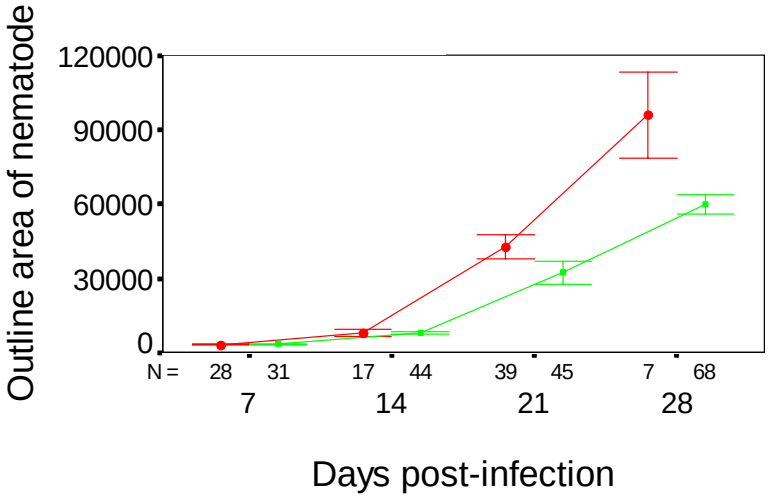


Fig. 7: Outline area in μm^2 of *M. incognita* on wildtype plants (●) and those expressing CEWc at <0.015 tsp (●). N = number of nematodes analysed. Values are means ± sem.

Second trial: The levels of *M. graminicola* that cause 10% loss of yield on upland rice at 60 days post-invasion is about 600 eggs/plant (Bridge *et al* 1990) but such plants do not have the uninfected initial growth phase we provided. The vegetative growth of these plants is considered later in Table 5. The levels of eggs recovered from the two transgenic lines 529 and 531 may indicate sufficient parasites were present to damage vegetative growth (Table 5). Infected wildtype plants did show significant less increase in tiller number relative to transformed plants ($P<0.05$, Table 5). However the lack of difference between dot blot positive and negative plants may reflect some expression occurred below the detection limits for the assay. However it cannot be certain that the difference in plant growth are due to nematode challenge.

These results allow a number of important points to be made. The level of resistance obtained was statistically significant for line 531 but not 529. The low level of expression found with Oc-IΔD86 was not overcome by replacing this cystatin with CEWc. The promoter CaMV35S provides low level of expression with both cystatins This suggest the low level of expression with Oc-IΔD86 (see earlier) is not due to homology dependent silencing. These low levels of expression do suppress egg protection by *M. incognita* (Fig. 5 and Table 2) but the level of resistance is not sufficient to justify field trial.

Line	dot blot +ve	dot blot -ve	wildtype	% reduction in eggs (resistance)
wildtype			1614 ± 247 (19)	
531	1247 ± 259 (9)*	2390 ± 106 (5)		48%
529	981 ± 168 (7)	1416 ± 275 (7)		31%

Table 2: Reproduction of one generation of *M .incognita* (eggs produced / g root) on wildtype and dot blot positive or negative transgenic rice plants. The egg number is of the order expected with very heavy infestation in the first generation under field conditions. The number in parenthesis is number of analysed plants. The difference between dot blot positive and dot blot negative plants for line 531 is statistically significant (P=0.008) whereas it is not for line 529 (P=0.20).

Resistance achieved with *TUB/ubi/CEWc* (S series plants)

One possible explanation of the poor levels of expression found in rice for the constructs considered above is the use of constitutive promoters. We have established (see section on promoters) that the 0.5kbp fragment of the tubulin promoter provides root-specific expression in rice. T_0 plants were generated at JIC. Severn T_0 transformed lines were western blotted to detect expression of CEWc in their root systems. Three lines expressed the protein and one line (S5) was western +ve at 0.1% tsp. Twelve seeds derived from T_1 plants that were vegetatively propagated siblings of the original S5 plant were provided by JIC. This generation necessarily contains both homozygotes and heterozygotes. They were germinated and western blotted (Fig 8).

Four lines were selected for further work. The conclusion was reached that western blotting is a reliable basis for detecting higher expressing lines using the TUB promoter but that it is not capable of identifying non-expressing plants. They may express at levels lower than can be detected by western blotting. The possibility that higher expressing lines represent homozygotes has yet to be tested. Our earlier work has shown that Tubulin promoter is considerably more active in giant cells induced by *M. incognita* than the general root system. Therefore an effective anti-nematode level of expression may occur where *M. incognita* feeds even when the level in uninfected plants is below that detectable in western blots (<0.01% tsp).

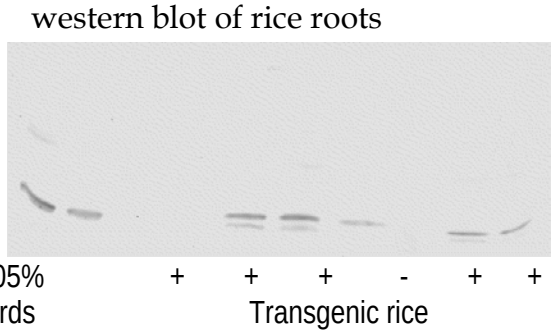


Fig 8: Rice plants transformed with the construct *TUB/ubi/CEWc*. 12 T1 rice plants were analysed for expression are shown in the above western blot for 6 lines. Two of the 12 plants have expression levels of c0.05% (left 2 above) and so present higher levels of expression than seen previously using CaMV35S as the promoter.

We introduced c2000 eggs of *M. incognita* per plant to established rice plants. They were added as egg masses hand-picked from females of *M. incognita*. Our plants had c5 tillers, a mean root mass of c7.5g at infection and were grown in pots containing 2.8kg soil. Therefore the inoculum challenge was c0.7 eggs/g soil. The inoculum is well below that of >5 J2 of *M. graminicola* /ml of soil at planting that provides intense nematode competition and overpopulation (Plowright & Bridge, 1990, *Nematologica* 36, 81-88).

The rice plants were harvested at 63days post infection. The levels of *M. graminicola* that cause 10% loss of yield on upland rice at 60 days post-invasion is about 600 eggs/plant (Bridge *et al* 1990) but

such plants do not have the uninfected initial growth phase we provided. Our wildtype plants had 2781± 902 eggs/plant and 1969 ± 480 egg/plant at harvest in the two assays. Space constraints prevented us from growing uninfected plants alongside the trial. The rice plants yielding eggs of *M. incognita* did not have significantly fewer tillers and either reduced herbage or root fresh weights at harvest (Table 3). Therefore we had achieved our intention of providing the rice plantlets with a challenge that was not very high relative to that found in the field. Therefore any differences in host status is not due to differential plant growth.

	Plants with eggs	Plants without eggs	P value
herbage weight (g)	75.0 ± 5.3	83.6 ± 5.3	0.27
Root weight (g)	14.8 ± 1.2	17.6 ± 1.6	0.17
Tiller number	6.8 ± 0.42	7.6 ± 0.49	0.27

Table 3: The leaf, root and tiller number at harvest of infected transformed rice from which eggs were either recovered or not present after 63 days of infection. Results establish that no significant reduction in vegetative growth explains the difference in host status. Comparisons are based on a t-test. Values are means ± sem.

The egg numbers per plant in the first bioassay were not normally distributed because many of the transgenic plants provided no eggs. The values were transformed logarithmically for comparison. The resultant mean values expressed arithmetically were 47.6 ± 53eggs/g root and 5.8 ± 2.9 eggs/ g root for the wildtype and transgenic rice respectively. The means were significantly different ($P<0.028$; t test). This suggest a level of resistance of 91% measured as reduction in egg production. However, if plants failing to provide eggs are discounted, similar mean values of 130 ± 37 eggs/ g root and 84 ± 25 eggs/ g root are obtained (NS; $P = 0.43$) for the wildtype and transgenic lines respectively. Therefore the major effect is that many of the transgenic plants had failed to support reproduction of *M. incognita* by 63 days post-infection. Egg production is not reduced in a quantitative manner for all plants. This suggests that many plants have a level of expression at feeding sites that is severely retarding growth and preventing any parasites on these plants from initiating egg production within the experimental period. A few plants fail to express that level of cystatin and show a low resistance level of c35%.

The second assay provided very similar results. Analysis based on logarithmic values provides values expressed arithmetically of 28.2 ± 26.0 and 3.8 ± 2.6 eggs/g root for wildtype and transformed plants respectively ($P<0.028$; t test). This suggest a level of resistance of 88% measured as reduction in egg production. However this analysis is again misleading as the principal difference in the two sets of data is the number of plants that failed to provide eggs of *M. incognita*. If only plants yielding eggs are compared, there is no difference in mean eggs per plant. The values are 85.5 ± 23.8 and 105.5 ± 26.4 eggs/g root for wildtype and transformed plants respectively (NS, $P>0.78$; t test).

The overall level of resistance for both assays is given in Fig. 9. The number of plants that failed to provide eggs were analysed further by chi-square analysis. The first bioassay revealed no difference in the proportion of plants without eggs when the four sets of transformed plants were compared ($P = 0.13;\chi^2$). Therefore all transgenic plants are grouped together for further analysis. These results showed a significant difference in incidence of plants without eggs relative to untransformed plants of the same cultivar ($P=0.015$; χ^2, Table 4). The results of the second bioassay were similar with more egg-free plants for the transgenic plants than expected from data for the wildtypes ($P<0.015$; χ^2 Table X). Combining the two assays establishes many more egg-free hosts for transformed relative to wildtype plants ($P = 0.00061$, Table 4, values in red).

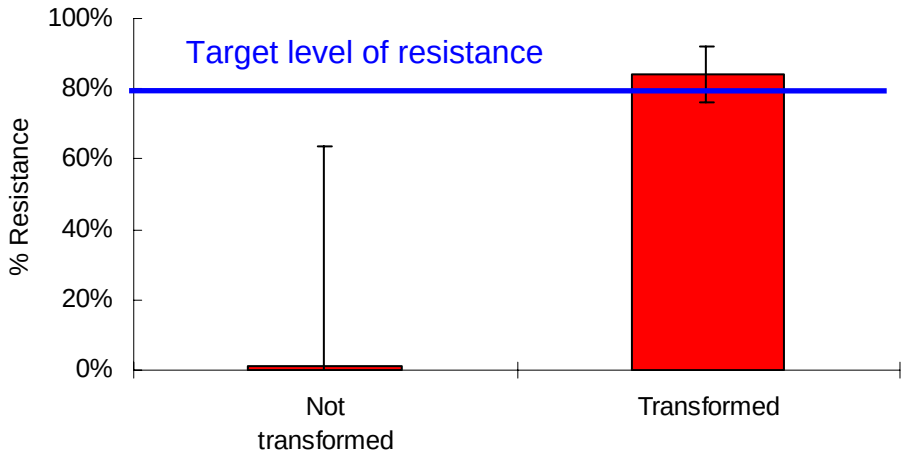


Fig. 9 The estimated level of resistance obtained in two trials of TUB/ubi/CEWc. The values are somewhat misleading as the main difference between transformed and untransformed plants is the proportion of root systems from which eggs were recovered (see text). Values are means ± sem.

Challenge	Transformed plants			Untransformed plants			P value
	no eggs	with eggs	Total Plants	no eggs	with eggs	Total Plants	
First	26	13	39	2	7	9	0.0148
Second	12	5	17	2	7	9	0.0153
Both	38	18	56	4	14	18	0.00061

Table 4: Number of rice plants with or without eggs of *M. incognita* associated with their root systems at 63 days post-infection with the nematode. Plants were either transformed or untransformed individuals of the same cultivar (ITA212).

4.5:Expression of cystatins

A	Infected			
Plant status	number of plants	tiller number at infection	tiller number at harvest	Ratio
Transformed expressing	20	2.75 ± 0.19	7.15* ± 0.56	2.84 *± 0.27
Transformed not expressing	20	2.75 ± 0.19	7.75* ± 0.57	3.12 *± 0.33
Wildtype	20	2.75 ± 0.19	5.40 ± 0.53	2.05 ± 0.18

B	Not infected			
Plant status	number of plants	initial tiller number	tiller number at harvest	Ratio
Transformed expressing	5	2.80 ± 0.37	8.20 ± 1.46	2.92 ± 0.44
Transformed not expressing	5	2.80 ± 0.37	7.20 ± 2.40	2.35 ± 0.68
Wildtype	5	3.40± 0.40	6.80 ± 1.59	2.08 ± 0.44

Table 5 **A)** The ratio of tillers at time of infection and at harvest (42 days later) for rice plants transformed and expressing or not expressing CEWc plus values for wild type plants. * Means ± SEM differ from wildtype (P<0.05 SNK).

B) As A) except plants not infected.

We have experienced unexpected problems in obtaining high levels of expression of cystatins in rice using two constitutive promoters. Previous work by others had established a maize cystatin can be expressed at 2% tsp in rice . An in depth study for a dicot had established that 1% tsp of a cystatin did not influence growth. We have shown no loss of yield for potatoes under UK field conditions when expressing CEWc at 0.4% tsp under control of a constitutive promoter (CaMV35S). These plants

provided 72 ± 9 % resistance to potato cyst-nematode.

Low expression may result from toxicity of CEWc to rice plants. Wildtype plus expressing and western negative transgenic plants were selected with the same tiller number as plants that were expressing low levels of CEWc under control of the promoter CaMV35S. Plants were infected or uninfected with *M. incognita* and tiller number measured. Tiller number was not significantly changed relative to wild type for uninfected plants expressing the cystatin. There was a significant increase in tiller number for cystatin expressing plants relative to wildtype but this can be correlated with the level of resistance achieved. Therefore there is no evidence that the low level of cystatin expressed constitutively in these experiments impaired the vegetative growth of the rice plants. The level of resistance provided by these plants is considered earlier (CaMV35S/ubi/CEWc).

Other effects may occur. The lack of high expression level of cystatins could arise if high levels of cystatin are detrimental to the plants in the callus stage of regeneration. If an effect does occur when promoters are not tightly regulated in callus regeneration, it is also possible that the effect occurs in developing seed. This would be consistent with the pattern of declining expression with generation of certain transgenic lines. The effect could also extend to root-specific promoters and so needs to be determined. Another plausible explanation of low expression levels centres on gene silencing. This too characteristically involves a decline in expression level over several generations. In addition, there is evidence to suggest that biolistic transformation as used for rice in the project is more subject to gene silencing effects that *Agrobacterium*-mediated transformation as used in our successful work on potato. In that crop, we screened c350 lines to identify 6 that provided a level of expression of 0.4% tsp and provided 50-70% resistance to potato cyst-nematode.

Further development of this project will centre on defining the most effective transgene (several new lines of transgenic rice are expected to be produced over the next 12 months). The possible problem of stable expression may not arise with a root-specific expression such as Tubulin if its tissue specific expression limits the effect or ensures low level of expression are adequate for controlling *Meloidogyne* because of higher expression in the giant cells. Once an effective construct such as the tubulin promoter driving a plant cystatin is established, a large scale transformation may be required for a selected rice genotype to generate a number of lines offering high level of expression. In addition it is necessary to establish homozygous lines from plants with high resistance and no other change in phenotype. This should lead to isolation of a line with an effective and stable level of expression.

4.6: Constructs developed but transgenic plants still being developed

It takes several months of molecular biology to assemble constructs and a minimum of 9 months to generate transgenic lines. An additional constraint is that plants are transformed at JIC between April and September. Constructs for which plants are currently being generated include TUB/ubi/maize. The maize cystatin is a replacement for the rice cystatin (Oc-I) that is sufficiently distinct to avoid any homology dependent silencing. Previous work by others have reported 2% tsp of this cystatin in rice.

In addition we have developed an engineered maize cystatin that omits the amino acid in the same position as the D86 removed from Oc-I to from Oc-IΔD86. The expectation is that cystatin will have an enhanced Ki relative of maize cystatin. The plants will be tested with (R7294) once they are received from JIC.

4.7: Biosafety issues

There has been recent concern among the general public over transgenic plants. Biosafety is centrally important to responsible technology transfer to the developing world. There are three main issues: a) the safety of foods b) environmental safety and c) beneficial implementation to subsistence farmers. The cystatins are being examined for toxicological safety. Results from the partially complete study suggest that the proteins do not represent toxicological hazard. Results to-date also suggest that they are not allergenic relative to standard reference proteins. In addition we intend to have no new

expression of cystatins in food. The cystatins we are using are already expressed in major foods such as rice and maize seeds. Cystatins are contained in saliva and so are consumed with every mouth of food swallowed by humans. We expect to demonstrate that food made from plants using our technology represents no risks to humans. The risk of transgene escape in pollen varies with crop and geographical locality. It cannot be completely prevented for a crop such as rice. Our current view is that there is no evidence to suggest that chance acquisition of the defence from rice is likely to provide a competitive ecological advantage to wild plants. This will be tested more fully in later stages of the work. There is no risk to above ground insects and their predators as the cystatin will not be present in aerial rice tissue. Therefore further biosafety studies will centre around pollen transfer of transgenes and possible effects on soil fauna and other soil organisms. Our preliminary view to be tested by later experiments is that these risks are not a concern.

Even critics of genetic engineering appreciate that improvements to farming will arise from the approach if the research is public-funded and for the public good. Our position is unusual in that the technology belongs to University of Leeds and that royalty-free licences have already been given to DFID to underpin development of the approach. Therefore this represents one of the best examples of how transgenic plants can be provided to subsistence growers without concern for a cost premium or a domination of the new technology by the interests of biotechnology companies. The work has been used by BBSRC as a strong example of the benefits of transgenic plants at The Royal Show in 1998 and an initiative to promote innovation in biosciences (Conference on 12th October 1998, Queen Elizabeth II Conference Centre, London and a BBSRC brochure entitled *Innovation in the Biosciences*).

5: OUTPUTS

5.1: Publications

Atkinson, H.J. (1996) Novel defences against nematodes. *Journal of The Royal Agricultural Society* 157, 66-76.

Atkinson, H.J. (1998) A Robin Hood approach to transferring appropriate plant biotechnology to the developing world. Science and Public Affairs Winter 1998, 27-29.

Atkinson, H.J., Lilley, C. J. Urwin, P. E. and McPherson, M.J. (1998) Engineering resistance to Plant-parasitic Nematodes. Chapter 15, pp 381-413 in "*The Physiology and Biochemistry of Free-living and Plant-parasitic Nematodes*" ed Perry, R.N. & Wright, D.J. CABI International Wallingford

Lilley, C.J. Devlin, P. Urwin. P.J. and Atkinson H.J. (1999) Parasitic Nematodes, Proteinases and Transgenic Plants *Parasitology Today* In press.

Urwin PE, Møller SG, Lilley CJ, Atkinson HJ, McPherson, MJ (1997a) Continual GFP monitoring of CaMV35S promoter activity in nematode induced feeding cells in *Arabidopsis thaliana*. *Molecular Plant Pathogen Interactions* 10, 394-400.

Urwin, P.E., Lilley, C.J., McPherson, M.J. and Atkinson, H.J. (1997c) Resistance to both cyst- and root-knot nematodes conferred by transgenic *Arabidopsis* expressing a modified plant cystatin. *The Plant Journal* 12, 455-461.

Urwin, P.E., McPherson, M.J. & Atkinson, H.J. (1998). Enhanced transgenic plant resistance to nematodes by dual proteinase inhibitor constructs. *Planta* **204**, 472-479.

Vain, P., Worland, B., Clarke, M.C., Richard, G., Beavis, M. Lin, H., Kohli, A., Leech, M., Snape, J., Christou, P. and Atkinson, H.J. (1997) Expression if an engineered cysteine proteinase inhibitor (Oryzacystatin-IΔD86) for nematode resistance in transgenic rice plants. *Theoretical and Applied Genetics* **96**, 266-271

5.2: Internal Reports

- Bimonthly reports produced by Dr Gaelle Richard for research planning meeting during her period of employment Jan 1996-December 1997).
- Monthly reports by Dr Jayne Green in her employment period : May 1998-December 1998).
- Annual report to DFID Plant Sciences Programme
- Oral reports to DFID Plant Sciences Programme Rice Biotechnology meeting.

5.3:Other Dissemination of Outputs

A numbers of research seminars have been delivered both nationally and internationally. A selection of key invited contributions and other key outputs are listed below.

Atkinson, H.J. (1997) *Transgenic Plants and Food Security,* In Agriculture & Forestry Section. British Association: Annual Festival of Sciences, 7-12 September 1997, University of Leeds.

Atkinson, H.J. (1998) BBSRC Meeting publicising launch of "innovation in the biosciences" October 1998

Atkinson, H.J. *et al* (1998) Nematode-resistant Crops save money world-wide In "*Innovation in the biosciences*". BBRSC booklet

Atkinson, H.J. *et al* (1998) In "BBSRC at The Royal Show" BBSRC Business July 1998.

Atkinson, H.J. *et al* (1998) BBSRC International Exhibit *Genetically Engineered Nematode Resistance for World Crops.* The Royal Show, July 1998

Atkinson, H.J. et al (1998) *Genetically engineered crop resistance to nematodes.* IX *International Congress on Plant Cell Tissue and Cell Culture*, Jerusalem 14-19th June 1998.

Atkinson, H.J. et. al (1998) *Genetically engineered resistance to nematodes*. International Congress of Plant Pathology, Edinburgh August 1998.

Atkinson, H.J. et al (1998) *Transgenic crops for protection from nematodes*, British Crop Protection Council Brighton Conference 16-19th November 1998

Radford, T (1997) (Science editor of The Guardian)"Revenge of the killer Potato" *The Guardian* March 13 1997.

Public Debate organised by Food Future "*Question Time*" 21/10/1998 Leeds Town Hall

6: CONTRIBUTION OF OUTPUTS TO DFID DEVELOPMENT GOALS

6.1: Contribution of Outputs to Project Goals

- We have developed rice with levels of resistance per single generation that is sufficient to protect rice crops from damage by *Meloidogyne incognita.*
- The work has been progressive in nature. It has incrementally raising the level of protection towards very high levels. High levels will ensure useful protection in a wide range of agricultural situations.
- We have established that root-specific promoters can provide useful expression levels. They ensure that no new cystatin is expressed in rice seeds and that green tissues of rice do not contain the new protein. This is a valuable contribution to biosafety in regard to both GM food and environmental impact on non-target organisms that feed on green seeds or leaves of rice.
- A range of constructs have been made to raise efficacy and base it on cystatin already expressed in the consumed part of food crops.
- The remaining principal issue is stability of expression through many generations of the rice crop. This is being addressed in current work both in Leeds and at JIC.

6.2: Follow-up indicated/planned

- The transgenic plants carrying constructs made in this grant that have yet to be tested will be challenged as they become available.
- Approaches such as a large scale transformation with an optimised constructs may be necessary to obtain lines of sufficient stability to underpin bulking and distribution to WARDA, IRRI and LARS.
- The biosafety issues surrounding the adoption of this technology requires to be resolved.

Pioneering work is intended in R6830 for potato but additional issues need to be addressed.

- Transfer of seeds and establishment of containment/field trails at WARDA will be initiated once 90% resistance against *Meloidogyne* has been confirmed with a plant cystatin. This also requires sufficient transgene stability to ensure bulking of seed through 1-2 generations does not involve loss of efficacy.
- Transfer to WARDA is not possible until national biosafety regulations for the Ivory Coast are implemented and a containment glasshouse provided by Gatsby Foundation is completed. Both should be in place by the end of 1999.
- Uptake of the outputs after extensive testing will be through WARDA and IRRI . Secondary trials should then be initiated through LARS.