Appendix 1C: FINAL TECHNICAL REPORT
Full and Durable Crop Resistance in Rice and Potatoes to Nematodes
DFID Plant Sciences Programme Project R7294
Final Technical Report: Start date 1/1/1999, end date 31/12/2001
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EXECUTIVE SUMMARY

The aim is to deploy plant genes expressed in food as transgenes to achieve full and durable resistance to nematodes with expression limited to roots systems. The work centred on a monocot (rice) and a dicot plant (potato). The approach has potential against a range of nematodes but the work has emphasised *Meloidogyne*. DFID and others have identified this genus as the most important nematode problem of developing world agriculture. The intention is to retain cultivars that are preferred by local populations and not change agronomic practices. The plants will also be used to champion the benefits of transgenic crops for poor growers.

The work on rice is collaboration with John Innes Centre in which they use the constructs we make to transform rice for us to evaluate. Constructs were made for biolistic transformation and to replace that approach with one that is *Agrobacterium*-mediated. On-going development of the latter approach at JIC to a variant that avoids selectable markers has resulted in R7294 necessarily concentrating on biolistically-transformed rice.

Rice expressing a cystatin from maize included lines (NE lines) that provided resistance levels of 83 $\pm$ 6% and so effectively met the level of resistance targeted at the start of the work. These plants grew normally. Co-transformation with the same construct and one expressing Chicken egg-white cystatin did not raise resistance further. Possibly a higher level or resistance will not be achieved by expressing just cystatins additively. Analysis of transcripts from lines generated in previous work confirmed that silencing occurred in some biolistically transformed lines. However an homozygous line were identified in T2 seed of the NE line. These plants supported little reproduction of *Meloidogyne* and expressed cystatin. T3 of this line was generated. Sufficient seed of T3 is already available for small-scale field trial and a visit is planned to China in May 2002 to set up these trials and parallel effort for potato. The objective is determine efficacy and establish if reliable field trial conduct and analysis with these plants. Once the selectable marker-free rice plants expressing cystatin are generated, they will replace current rice plants in field evaluation.

The activity of promoters of a root specific tubulin (TUB-1) was studied in rice. It showed very little activity green tissues and was not very active in older root tissues. However it was highly active in the giant cells induced by *M. incognita* on rice. This enables a cystatin to be targeted to the feeding nematode with little activity elsewhere in the plant. Therefore the protein will not be present in rice used as food. This adds considerably to the inherent biosafety given that maize cystatin occurs in food and is neither a human toxin nor allergen.

An effort parallel to that on rice was completed for potato. A number of constructs we made and three field trials were carried out in the UK against *Globodera* which is an important pest in Bolivia. A Bolivian government temporary moratorium prevented transgenic trial in that country. Trials established that plant cystatins are as effective as that from chicken egg white which has been used as a standard for comparison. The second trial showed that the three root preferential provided as high a level of resistance as when a constitutive promoter controlled expression. Additive resistance was established using a cystatin to improve the partial natural resistance of both an Andean (Maria Huanca) and an UK cutlivar (Sante) to full resistance in a containment and UK field trial respectively. This establishes a paradigm for additive resistance using different genes that act distinctly against nematodes. Further additive constructs were made and the first field trialled in 2001 but analysis is not yet complete. The transgenic Desiree plants that showed resistance to *Globodera* were also resistant to *M. incognita* in containment. These plants did not show reduced growth in either the field or containment. Bolivian *S. tuberosum andigena* cultivars were transformed but their expression levels were lower than obtained for the hybrid Maria Huanca.

Promoter reporter constructs were made, expressed and studied in potato. Results were similar to those recorded for rice. The three promoters showed little or no activity in green tissues but all were active in the giant cells induced by *Meloidogyne*. This effect was confirmed with one promoter within the syncytium induced by *G. pallida.* Therefore promoter/cystatin constructs could be deployed within pest management strategies to provide a range of nematode resistant crops for subsistence farmers.

The perceived hazard of transgenic potato release into centres of biodiversity for solanaceae was addressed. Male sterility was achieved by delivering an RNAase or a ribosomal inactivating protein from maize seed under control of a promoter specific to male parts of the flower (tapetal specific promoter). Also distinctive forms of potato were established for future transformation based on both flower colour and leaf shape. Such plants would support farmer choice to grow or avoid GM plants. Phenotypic markers are important where literacy levels ensure written labels are inadequate. Such plants would also allow socio-economists to measure uptake by growers in the future.

Rice

Constructs for biolistic transformation.
Two constructs were developed to overcome a putative problem with gene silencing in the previous "S" series Tub/ubi/CEWc/nos constructs (see final report to R6453).. They both expressed maize cystatin under control of TUB with one co-transformed with the above Chicken egg white cystatin (CEWc).

Constructs for *Agrobacterium*-mediated transformation
Transgenic rice was provided by JIC for two of the constructs. The first designed NE expressed a maize cystatin under control of TUB-1 promoter. The second (NG) was a co-transformation of maize cystatin and Chicken egg white cystatin, both under control of TUB (Table 1). These constructs still contain an antibiotic selectable marker, as agreement with Syngenta to use their sugar selectable marker replacement had not been concluded when they were made.

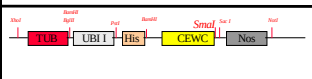
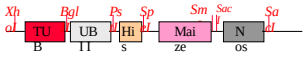
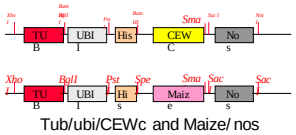
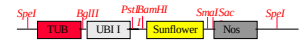
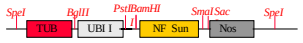
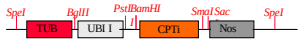
Construct	Map	Expression analysis
S	 Tub/ ubi/ CEWc/nos	Western analysis T1 0.1% expression T2 0.05% expression
NE	 Tub/ubi/maize/ nos	Western analysis T0 0.05% expression
NG	 Tub/ubi/CEWc and Maize/ nos	Western analysis T0 0.1-0.05% expression
	 Tub/ubi/sunflower/ nos	Construct made, confirmatory sequencing being completed
	 Tub/ubi/NF Sunflower/ nos	Construct made, confirmatory sequencing being completed
	 Tub/ubi/CPTi/nos	Construct made and confirmed

Table 1: List of constructs developed for nematode resistance in rice during the course of R7294.

Three further constructs were assembled for *Agrobacterium* transformation at JIC (Fig 1). However the approach being used at that institute has evolved and so the constructs have not been used in transformation. They are being re-made to new JIC requirements within R8031. The new approach avoids the need for a selectable marker.

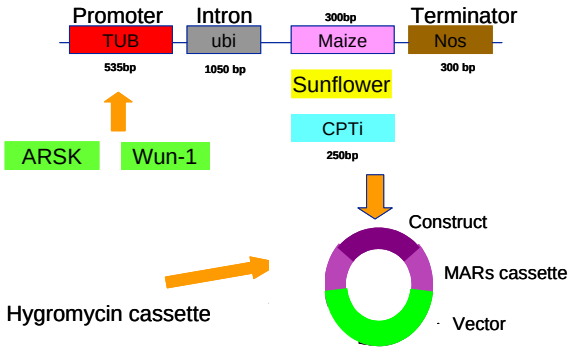


Fig 1: Summary of approach for constructs to be used in *Agrobacterium*-mediated transformation of rice.

Biolistically transformed rice with nematode resistance
Rice was transformed with a cystatin normally expressed in maize kernels (NE plants). The aim was to provide a plant cystatin from a common food that would overcome any possible homology-dependent silencing caused by use of a rice cystatin. The cystatin was placed under the control of the tubulin promoter with a ubiquitin intron (to aid expression). The construct *Tub/ubi/maize/nos* was developed at Leeds and rice transformed at the John Innes Centre. Rice plantlets were sent back to Leeds for analysis and bioassays.

Challenge of transgenic rice with *M. incognita*
Challenge of rice was modified in a successful attempt to reduce variation recorded in our previous work. The *Meloidogyne* J2 were introduced in cotton wool beneath the root system and plants irrigated to maintain a constant low pF (Fig 2).

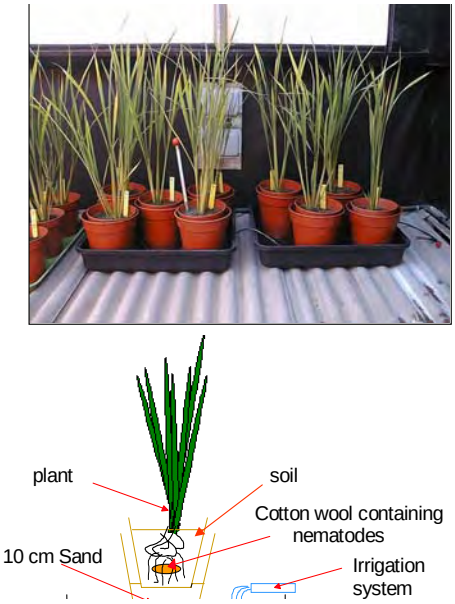


Fig.2: Revised bioassay conditions with a water reservoir provided by an irrigation system to maintain a constant moisture deficit.

A total of 25 rice NE lines were analysed by western blots and 6 positives were selected for challenge by nematodes. The challenge revealed a high level of partial resistance of up to 83 ± 6% to *M. incognita* after one generation of the

nematode (Fig 3). The two best lines were designated NE11 and NE30.

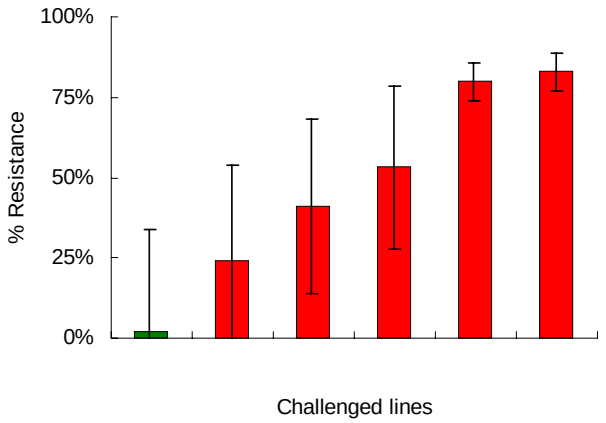


Fig 3: Resistance to *M. incognita* of 5 lines of rice expressing maize cystatin under control of TUB-1 (red bars) relative to multiplication of the nematode on wildtype rice (green bar).

At harvest the rice plants were measured for plant height and tiller number. The tillage of NE11 was similar to that of the wild type but NE30 demonstrated slightly lower tillage. Both NE 11 and 30 demonstrated slightly lower plant height as compared to wild type (Fig 4). The tiller number of one high expression line but not the other was significantly lower than for wildtype but the effect was not large.

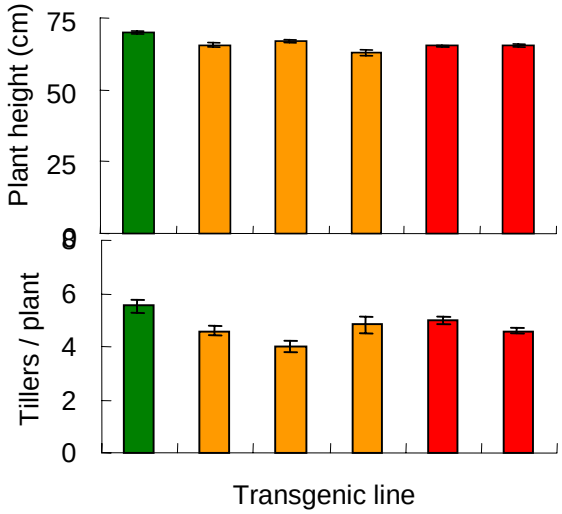


Fig 4. Comparison of plant height and tiller number for wildtype rice (green) and five lines expressing maize cystatin providing moderate resistance (<50%, orange bars) or high resistance (>80%, red bars).

Co-transformation of *Tub/ubi/maize/nos* and *Tub/ubi/CEWc/nos* was carried out to investigate if expressing two cystatins enhanced resistance. The expectation was that this would raise expression levels. Four lines were generated by this method and all were confirmed to be western positive. One of the lines, NG4, showed similar resistance and characteristics to that of NE 11 and NE30 but a higher level of resistance was not detected (Fig 5). Possibly some increase in resistance may have occurred if many lines had been produced.

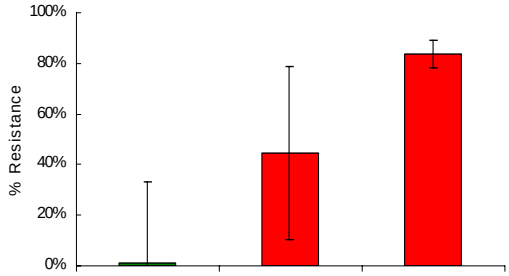


Fig 5: Resistance to *M. incognita* of 2 lines of co-transformed rice expressing both maize cystatin and CEWc under control of TUB-1 (red bars) relative to multiplication of the nematode on wildtype rice (green bar). The better transgenic line is designated NG4.

Silencing analysis of rice transformants from previous work

ITA212 rice cultivars from R6453 expressing Tub/ubi/CEWc/ nos (S series) showed a loss of expression of the cystatin that was interpreted as evidence of gene silencing. Bioassays established that a higher proportion of Tub/ubi/CEWc/nos plants supported no nematode reproduction than occurred on untransformed controls (Table 2; $P<0.001$). However the small number of plants transformed supporting nematode reproduction had 84 ± 25 and 106 ± 26 eggs/g root after 42 days in the first and second challenges. These were similar to values for untransformed controls in the two trials of 103 ± 137 and 86 ± 24 eggs/g root respectively. Therefore a minority of transformed plants showed no resistance. This could not be explained by segregation as all the plants for these challenges where obtained vegetatively by splitting them from two originally western positive plants.

This project has studied potential silencing further to develop reliable ways of detecting and avoiding the effect in NE and NG plants. Western blots established a progressive decline in expression levels of the S series plants from 0.1% tsp in T0 to 0.05% tsp in T1 plants. Analysis was carried out to determine if post-translational silencing occurs in S series plants. Post-translational silencing involving production of aberrant short mRNA is difficult to detect. Total RNA has been prepared from several plants all of which have been vegetatively propagated to some degree (plants have been split 1,2 or 3 times).

RT PCR was carried out in order to investigate any abnormalities in the cystatin message length. It revealed a correlation between those that proved western positive as mature plants and presence of an RNA message of the expected size (Fig 6). Many other plants had no message and were also western blot negative. In addition some plants that were negative for expressed protein were positive by RT-PCR but showed messages of abnormal length. We interpret this experiment as indicating that silencing is occurring in this vegetatively propagated line.

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

Table 2: Number of rice plants (S series transformants) with or without eggs of *M. incognita* associated with their root systems at 63 days post-infection with the nematode. There were two trials. Plants were either transformed or untransformed individuals of the same cultivar (ITA212). Relatively more transgenic plants showed no presence of eggs of *Meloidogyne* than occurred for controls.

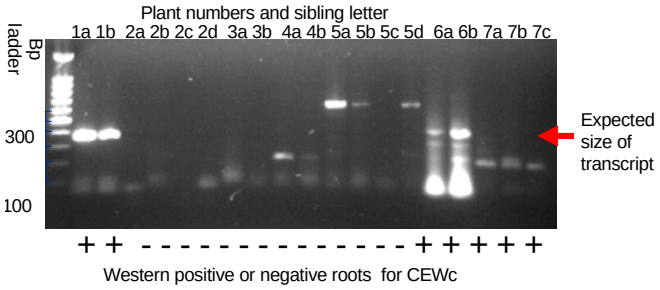


Fig 6: RT-PCR analysis of transcripts provided by plants generated from one transgenic plant expressing CEWc under control of TUB and the correlation between a message of the expected size and detection of CEWc by western blotting. Of particular interest is the occurrence of plants with a transcript size that differs from that expected. Numbers represent the original plant from 7 seeds and the letters are the vegetatively produced plants.

The above results led us to examine the histogram of different levels of resistance shown by the plants expressing maize cystatin (NE and NG plants) that were generated in this project. The moderate level of resistance in some plants and the high variance of some of these lines (Fig 3) could represent high levels of resistance in some plants and silencing in others. Comparison of the histograms for number of plants per line against eggs produced by *Meloidogyne* lines is of interest. Two highly resistant lines (NE 11 and 30) and that from co-transformation (NG4) showed similar histograms that differ from that of the wildtype as expected (Fig 7). However plants of line NE2 do not provide a normal distribution. There are clearly 2 populations of plants one with high and another with little resistance. This distribution may indicate silencing occurring in plants in this line so recruiting plants from the high to low category of nematode resistance.

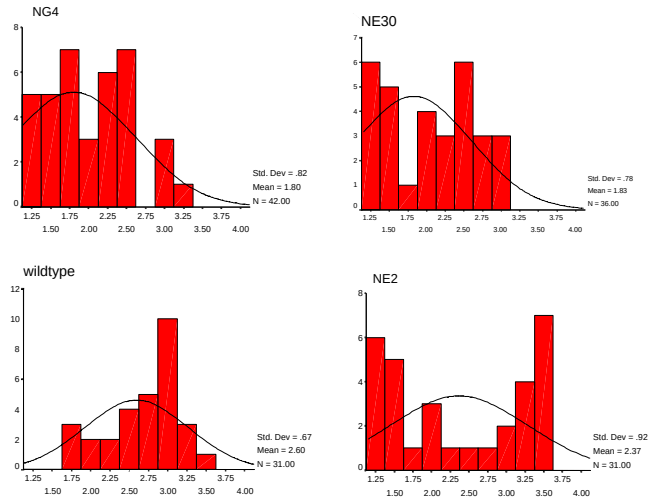


Fig 7: Histograms of number of plants against $\log_{10}$ eggs of *Meloidogyne*/g root for 4 lines of rice plants. Line NG4 expresses both maize and CEWc cystatin whereas NE30 expresses just maize cystatin. Both provide overall levels of resistance of >80%. The histograms should be compared with that for the wildtype.

NE 11 and NE30 plants were challenged and egg counts made for only 50% of the root system. Those plants with under 30 eggs per harvested root mass were grown on after challenge. The remaining half root systems were grown in soil with aldicarb to prevent nematode damage. They were left to go to seed, which was then harvested.

Homozygosity was determined by germinating 20 harvested seeds for each line in the antibiotic hygromycin and an equal number in water. The lines carry a selectable marker gene that confers resistance to the antibiotic. Lines were considered to be homozygotes if the null hypothesis for a difference in their germination frequency was accepted. Five lines tested had NE 11 parentage (TUB/ubi/maize/nos). Three were homozygous lines. Similarly two lines from the co-transformed NG series (TUB/ubi/maize/nos plus Tub/ubi/CEWc/nos) were analysed and one was determined as a homozygote (Table 3).

The plant lines showing homozygosity are being grown through successive generations and will be re-tested for a) homozygosity and b) expression level by western blots. Plant lines showing expression will be not be re-challenged at each generation with nematodes because this is time consuming. These bioassays will be carried out on generation 5. However we now assume that if a generation produces seed that still expresses cystatin then the parental generation is suitable for initial field trials. We have sufficient T3 seed on that basis for a field trial in China within R8031. The aim is to use this transgenic resource until the new transgenic lines being generated by JIC have been evaluated in containment and shown to be of value. The value of this approach is the gaining of information on the reliable conduct of such trials.

This will enhance the rate of technology transfer later with the new lines.

Line & plant #	germination in Hygromycin	water	Homozygote	Mean eggs/plant	RT	Western
NE11/1125						
146	20	20	yes	20	+ve	<0.1%
136	12/20	15/20	no	5	+ve	<0.025%
157	16/20	11/13	no	20	+ve	<0.1%
156	20	19/19	yes	2	+ve	<0.025%
154	20	17/18	yes	0	+ve	<0.025%
NE30/1183						
216	20	19/20	yes	10	na	<0.05%
220	16/20	17/20	no	22.5	+ve	<0.1%

Table 3: The proportion of twenty seeds germinating in hygromcyin orwater for seven rice lines. Those lines germinating equally frequently in both media are assumed to be homozygotes.

Promoter/reporter studies

The temporal and spatial expression of three promoters were investigated in rice plants using a promoter-β-glucuronidase (*gusA*) reporter gene and biolistic transformation. The objective was to define promoter activity where the parasites occur on roots throughout an infection time course. The acquisition of this data proved very time consuming but a publication is now in press (Green *et al*., 2002).

The promoters studied were ubiquitin-1 (UBI-1) of *Zea mays*, Cauliflower Mosaic Virus 35S gene (CaMV35S) and a tubulin gene (TUB-1) of *Arabidopsis thaliana*. The TUB-1 promoter provided 7.32 fold more GUS activity in roots relative to tillers. This was significantly different from the corresponding value of 2.82 fold for CaMV35S but not from that of 4.55 fold for UBI-1, activity of both promoters was higher in the root tips and zone of elongation than mature roots (Table 4). This younger root tissue represented a declining proportion of the expanding root system with time. Older tissue expressing GUS under control of the TUB-1 promoter showed a steeper decline in activity with time than occurred with the UBI-1 promoter. Nematode infection did not alter the overall pattern of expression from the two promoters (Fig 8) except that the giant cells induced by *Meloidogyne incognita* retained TUB-1 promoter activity as roots matured (Fig 9). *Pratylenchus zeae* invaded older root regions than *M. incognita* and no changes in promoter activity were detected where it fed (Fig 9). The results suggest the TUB-1 promoter has characteristics that favour its use for delivering anti-feedants, such as cysteine proteinase inhibitors, to *M. incognita*.

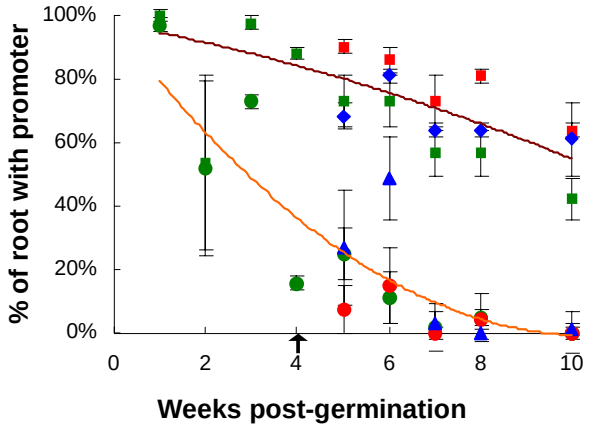


Fig. 8: Changes in promoter activity in rice roots expressing a *gusA* reporter gene under control of UBI-1 no infection (■), UBI-1 *Meloidogyne* infection (■), UBI-1 *Pratylenchus* infection (◆), TUB-1(●)no infection, TUB-1 *Meloidogyne* infection (●) and TUB-1 *Pratylenchus* infection (▲). The arrow indicates the time of nematode infection.

Root type	Root Zone	Mean % ± SEM	% change per week
		Ubiquitin	
Lateral	tip	97 ± 1	+0.9
	elongation zone	82 ± 3	+3.5
	mature	66 + 4	+4.2
Main	tip	90 ± 3	+2.4
	elongation zone	67 ± 4	+2.5
	mature	30 ± 4	+1.2
		Tubulin	
Lateral	tip	82 ± 3	-3.5
	elongation zone	54 ± 4	-1.8
	mature	35 ± 4	-4.4
Main	tip	69 ± 4	-11.3
	elongation zone	76 ± 3	-11.6
	mature	10 ± 3	-1.5

Table 4: Mean proportion of root tips or zones of elongation showing GUS reporter activity plus the proportion of mature root lengths with this activity for Ubiquitin and Tubulin promoters. Results are for both lateral and main roots

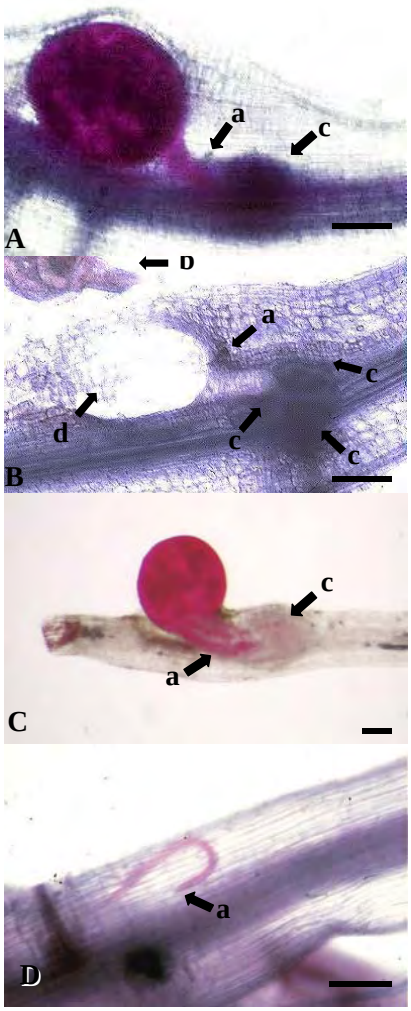


Fig 9: *M. incognita* and *P. zeae* in rice plants 28 days post-infection.
A) A length of whole root from a TUB-1 GUS plant showing an enlarged saccate female of *Meloidogyne.* The root shows GUS staining plus acid fuchsin staining of the nematode. The head of the nematode (a) is associated with a region of higher promoter activity (GUS staining) within the giant cells (c) from which the nematode feeds. The scale bar is 200µm.
B) A longitudinal root section of a TUB-1 GUS plant at the site of parasitism by *Meloidogyne*. There is higher GUS activity in giant cells (c) than other plant cells around the feeding site. The female (b) has been dislodged from the root during section preparation but the former locations of its body (d) and head (a) *in planta* are evident. The scale bar is 200µm.
C) *M. incognita* in wild type control rice plants at 28 days post infection, the enlarged saccate female is stained by acid fuchsin. The head of the nematode (a) extends to the giant cells (c) which lack staining by GUS reporter activity in contrast to Fig.9A. The scale bar is 100µm.
D) *P. zeae* in a rice plant expressing GUS under the control of the TUB-1 promoter. The head of the advancing nematode (a) is not surrounded by GUS expression. The scale bar is 200µm.

Potato

A range of cultivars has been considered of value to the project. (Table 5).

Sub-species cultivar	Transformed	Planting as % national acreage in Bolivia	Rank in national acreage
S. tuberosum andigena			
Waycha	No	14	1
Imilla Negra	No attempt	9	4
Imilla Blanca	No attempt	not known	not known
Runa Toralapa	No attempt	4	10
S. tuberosum tuberosum			
Desiree	Yes	10	2
Hybrids			
Maria Huanca	Yes	not known	not known
Gendarme	Yes	3	12
Revolucion	Yes	1.5	16

Table 5: Summary of potato cultivars within the project

Development of additive constructs for transgenic potato
Constructs for additive resistance have been completed (Fig 10). Two approaches are being followed to ensure more than one anti-nematode protein is produced from one transgene. When additive approaches are required, we are providing a peptide linker from galactose oxidase (GO) that is not readily cleaved *in planta.* In addition we have introduced a cleavage site in to GO. Providing this proves effective, this will allow a one construct to delivers several protein products. This simplifies transformation requirements when multiple products are required.

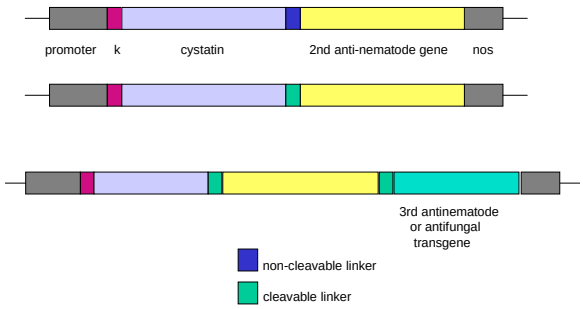


Fig. 10: Summary of constructs using a non cleavable linker to deliver two or more anti-nematode proteins from one transgene or a cleavable linker from the same linker to deliver 2 or more proteins.

Table 6 summarises several constructs within the transformation programme at the start of 2001. Others have been made since then and they will be available to DFID PSP once their utility is established.

UK trials in 1999

The field trial work in each year was done jointly by staff in this project and a grant awarded by SEERAD. This ensured an economy of effort for a very labour intensive activity.

We prioritised replacement of CEWC from an animal genome (chicken) with genes expressed naturally in the consumed parts of plants. The 1999 field trial tested transgenic lines of Desiree that expressed plant cystatins. We used a form of a rice seed cystatin (Oc-I) that we have engineered to be a more potent inhibitor with a lower Ki (Urwin *et al.*, 1995, *Plant J.* 8: 121-131). We also used an effective native cystatin from sunflower seeds that we isolated in work for DFID from published protein sequence (Kouzuma, Y. *et al.*, 1996, *J. Biochem.* 119: 1106-1113). The *ocIΔD86* was modified to provide potato codon usage and so favour expression. The cystatins were both cloned into vectors placing them under the control of the CaMV35S promoter. Molecular analysis was again carried out to ensure that plants expressing the cystatin were selected for field trials.

Transgene type	Constructs
Cystatins	CaMV35S-Sun(pot) CaMv35S- OCIΔD86(pot)-KDEL CaMV35S-Pineapple cystatin (pot)
Cystatins in additive defences	CaMV35S- OCIΔD86(pot)-GO-serine proteinase inhibitors-nos CaMV35S- OCIΔD86(pot)-GO-Sun(pot)-nos CaMV35S- OCIΔD86(pot)-GO(cleavable)-Sun(pot)-nos
Anti-invasion gene	PsMTa-anti-establishment gene-nos 35S- anti-establishment gene-nos ARSK- anti-establishment gene-nos
Male sterility of potato	TA29-RIP-nos TA29-Barnase-nos
Miscellaneous	CaMV35S-GFP-KDEL CaMV35S-GFP TUB-GUS ARSK-GUS RPL16-GUS

Table 6: Summary of constructs in transformation programme. Some of these constructs were generated in the course of other work.

Lines were regenerated as plantlets in tissue culture and screened by western analysis. A sub set of lines were selected with expression levels of >0.3% tsp as cystatin in roots for field trial. The presence of transcripts was confirmed for these lines by northern analysis.

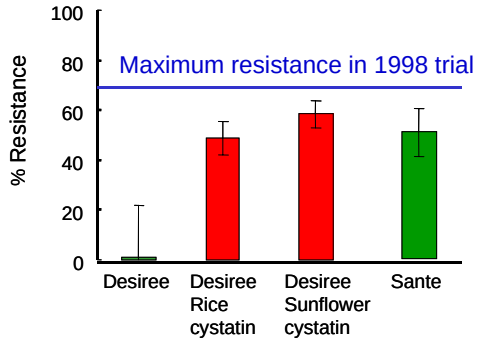


Fig.11: Partial resistance conferred on susceptible cultivar Desiree by expression of two plant cystatins relative to that of a naturally partially resistant cultivar. Sante.

Substantial levels of resistance were observed but they were less than in earlier work (1998 field trial). Fig.11 shows the resistance levels as percentage of that achieved for cv Sante. The values were 118 ± 15 % and 115 ± 11% for the best line expressing either Oc-IΔD86 or sunflower cystatin respectively. Both these transgenic PI-expressing lines showed levels of resistance similar to that shown by cv Sante.

UK Field trials in 2000

Cv Desiree was transformed to express OcIΔD86 under control of three root specific promoters. The aim was to trial 4-5 lines per construct but only 2 TUB/OcIΔD86 lines were positive in northern blots. Northern blots for the lines selected as positive are shown in Fig. 12.

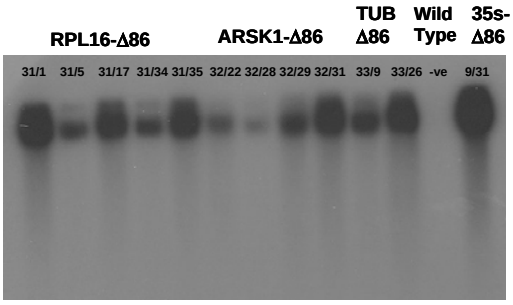


Fig 12: Northern blots of roots of lines used in field trials. Lines designated 9, 31,32 and 33 correspond to expression of OcIΔD86 under control of CaMV35S, RPL16, ARSK and TUB1 respectively.

The levels of expression of cystatin were low. This can be correlated with localised expression in roots (see later). Once this was appreciated some lines were confirmed as expressing cystatin by analysing their root tips (Fig 13).

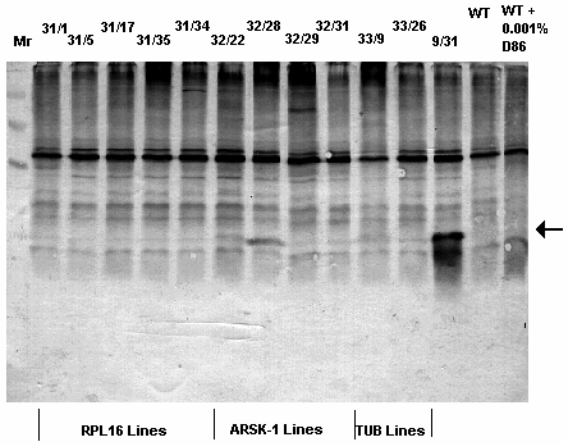


Fig. 13: Western blots of potato root tips showing positive detection of low levels of protein in some lines. Lines designated 9, 31,32 and 33 correspond to expression of OcIΔD86 under control of CaMV35S, RPL16, ARSK and TUB1 respectively. The arrow indicates the expected Mr of the cystatin.

The results show each root specific promoter provided levels of resistance that are significantly greater than achieved with CaMV35S (Fig 14). The level of multiplication of *G. pallida* on control Desire was 21.4 fold. This establishes that the conditions were favourable for the nematode. In contrast to the 1999 field trial, cv Sante provided a high level of resistance. It shows a variable partial resistance to *G. pallida* depending on the virulence of the population that challenges it.

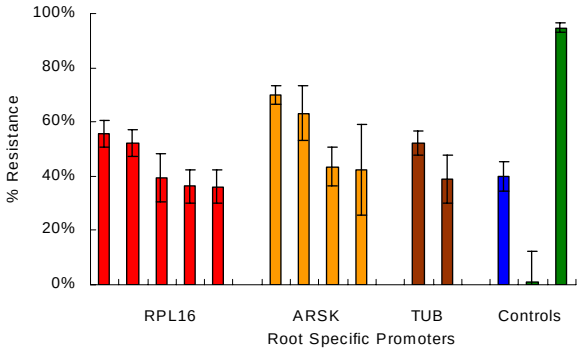


Fig 14. Transgenic field trial comparing resistance to *G.pallida* for lines expressing OcIΔD86 under control of three root specific promoters (RPL16, ARSK and TUB). Controls are OcIΔD86 under control of CaMV35S (blue), and wildtype cultivars Desiree (fully susceptible) and cv Sante (partial resistance, higher green bar). The latter provides a high level of resistance to non-virulent forms of this potato cyst-nematode.

UK Field trials in 2001

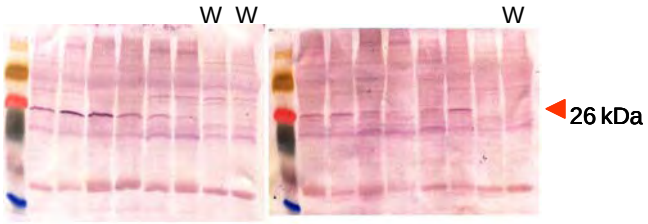


Fig 15: Western blots of transgenic potato lines expressing CaMV35S/Oc-IΔD86/GO/Sunflower from which 5 were selected for field trial in summer 2001.The dual cystatin has a molecular size of 26kDa.

Lines transformed with three constructs expressing two proteinase inhibitors linked by the GO peptide linker. Expression was under control of CaMV35S and up to 5 lines/construct were selected for evaluation in the field. Fig 15 gives an example of the results.

The soil has been harvested and dried but cyst and eggs counts have yet to be made due to pressure of work on other aspects of the work. The counts will be made in late spring 2002.

Evaluation of transgenic potato against *Meloidogyne*.

The next step was to demonstrate that the plants with resistance to *G. pallida* also showed resistance to *Meloidogyne incognita*. This is important for subsistence farmers for which *Meloiodgyne* is the main nematode pest in the mesophilic valleys of Bolivia and other warm soils e.g. within India. Dual resistance is also important for instance in the lower valles region of Bolivia.

Therefore the better lines from the field trial (RPL16 lines 31/1/31/4, 31/35, ARSK lines 32/22, 32/31, TUB lines, 33/9, 33/26 and the CaMV35S line 9/31) were used for challenge with *M. incognita* at 25 ± 3 C in a containment glasshouse. There were no significant differences between the root specific promoter lines and so the pooled results for each promoter are provided in Fig 16. *M. incognita* produced 1328 ± 181 eggs/plant at harvest on controls. This is not a damaging population and so no effect on potato plant growth was anticipated.

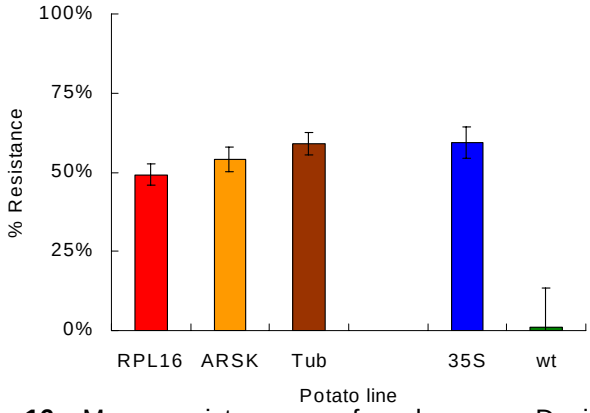


Fig 16: Mean resistance conferred on cv Desiree against *Meloidogyne incognita* by OcIΔD86 when it was under control of three root active promoters and a constitutive promoter (CaMV35S). Comparison is with untransformed Desiree.

There was no correlation between resistance levels achieved against *Globodera* and *Meloidogyne* (Fig 17). The best level of resistance to *G. pallida* was provided by a line under control of ARSK. Lines under control of TUB were slightly more effective for *Meloidogyne* than *Globodera* but the differences are not substantial.

As in the field trial, there was no evidence of a growth penalty from expressing the cystatins although again RPL 16 plants ranked lowest overall in vegetative growth and so expression of a cystatin under control of this promoter may impose a slight growth penalty (Fig 18).

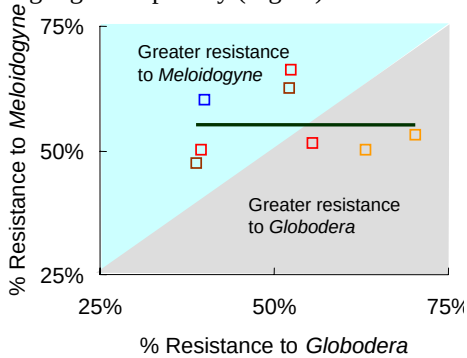


Fig 17: Mean resistance conferred on cv Desiree against *Meloidogyne incognita* by OcIΔD86 when it was under control of three root active promoters, RPL16

(red) ARSK (orange) and TUB (brown) and a constitutive promoter, CaMV35S (blue) when compared against untransformed controls. There is no correlation between resistance levels for the two nematodes

.

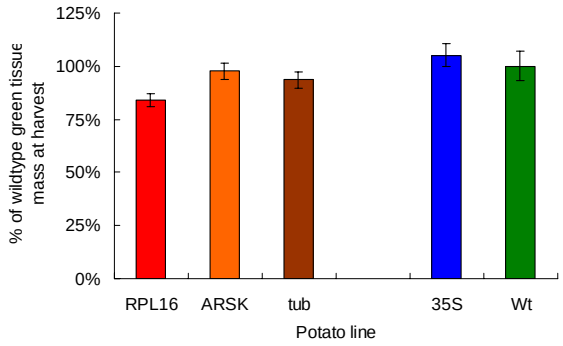


Fig 18: Vegetative growth of potato lines during challenge by *Meloidogyne*.

Bolivian cultivars: Field trials were carried out in the UK in 2000 summer with wildtypes of cv Waycha, Gendarmé and Maria Huanca. The aim was to determine if lines could be trialled and selected under UK conditions prior to trials in Bolivia. The advantages would be a) selection of only effective lines for Bolivia, b) progress while conditions for transgenic trials remain uncertain in Bolivia and c) an ability to confirm to those that oppose use of the technology in Bolivia that only lines pre-trialled in UK were being deployed in Bolivia. The plants proved to grow satisfactorily under UK sites conditions.

Transgenic Bolivian cultivars:
Gendarme and Revolucion lines were screened with an ELISA having a detection limit of 0.03% tsp but high levels of expression were not obtained. Some lines of cv Maria Huanca were detected as expressing the cystatin (Fig 19). The best line from these experiments (MH10) was screened in containment. DTER would not vary our field consent to add Bolivian cultivars to those already listed. The results from western blotting suggest that expression levels in the Bolivian cultivars were less than obtained previously with cv Desiree.

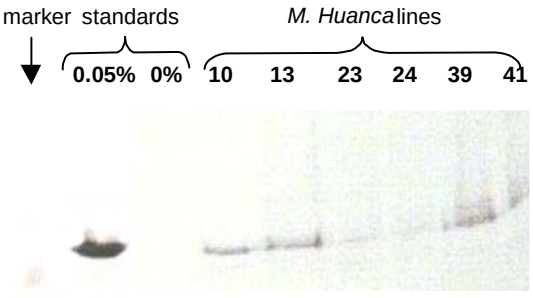


Fig 19: Western blot confirmation of low expression levels of for the transformed Bolivian hybrid cultivar Maria Huanca. Expressing CEWc under control of CaMV35S. Levels of expression were low.

Trials with natural partial resistance
Trials were carried out with the natural partially resistant cultivars cv Maria Huanca in containment and with cv Sante field in the UK. They are both partially resistant to *G. pallida* but Sante is not grown in Bolivia. The aim was to determine if natural and transgenic resistance provided additive effects. Wildtype cv Maria Huanca and its transgenic line 10 (see Fig 19) were challenged with 40 viable eggs/ soil of *G. pallida* in containment. The results for this experiment and that in the field for Sante established that additive resistance did occur for both cultivars. In both experiments the transformed, partial resistant cultivars prevented multiplication of *Globodera* and the population was no higher at harvest than at planting (Fig 20).

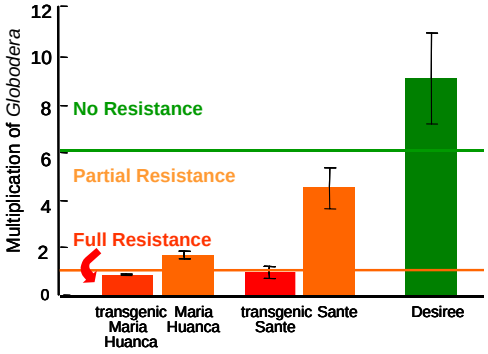


Fig 20: Improvement of cv Maria Huanca in containment and Sante in the field from partial to full resistance to *Globodera* when expressing OcIΔD86. Multiplication on the susceptible cv Desiree in containment is shown. Multiplication in the field is typically c20x.

This is the first demonstration of such additive approaches and offers the important opportunity for complete nematode control in subsistence farming in Bolivia. It is uncertain whether or not this high level of resistance would be sufficient to ensure wide uptake by growers of a variety that has yet to find favour.

Gus reporter studies with potato: This work extended that for promoter activity with rice. Again it used a GUS reporter system. A 1kb TUB-1 was used plus the two other promoters that showed root-preferential expression in *Arabidopsis* (see Fig. 15). The pattern of expression of all three promoters proved activity was limited to some root tissues with no or very little expression in green tissues. Fig. 21 and 22 summarise the results for ARSK and TUB-1 respectively. All three promoters also showed expression in the giant cells induced by *M. incognita* (Fig 23). This localised expression was also detected in syncytia induced by adult female *Globodera pallida* (Fig 24).

Overall the results provide a similar conclusion to that reached for work with rice and TUB-1. All three promoters deliver expression in feeding cells of *M. incognita* but expression elsewhere in the plant is highly limited. This makes detection of expressed protein more difficult in experiments but adds considerably to the biosafety of the approach in future uptake of the technology. Eliminating expression from food products provides a level of biosafety

that adds to that provided by inherent lack of toxicity of expressed proteins such as cystatins.

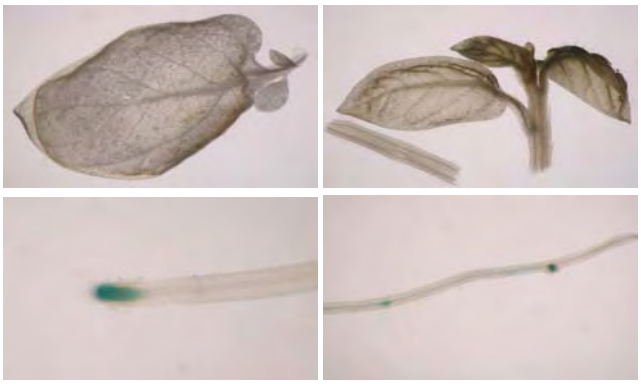


Fig 21: The pattern of promoter activity detected in potato plants for ARSK using GUS as a reporter giving a blue coloration where the promoter is active. It is not active in leaves, stems or leaflets and shows restricted activity in roots at their tips and lateral buds.

Fig 22: The pattern of promoter activity detected in potato plants for TUB-1 using GUS as a reporter giving a blue coloration where the promoter is active. It is active in stems but not leaves and shows variable activity in roots close to root tips.

Fig 23: *Meloidogyne* females (brown spheres) in potato roots with their giant cells showing GUS reporter activity (blue coloration) when under control of left ARSK, centre, RPL16 and right TUB-1 promoters.

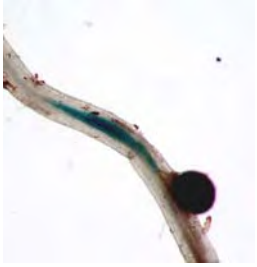


Fig 24: *G. pallida* female parasitising potato roots with its feeding cell showing GUS reporter activity (blue coloration) under control of the ARSK promoter.

Development of male sterile potato plants

The perceived risk of cross fertilisation of pollen from transformed potatoes to wild type solanaceae in South America was addressed in the project. Male sterile constructs have been developed using a tapetum specific promoter (TA29) and a pollen specific promoter. Both of these regulated the expression of either an RNAse (barnase) which is known to kill plant cells or a ribosomal inactivating protein (RIP) originally from maize kernels. Fifty lines of potato/construct were screened for normal vegetative growth and loss of viable pollen. This involves assessment of pollen tube formation and fluorescent staining for pollen viability. (Fig 25).

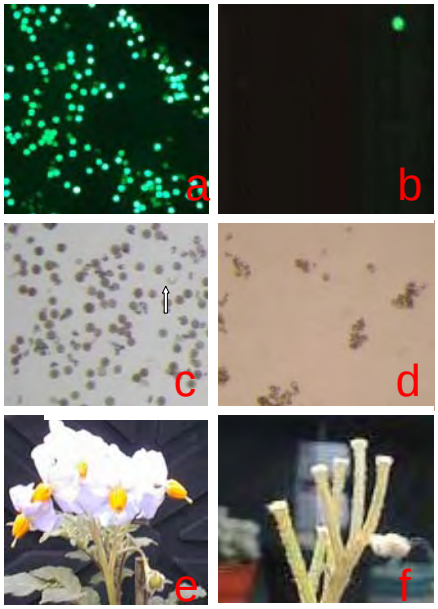


Fig.25: Left from male fertile plant, **a**, viable pollen taking up a fluorophore, **c** viable, pollen with a pollen tube arrowed **e**, berry formation. **right from male sterile plant; b** non-viable pollen (rare fluorophore uptake), **d**, no pollen tubes **f**, flower drop without berry formation.

Analysis of pollen grains able to produce tubes or take up a vital dye was reduced to <1% in contrast to control levels of 40% and 55% respectively (Table 7).

Construct	#	tubes	#	FDA +ve
RIP	1110	5	1350	4
Barnase	150	1	500	1
Control	10	4	200	110

Table 7: Number (#) of pollen grains examined and able to form tubes or take up the vital fluorescent dye FDA when expressing a RIP or barnase (an RNAase) relative to values for control potato plants.

Field markers for transgenic lines

Labelling plant products for the developed world markets is an essential part of consumer choice. In the developing world there are a number of problems that relate to this issue and its practical utility. High female illiteracy in Bolivia is known to hinder health care objectives and FAO have concern over pesticide safety when labels must be understood to avoid risk. There is often no labelling of any products in rural markets and it would be difficult to enforce or provide the infrastructure for this.

We explored approaches for identification of transgenic lines in the field. We obtained an unusual, white flowered variant of Waycha from SEPA in Bolivia (Fig 26). A plant passport

was obtained for it and it is available for future transformation in the UK. Growers can distinguish Waycha tubers from others. Therefore the white flower could be used as a field indicator that a GM variant of Waycha is being grown. This would allow for positive confirmation of GM or conventional cultivars by growers.

Secondly we were provided by Dr Jeffries of SASA with abnormal leaf variants of cvs Desiree and Romano. Their characteristics were confirmed in our glasshouses for possible future use in Bolivia as transgenic cultivars with a visually distinct phenotype (Fig 27). Desiree is widely grown in Bolivia but Romano is not. However the two cultivars are very similar and share tuber characteristics. The crucial difference is that Romano rarely flowers in UK conditions. It also fails to flower in Bolivia, then it offers a biosafe alternative to Desiree. The latter cultivar fruits readily when it flowers in Bolivia.

Fig 26: Left: normal purple flower of Waycha. **Right**, white flowered variant introduced to UK as a basis for a transgenic Waycha distinguishable from an untransformed plant in the field.

Fig 27: Lines of Desiree showing normal (upper) and elongated leaf shape (lower).

Cultivars that do not flower or have been rendered male sterile, contribute to biosafety of transgenic potato donation to subsistence farmers. Those that possess distinct flower colour or leaf shape provide a check for growers of their choice to adopt or avoid transgenic plants. They also assist socioeconomists to assess future uptake during field visits.

Public appreciation of GM potential for Bolivia

This work was carried out by Dr Javier Franco at PROINPA to determine attitudes within Bolivia (Table 7). It reveals widespread support for GM crops and helped establish that in future we should secure population support for experimental field trials in land close to them. Unfortunately funding constraints and some uncertainty whether or not a temporary embargo on GM trials with potatoes is to be lifted by the Ministry of Agriculture has prevented field trial of the potato plants.

ACTIVITIES	events	#	% RESPONSE TO POTATO GM RESEARCH		
			positive	negative	Do not know
Conferences	12	221	92	0.02	7.8
Debates	2	500			
Farmer field visits	22	857	55	0	45
Television Interview	4	GP			
Radio Interview	5	GP			
Press interview	4	GP			
Institutional survey by hand-out	43	-	45	0	55
Dossier	1	GP			
Techniques Documents	3	GP			
Triptychs	1	GP			
Radio Spot	1	GP			
Bulletin	1	GP			
Slides for farmers	1				
Data show presentations	15				
Demonstration aid	1	857			

Table 8: Summary of public perception of GM potato at different levels of society in Bolivia by Dr Franco and colleagues of PROINPA. # = total number of attendees or general circulation to GP = general public.

Control of Andean weevils

Soybean kunitz trypsin inhibitor has been shown to be effective against Andean weevil (see final report for small grant R7886).

Dissemination

Publications

Atkinson, H.J. Molecular approaches to novel crop resistance against nematodes pp 569-598 in *The Biology of Nematodes* (edited Lee D.L.) Taylor and Francis, London, 635pp (USBN 0-415-2711-4)

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Atkinson, H.J., Green, J. Cowgill, S. Urwin, P., Franco, J. and Witcombe, J. (2001). Developing a paradigm for safe adoption of GM crops with a poverty focus: a specific example of nematode resistance for potato in Bolivia. In Conference proceedings "*Sustainable agriculture in the new millennium – the impact of biotechnology on developing countries*" Brussels, May 28-31, 2000. In Press, FOE, Europe.

Green J., Vain P., Fearnehough M,' Worland' B., Snape J. and Atkinson H.J. (2002) Root specific expression analysis of the *Arabidopsis thaliana* tubulin-1 promoter and the

constitutive rice ubiquitin promoter in rice plants for nematode resistance. *Physiological and Molecular Plant Pathology, in press.*

Urwin, P.E., Green, J. and Atkinson, H.J. (2000) Resistance to *Globodera* spp. in transgenic *Solanum tuberosum* cv. Désirée that express proteinase inhibitors *Aspects of Applied Biology* 59, 27-32 2000.

Urwin, P.E. Troth, K.M. Zubko, E.I. and Atkinson, H.J. (2001) Effective trangenic resistance to *Globodera pallida* in potato field trials. *Molecular Breeding*, 8, 95-2001.

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Atkinson, H.J. (2000) Developing a paradigm for safe adoption of GM crops with an poverty focus: as specific example of nematode resistance for potato in Bolivia. Consultation of Environmental Protection Dept. of DFID with research programme managers, project leaders and advisers on 19th December 2000 at 94 Victoria St, London..

Presentations

Atkinson, H.J., Green, J. Cowgill, S., Urwin, P., Franco, J. and Witcombe, J. (2000). Developing a paradigm for safe adoption of GM crops with a poverty focus: a specific example of nematode resistance for potato in Bolivia. In Conference proceedings "*Sustainable agriculture in the new millennium – the impact of biotechnology on developing countries*" Brussels, May 28-31, 2000.

Atkinson, H.J. and Green, J. (2000) The case in favour of transgenic, nematode resistant potatoes for Bolivia. In DFID PSP +CPP/CIP Conference: Biosafety of GM Potato in the Developing World, Manchester, June 2000.

Atkinson, H.J. (2000) *Transgenic nematode resistant potatoes for subsistence farmers*. Research Seminar, International Potato Centre, Lima 3rd October 2000.