Bt Cowpea with Protection against Pod-borer for Transfer to Africa

2007 FS 003

Final Report to the Rockefeller Foundation

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Final report

The project was expected to produce the following deliverables and achieve during this grant term the following milestones:

1) to create up to 100 independent events of transgenic cowpea expressing the designated Bt gene;

2) from these to select those that are homozygous for the gene and show strong and stable expression of the gene through succeeding generations;

3) to select the one or two most potent events through testing against pod borers in Australia in greenhouses;

4) to set the stage, get permits, and export seed of the best events to one or two suitable countries in West Africa;

5) to carry out training of West African cowpea breeders to allow them to back cross the gene into local varieties and eventially to carry out confined field trials in their own countries;

6) to subcontract some bio-safety studies involving gene flow and ways to monitor and/or prevent it.

Summary of Achievements

Over 260 independent transgenic events containing the reconstructed Cry 1Ab gene were produced. All lines were characterized at a molecular level including gene copy number and Bt protein level by ELISA and western blotting. One hundred and four events were taken through to homozygosity using quantitative real time PCR and progeny testing. Of these homozygous lines, 60 events were high expressors and showed stable transmission of the transgene over several generations. In some cases gene stability has been demonstrated over seven generations. A balance between high expression and vigorous phenotype had to be struck when it was discovered that there was an inverse relationship between level of expression and plant vigour. Twenty five events were selected for field trialling. The first and second of these were conducted in Puerto Rico which permitted us to select lines for a confined field trial (CFT) in Nigeria in 2009 and 2010. Permits have been obtained for CFTs in

Nigeria in 2009, 2010 and 2011 and for Burkina Faso in 2011. A permit is close to grant for Ghana in 2011 or 2012. The cowpea breeder in Nigeria is already trained in backcrossing the transgene into locally adapted germplasm and is in the third season of such introgression. He has selected one line which is showing promising *Maruca* resistance and good agronomic performance. The Nigerian cowpea breeder is in the process of training the breeders in Burkina Faso and Ghana in the skill of transgene introgression. Biosafety studies were subcontracted out to ICIPE and Dr Remy Pasquet has already reported separately to Rockefeller Foundation on the outcomes of that research.

Introduction

Cowpea, *(Vigna unguiculata),* has an annual production of 8-10 million tonnes, and is one of the most important food grain legumes in the tropics. Africa, accounts for over 60 percent of the world production. The major cowpea producing countries in Africa are Nigeria, Niger, Mali, Senegal, Burkina Faso and Ghana. While cowpea leaves, green pods and green peas are consumed as human food, it is the protein-rich grains that constitute the main food product of the crop.

In spite of the significance of cowpea as a food crop to millions of people on the continent, grain yields are low at 0.3 tonnes/ha due to several biotic and abiotic stresses. The adverse effects of some of these yield-limiting factors could be ameliorated through better agronomy, while others require manipulation through breeding.

Losses due to insect pests alone have been documented to sometimes exceed 90 percent. There are cases where modest levels of resistance have been developed in cowpea varieties against some of the insect pests, but there is no cultivated cowpea germplasm with demonstrable resistance against *Maruca* pod borer (MPB), a serious field pest of cowpea. Insect pests, especially lepidopterans, are known to be controlled by *Bacillus thuringiensis* (Bt) – a ubiquitous, soil-dwelling, spore-forming bacterium – when applied topically on crops as spore formulations. However, Bt sprays are often washed away by rain, degrade under solar ultra violet radiation and are not optimally targeted against certain insect pests that live within plant tissues. The limitations associated with the use of conventional methods in effectively dealing with cowpea's pest problem makes the application of biotechnological procedures for overcoming the constraints to cowpea production particularly attractive. With advances in molecular and cellular biology, it is now possible to engineer into plant genomes the genes that encode expression of crystal proteins, thus providing the plant with built-in protection against lepidopterans such as

MPB. This effort is currently under exploration by a coalition of institutions to reduce grain yield losses in cowpea in Africa. If the Bt gene, which confers resistance to the pod borer, is transferred into improved cowpea varieties, the need for insecticidal sprays to control the pod borer will be reduced and smallholder farmers can increase their yields by over 20% and greatly enhance their food security and economic status.

Background

The *Maruca* pod borer is known to be susceptible to the crystal (Cry) proteins produced by certain strains of *Bacillus thuringiensis* (Bt)[1]. A Bt gene for the appropriate Cry protein, when introduced into cowpea, is therefore predicted to control this pest and would provide in-built plant protection technology that could become available to farmers via the seeds and be expected to have a major impact on food security in sub-Saharan Africa. During the Rockefeller Foundation project (2006 FS 004) we have adapted an *Agrobacterium* mediated transformation protocol for cowpea developed earlier [2] so that we can use a selection system that is acceptable to regulators. We have constructed a Bt gene supplied by Monsanto to AATF so that the gene will work in the reproductive organs of the cowpea that are attacked by the podborer [3]. We produced the first set of transgenic lines and tested them for protection against another caterpillar known to attack cowpea and which acted as a surrogate for *Maruc*a until we found *Maruca* and set up the assays for this pest in Australia. The major tasks for the new project were to produce large numbers of transgenics so that we could select one elite line for sending to Africa for the introgression program and generation of varieties for different African environments and for Remy Pasquet to complete baseline studies on the potential weediness of Bt cowpeas.

Methodology;

1. Produce over 100 different transgenic cowpea lines with the Bt gene

2. Select high expressing lines that transmit the gene to the next generation

3. Make homozygous lines suitable for *Maruca* testing in Australia and Africa

4. Evaluate effect of predators on potential weediness of wild cowpea as part of Biosafety Studies

5. Evaluate whether cleistogamy will contain the transgene and help protect against gene flow to weedy cowpea

Expected Outcomes;

To have tested the efficacy of selected Bt cowpea lines using a *Maruca* assay developed in Australia and supplied seeds to our plant breeding colleagues in Africa when regulatory approval was obtained to do the tests in Africa. This would supply the germplasm with resistance to pod borer, which could then be used to supplement the existing cowpea breeding programs in sub-Saharan Africa. We would obtain baseline knowledge of the potential for cowpea with enhanced fitness to become weeds. Cowpea gene technology would be transferred to African national institutions as well as IITA.

Plans for dissemination;

In the new project, we would provide well characterized lines of transgenic cowpea in a genetic background that will expedite a rapid backcrossing program by African plant breeders into adapted germplasm for multiple agro-ecological zones. This germplasm would include lines that would help contain the gene and greatly reduce the chance of gene flow to wild and potentially weedy cowpeas. We would train African scientists in gene technology and trait introgression and provide information to regulators on likely weediness of Bt cowpeas and their relatives.

In year one of 2007 FS 003 we produced over 50 transgenic cowpea lines with the reconstructed Bt gene. A total of 52 T_0 lines were established in the glasshouse and 50 of 52 lines were fertile and produced seed for the next generation (T_1 seeds).

We observed variable levels of expression ranging from very low levels to levels that exceed those found in Bollgard II cotton. From the 52 T_0 lines we selected six high expressing lines by polymerase chain reaction (PCR), enzyme linked immuno sorbent assay (ELISA) and by western blot analyses. With the availability of T_1 seed we began the process of selecting lines that were homozygous for the Bt gene with a particular focus on the high expressers. Some lines had progressed to the T_3 stage and we expected that we would obtain several homozygous lines during 2009.

We were not able to find the *Maruca* podborer in Australia in 2007 due to continuing drought. As a result we proposed, together with AATF, to do a confined field trial in Puerto Rico in 2008 to assess whether the high expressing Bt lines protect against *Maruca* as they have done in laboratory bioassays against the insect pest *Helicoverpa armigera*, the African boll worm. Although the trial was most useful in that it allowed us

to assess potential agronomic performance of the lines, we did not obtain data on efficacy against *Maruca* because of very low insect pressure.

We proposed to host Dr Mohammed Ishiyaku in 2007. Unfortunately this was not possible because he had not been able to obtain a visa to visit Australia. Instead I visited his facility in Zaria, Nigeria together with Larry Beach so that we could progress discussions with him about introgression and the breeding of new varieties. I was not optimistic that he would be able to visit Australia in 2009 based on information obtained from the High Commission in South Africa and Professor Ishiyaku's steep commitments in Nigeria.

Details of Progress

Produce up to 100 transgenic cowpea lines expressing the reconstructed Bt gene

During the 2007/2008 year we produced 52 independent transgenic lines in the background of the advanced breeding line, IT86D-1010. This was possible because of the enhanced efficiency we have observed by using the *npt* II gene controlled by the sub-clover stunt virus promoter.

These lines were established in the glasshouse and 50 of the 52 lines produced T_1 (next generation) seeds. The number of seeds produced varied from event to event depending upon the season at which they were transferred from the tissue culture chamber to the greenhouse. Usually a minimum of 6 seeds were produced in the T_1 generation and sometimes much more. The period between March and October is not ideal for cowpea seed production in Canberra under our glasshouse conditions and we ascertained that it is preferable to move plants during this part of the season to the Canberra Phytotron where night temperatures are higher and the resultant plants produce seed more quickly. In subsequent years we followed the protocol of establishing plants in the Phytotron during the Canberra autumn/winter season.

Select high expressing lines that transmit the Cry gene to the next generation

All 52 primary (T_0) transgenic cowpeas were analysed, first for the expression of the *npt* II gene by dot blot analysis. As expected all the lines were positive for expression of this transgene. This reassured us that there were fewer escapes in the transformation system than we had expected from earlier work. The plants were then analysed for the presence of the Cry1Ab gene by PCR and 50 of the T_0 plants were found to be positive. The activity of the Cry1Ab gene was analysed by ELISA and western blot. The majority of the plants expressed the Cry1Ab gene although to varying levels as is typical of such experiments with

transgenic plants. Fifteen strongly expressing lines were particularly interesting although several other lines with intermediate to moderate levels of expression were useful in insect bioassays. The Cry1Ab protein was found as a 65,000 molecular weight protein as expected although several cleavage products were also detected (see Fig. 1). The cleavage products were not detected in all plants but were apparent in several (see profile for line 632A in Fig. 1). The significance of these cleavage products is unknown but similar low molecular weight forms of Cry1Ab have been observed in other transgenic crops such as corn and cotton as well as in *B. thuringiensis*.

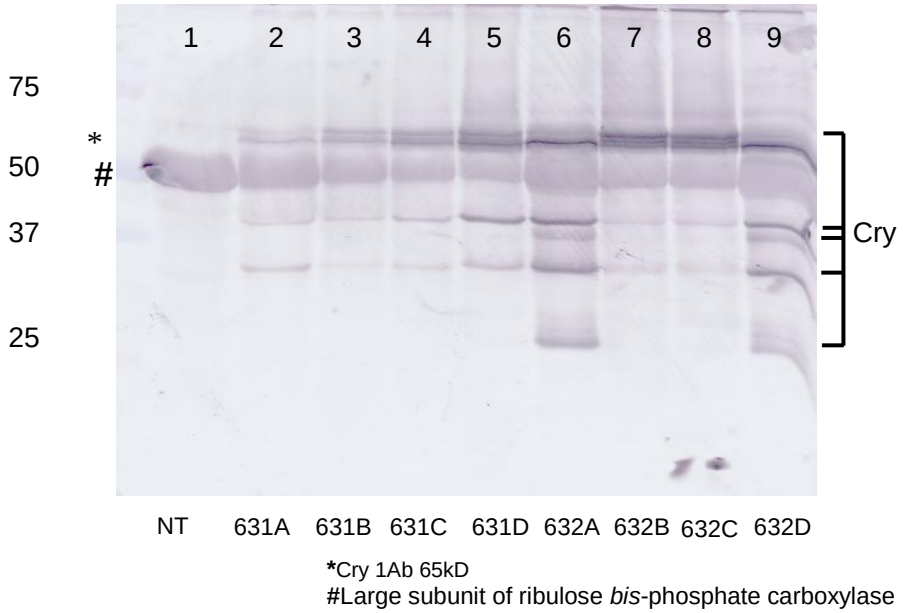


Fig. 1. Cry1Ab protein in eight independent lines of Bt cowpea

We also estimated the segregation ratio at this stage but because of the low number of seeds usually produced in the T_1 generation we could not place a lot of credibility on the segregation ratio. At this T_1 stage we were most interested in gene transmission as well as its level of expression. The T_0 plants were frequently not as productive as their T_1 progeny because of the extended period of tissue culture and the stress of being transferred from a tissue culture chamber to the greenhouse. It has been our experience that the T_1 plants are much more vigorous than the T_0 progenitor line. That is, the plants recover from tissue culture effects once they have been through one seed generation. We usually wait for the T_2 generation, when we can routinely produce 50 to 80 seeds on each plant, to make a firmer conclusion about the segregation ratio. We then grow on at least 30 individual T_2 lines from each T_0 parent.

Select lines that are homozygous for the Bt gene

As indicated in Milestone 2 we frequently did not obtain sufficient seed in the T_1 generation to make a definitive conclusion about segregation ratio and thus grew on the T_1 lines to produce T_2 seed. It was our experience that we could more reliably determine segregation ratio in the T_2 progeny and we selected 15 high expressing lines out of 50 that warranted production of T_2 and T_3 seeds in order to obtain homozygous lines for a first field trial in Puerto Rico. We had not obtained any homozygous lines by the end of December 2008, although in 2009 we did obtain several homozygous lines.

Train Dr Mohammed Ishiyaku in the techniques of Bt gene introgression

There are several plant breeders in West Africa who could receive training in how to handle transgenics and prepare for the introgression work that will be needed to produce varieties for different agri-ecological zones throughout Africa. Dr Ishyiaku was identified from the Nigerian national system in Zaria as the first candidate to be invited. Dr Ishyiaku was invited to Australia for a period of training. However, we have experienced some difficulty with the Australian High Commission in Africa (based in South Africa). Hence he was unable to arrive in 2008. We sought the help of AATF and ascertained that while he may be able to obtain a visa it is unlikely that he can fit this into his busy schedule as he is now Dean of Students. As an alternative to the training of Dr Ishiyaku we undertook to invite Nigerian Regulators from the Department of Environment to obtain training in Biosafety and Risk Assessment procedures (see later). We also hosted Dr Ivan Inglebrecht from IITA and shared our joint experiences in cowpea transformation.

Other Significant Events in 2007/2008

The early stages of this work for 2007 were described at the Rockefeller Biotechnology Conference in Maputo, Mozambique in 2007 and at the Cowpea Meeting in Accra November 2006.
Significant visitors to the Bt cowpea project in Canberra included Dr Mpoko Bokanga, the Executive Director of AATF, Francis Nang'ayo, manager of Regulatory Affairs at AATF and Nancy Muchiri, Manager of Communications at AATF. They spent substantial time visiting the project but they also visited the Australian regulators and communicators to discuss gene technology in Australia. Their visits included Food Standards Australia New Zealand and the Office of the Gene Technology Regulator as well as Biotechnology Australia and AgriFood Awareness Australia. These visits proved very useful to the researchers in Canberra and also to the visitors from AATF. The visitors gained firsthand experience of the

regulatory system in Australia and became familiar with the Bt plants in the greenhouse.

Awareness of the Bt Cowpea Project was also raised in the Australian Development Community which funds relatively little work in Africa. We have convinced the Australian Academy of Technological and Engineering's (ATSE) Crawford Fund to sponsor a Regulator from the Nigerian Ministry of the Environment to study the regulatory system in Australia.

> *Email from Tony Fischer:ATSE Crawford Fund:*
> *Dear TJ*
>
> *My ACT CF Committee has given the green light on support for Mr Ebegba to come to Australia in 07-08 as per the application you sent some time ago in the name of Mr Dore ($9187.50). Could you simply update that application and send to me, while moving forward on the assumption that the money will come to you in due course.*
>
> *Cheers*
>
> *Tony*

We invited two senior members of the Federal Ministry of the Environment in Nigeria (Mr R Usman and Mr R Ebegba) to visit their counterparts in Australia in late 2008.

A fourth significant visitor to the project during the year was Dr Willy De Greef who spent one week going over the details of the project and providing advice to the researchers and the project management team on protocols and procedures that will need to be documented prior to the development of the regulatory package as part of the application for de-regulation in Africa. Dr De Greef's visit was useful because of his extensive experience with other crops and his in depth knowledge of the European regulatory environment.

Another significant event in late 2007 was a visit that I made with Larry Beach to Nigeria to observe the crop during the growing season and to visit plant breeders and the regulators. We spent one week travelling from Ibadan to Kano and also visited Dr Mohammed Ishiyaku in Zaria as well as representatives of USAID in Kano and Abuja. In Abuja we met with several members of the National Biotechnology Development Agency and Federal Ministry of Environment as well as SHESTCO. This visit was an extremely important opportunity to brief the Nigerian community about

the status of the project and for us to gain a perspective of some of the issues that a Bt cowpea would face during the early stages of containment and confinement trials in Nigeria. There is still much to be done in this area but we were impressed with the high level of interest that the decision-making community in Nigeria is showing in Bt cowpea.

Finally, a Project Advisory Committee (PAC) for the Bt cowpea project had its first meeting in Washington DC during February 2008. This committee is building on work that has been done by Francis Nang'ayo, Jacob Mignouna and Mpoko Bokanga. The PAC plays an important role in providing guidance for the project and in solving some of the hurdles that will need to be jumped prior to the conduct of trials in Africa.

The Methodology for 2009 included:

1. The production of another 70 T_0 events
2. Molecular characterisation of selected high expressing lines
3. Establishment of a lab colony of Maruca vitrata in Canberra
4. The selection of homozygous lines for field trials
5. Drafting of an application for a field trial in Nigeria for 2009
6. Selection of lines suitable for a Field Trial in Nigeria

Summary of progress for 2009

More than 150 transgenic events containing the Cry 1Ab chimeric gene were produced and almost all were shown to transmit the transgene to subsequent generations. Molecular characterisation was achieved and a modification of PCR called quantitative PCR (Q-PCR) was adapted to speed up the selection of single copy insertions and the subsequent determination of zygosity levels. This meant that we could select for homozygous lines in the T_2 generation and obtain T_3 seed in good quantity (300-500 seed) and in a reasonable period of time following the generation of new events. This allowed us to conduct a second field trial in Puerto Rico with genetically homogenous material and to learn that there appears to be an inverse relationship between Bt protein level and plant vigor in the field. This has meant a shift of focus from concentrating on Bt protein level to achieving a balance between Bt protein amount and a vigorous phenotype. A glasshouse based screen for a strong phenotype proved useful in selecting lines that performed well in the field. Lines were selected for field trials based on growth in the glasshouse and they possessed the same relative ranking when grown in the field.
A lab colony of *Maruca vitrata* was established in Canberra. This means that we could assay the Bt plants for efficacy in the lab prior to selecting lines for the field.

The production of another 70 transgenic T0 events

By this stage we had a total of 163 transgenic lines that were moved to the glasshouse. They were screened by PCR and by quantitative PCR to estimate gene copy number insertions.

Six new lines were selected for CFT 2 in Puerto Rico by a combination of PCR, western blot, Q-PCR, segregation analysis and glasshouse "phenotyping" as described in 2 below. We concentrated on screening all of these lines for single copy of the Bt gene, homozygosity and vigorous phenotype in the greenhouse.

We also selected a set of additional lines for the first CFT for Nigeria. These lines included two being tested in Puerto Rico so that comparisons could be made between different environments and between trials.

The continued production of new lines with the existing construct slowed while we evaluated the phenotypes from the 163 lines already in hand. Based upon the outcomes of a strategy meeting in Purdue in August 2009 we consolidated the data from the existing lines and pursued the generation of a new construct that targeted the Bt protein to the chloroplast compartment in order to evaluate the effect of sequestering the protein on the stunting phenotypes that we were observing. This strategy is based on data emerging from potential commercial lines of Bt soybeans being prepared for release in Brazil and that were described in US patent application WO/2009/064652 and by Miklos and colleagues [4].

Select high expressing lines that transmit the Bt gene to the next generation via molecular characterisation and phenotyping

Although we used Southern blot analysis to test for gene copy number and the presence of backbone sequences as a routine, this is very laborious and time-consuming because the primary transgenics are small and do not produce a lot of leaf in the T_0 generation. We have to wait until T_1 in order to collect enough leaf to do Southern blots. Following discussion with Joe Huesing and Larry Beach about the procedures used in industry to overcome this bottleneck I visited Monsanto and Third Wave Technologies in Madison to evaluate quantitative PCR and Invader Technology. During 2009 I spent a week in Madison and gained considerable insight about the value of these moderately high throughput systems of molecular analysis. The results in the lab in Canberra with Q-PCR have been most useful. We are able to do gene copy number on T_0 material which allows us to discard lines with multiple inserts at a very early stage. This saves on greenhouse space and speeds up the

production of homozygous lines with single copy inserts. We also explored the use of Invader Technology to complement the results with Q-PCR.
It is now possible to screen the T_1 generation plantlets for zygosity using Q-PCR which allows us to select the homozygous parent from that generation. The T_1 plants can then be relied upon to produce 25-50 seed which means that in the third generation we produce at least 1000 homozygous seed.
As a result of the CFT 1 in Puerto Rico we became aware that the high expressing lines were showing signs of stunting especially when the lines were converted from hemizygous to homozygous. This raised the question about how to screen the plants before we went into the field. With the advent of new phenomics tools which allow us to monitor plant growth in real time we used the Lemna Tec Scanalyser to measure growth rates of selected transgenic lines against the growth of the non-transgenic parent line IT86D-1010. As can be seen from Fig 2 we found that there was considerable variation between the vigor of the lines.

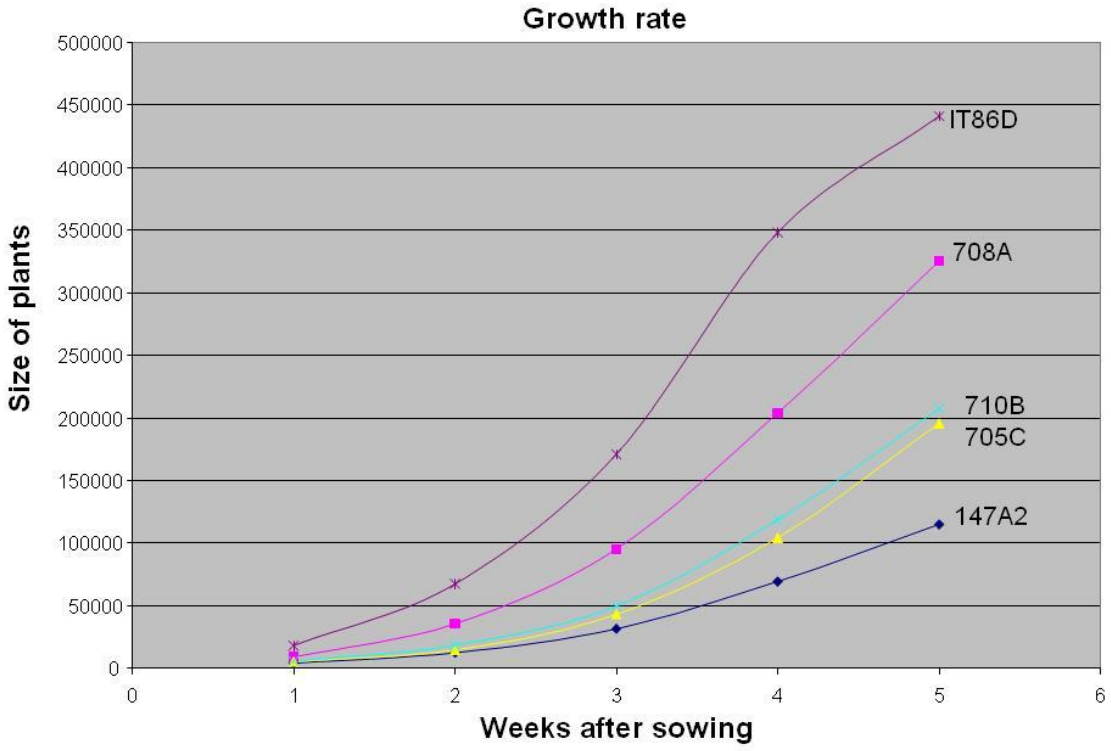
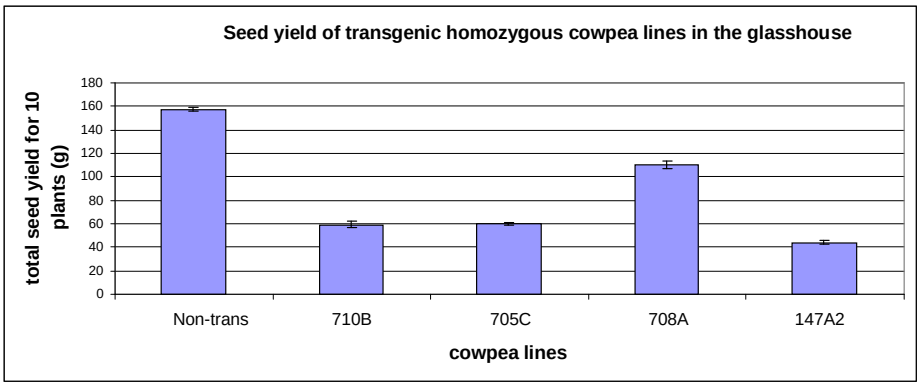


Fig 2. Growth rates of several transgenics compared to the non-transgenic parent line, IT86D-1010

The growth rate differences were reflected in seed "yield" per plant and from the results from CFT 2 in Puerto Rico it was clear that the technique of measuring plant growth in the greenhouse could be used a reliable predictor of how the plants performed in the field.

Is there a correlation of the growth patterns of transgenic lines grown in the glasshouse and the field?



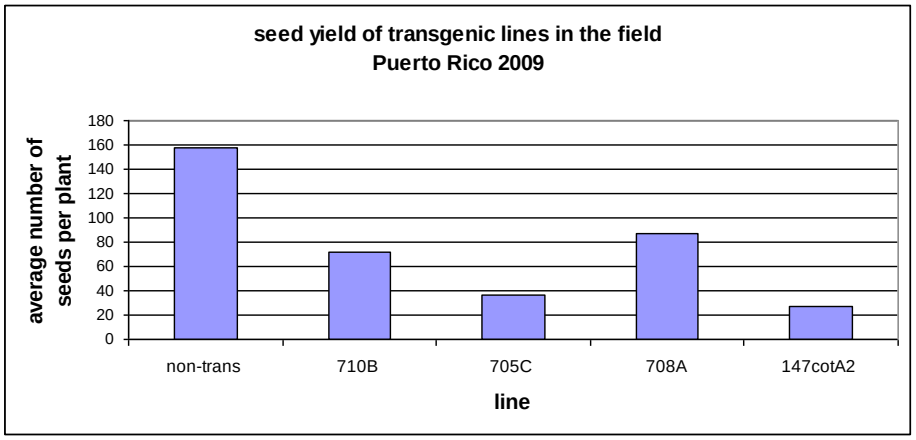


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Fig 3.There was a close correlation between growth in the glasshouse and in the field as measured by seed "yield" per plant.

Field Trials

With Dimuth Siritunga and Fernando Gallardo at University of San Juan and Jeff Stein at PBS and Larry Murdock at Purdue University two confined field trials (CFT) were conducted at Adjuntas under the control of APHIS. Permission was obtained for the import of 14 and 6 transgenic lines containing the Cry1Ab gene as well as the non transgenic parent line IT86D-1010. All the lines in CFT2 were tested for, and confirmed to be, homozygous. The trial design and the

associated buffer zone for CFT 2 are shown in Fig 4.

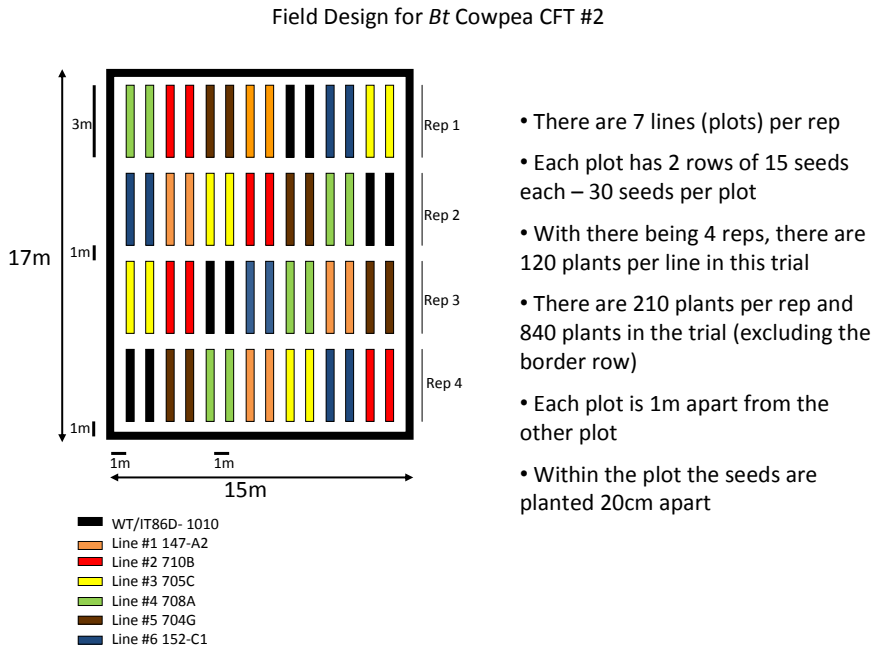

Fig 4. Design and layout of CFT in Puerto Rico

Seed were sown in the field on June 1 2009 and germination was monitored over the following two weeks. The percentage germination varied from 63 to 89% with IT86D at 76%. In summary, germination was better than in CFT 1 which may reflect the fact that seed did not have to be tested for the gene by shaving off any flour.

Fig 5. The Puerto Rico CFT plot about the time that germination was monitored.

We made a first assessment of the efficacy of these plants when they were flowering in early August 2009. Mpumi Obokoh, Joe Huesing, Larry Beach, Venu Margam, Mohammad Ishiyaku and Jeremy Ouedrago were in attendance. Growth of the different lines was variable as expected but in general the breeders were happy with agronomic performance of lines 708A and 710B. The most disappointing result was the absence of Maruca so that we could not get any efficacy data. It was thought that the season was too dry for successful mating and survival of the insects. Fernando released large numbers of lab-reared adults but they did not successfully infest the plots, largely because of intense bird predation and again because of the low humidity. By the end of November we harvested the seed and obtained yield comparisons between the transgenics and the non-transgenics.
An application to conduct a field trial in Nigeria in 2009 was successful. Seed of four homozygous lines were exported to Ahmadu Bello University and IAR, Zaria, Nigeria and a fully replicated trial was established by Dr Mohammad Ishiyaku and colleagues.

Establishment of a lab colony of *Maruca vitrata* in Canberra

We previously used *Helicoverpa armigera* as a surrogate for *Maruca* in lab-based insect bioassays. This was useful in the short term but was not a long term viable option. With the help of colleagues in the Northern Territory Government and CSIRO Entomology we now have a breeding colony of *Maruca vitrata* in the lab in Canberra. We used the colony in bioassays of flowers, young pods and whole plants (see Figs 6 and 7).

Fig 6. Larvae found in the flower of a transgenic (left) and non-transgenic (right) cowpea. Note that few larvae survived on the transgenic plants and the survivors were 10% of the size of larvae on the non-transgenic parent line.

Fig 7.Examination of cowpea plants following a whole plant bioassay with *Maruca*

Biosafety Studies including Gene Flow-Conducted by Remy Pasquet at ICIPE

Remy Pasquet provided his report to Rockefeller Foundation in 2009 and this complemented one of his earlier reports to the Foundation as part of 2004 GI 001, as they were related.

Summary of outcomes in 2010

We have completed the molecular analysis of all the T_0 transgenic Bt v 1.0 cowpea plants (now totaling 265 T_0 events) and have information on the level of Bt in the leaves of all of them which was completed in the 2010 year. We have another 40 homozygous lines characterized for their gene copy number and selected several lines that are vigorous but still have detectable levels of Bt toxin. We still have 69 T_1 families in the testing regime and we predict finding, during 2011, several likely lines for field trialling in 2012. We have conducted a second field trial in Nigeria. We have much improved proof of concept information on the efficacy of a Bt gene for the control of *Maruca* caterpillars in cowpeas compared to what we had from CFT 1 in Nigeria or the CFTs in Puerto Rico. We have generated a set of Bt-CTP (Bt v2.0) cowpeas and are putting them through an intense program with a view to having several lines available for field testing in 2012.

We expect to have a response from Monsanto in 2011 on the likelihood that we can gain access to a second Bt gene with a different mode of action that will greatly enhance IRM for cowpea.

Completing the *Maruca* bioassays on selected homozygous lines of Bt cowpea

Maruca larvae were raised on a meridic diet based on that developed by Jackai and colleagues [5]. Second instar larvae were placed on cowpea flowers in 32-well dishes and monitored after 3-7 days. Where necessary, flowers were replaced every 48-72 hours. In the case of flowers from most transgenic lines, this was not necessary as larval mortality was 100% within 48 hours although in lower-expressing lines we observed lower mortality, ranging from 40 to 70% (see Table 1).

Table 1. Mortality of 2nd Instar *Maruca* Larvae on Flowers of Selected Cowpea Lines Expressing the Cry 1Ab Gene

Cowpea line	Mortality (%)
1. IT86D-1010	0
2. CP147A2	100%
3. 704E	100%
4. 708A	100%
5. 705C	100%
6. 710B	100%
7. 212D	70%
8. 721C	70%
9. 218B	40%

These lines allowed us to prepare dose-response curves to Cry1Ab toxin levels in flowers (See Fig 8). We conclude that lines containing over 1.5ng Cry 1Ab/mg Fresh Weight of flowers (as in Line 212D) give 100% mortality. Line 709A has approximately three-fold the minimum level of Bt protein needed for full mortality.

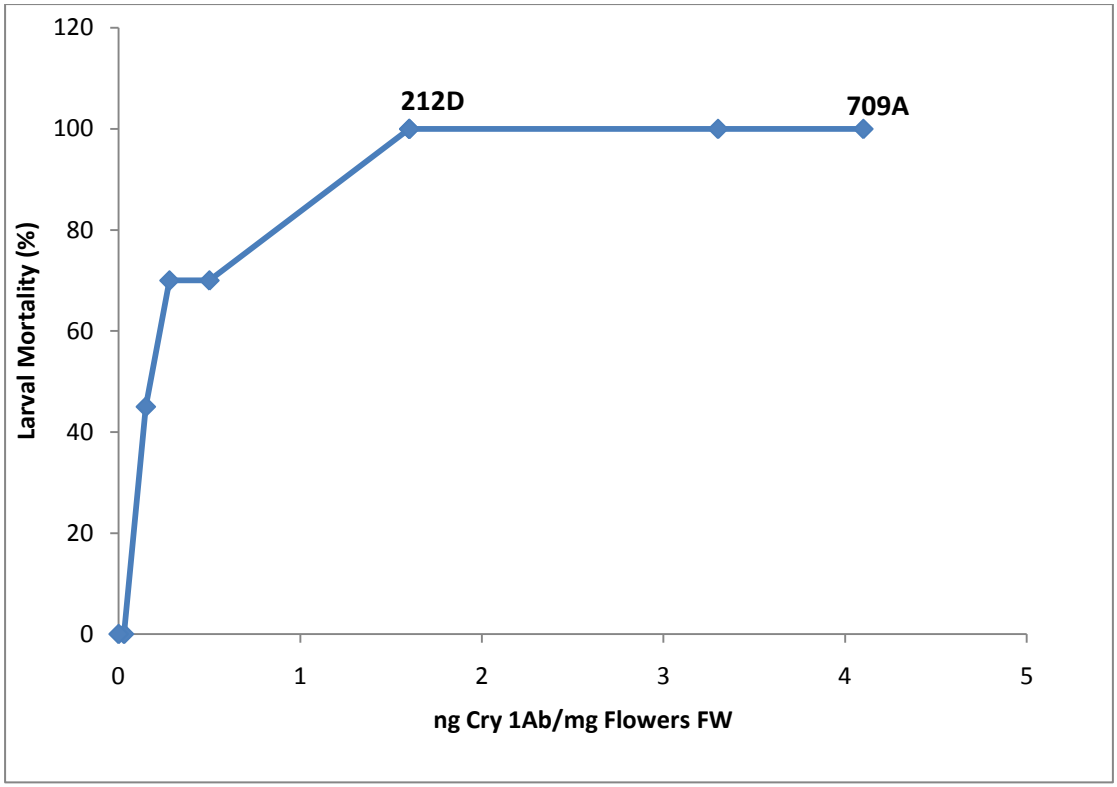

Fig 8. Dose response of *Maruca* larvae to Cry 1Ab in flowers

Export seed of the most vigorous and highest expressing Bt cowpea lines for field trials in Puerto Rico and Nigeria

During 2010 we planned to select 5-10 lines expressing different levels of the Cry 1Ab toxin in their reproductive organs for a second field trial in Nigeria and a possible third in Puerto Rico. The PR trial was to be conducted by Professor Dimuth Siritunga, Department of Biology, University of Puerto Rico Mayaguez overseen by Jeff Stein, Biosafety Advisor, Donald Danforth Plant Science Center with entomological advice from Dr Venu Margam of Purdue University. We applied to USDA APHIS for permission to send the Bt cowpea seed with the appropriate phytosanitary certificate directly to the University of Puerto Rico at Mayaguez. During the year it transpired that IAR in Zaria became much more attractive as a test site largely due to the efforts of AATF (Mpumi Obokoh and colleagues), USAID (Jeff Stein) and Purdue University (Larry Murdock, Joe Huesing and Venu Margam). The Puerto Rican CFT was dropped in favour of IAR Zaria under the leadership of Professor Mohammad Ishiyaku with agreement that Drs Margam and Huesing would assist Dr S Misari with the entomology. The details of the trial design, buffer zones and ideas on the provision of an insect nursery in which *Maruca* would be multiplied to provide a high level of infestation to fully test the plants were developed at a meeting of the cowpea community in

2009 in West Lafayette and implemented by contracting IITA in Ibadan to supply second instar larvae of *Maruca*. This reduced the risk of a low natural infestation and perhaps more importantly meant that infestation could take place in the field under netting so that the risk of massive damage due to thrips and pod sucking bugs could be reduced or even eliminated. The netting arrived about a month after sowing which was less than ideal but it was still quite helpful in lowering damage by non target pests.

A large number of lines was screened in Canberra by ELISA, dot blot and by PCR to identify lines that were, single copy, no vector backbone, expressing a range of levels of Cry 1Ab, homozygous for the transgene and exhibiting a phenotype similar to the line used for transformation (IT86D-1010). This screening and selection process has become very stringent and many lines fail the combination of tests especially the phenotype test. We are thus faced with trading off high level of expression for a good phenotype. We sent sufficient seed for four replicates of three lines to be tested in the field. See Fig 9 for layout.

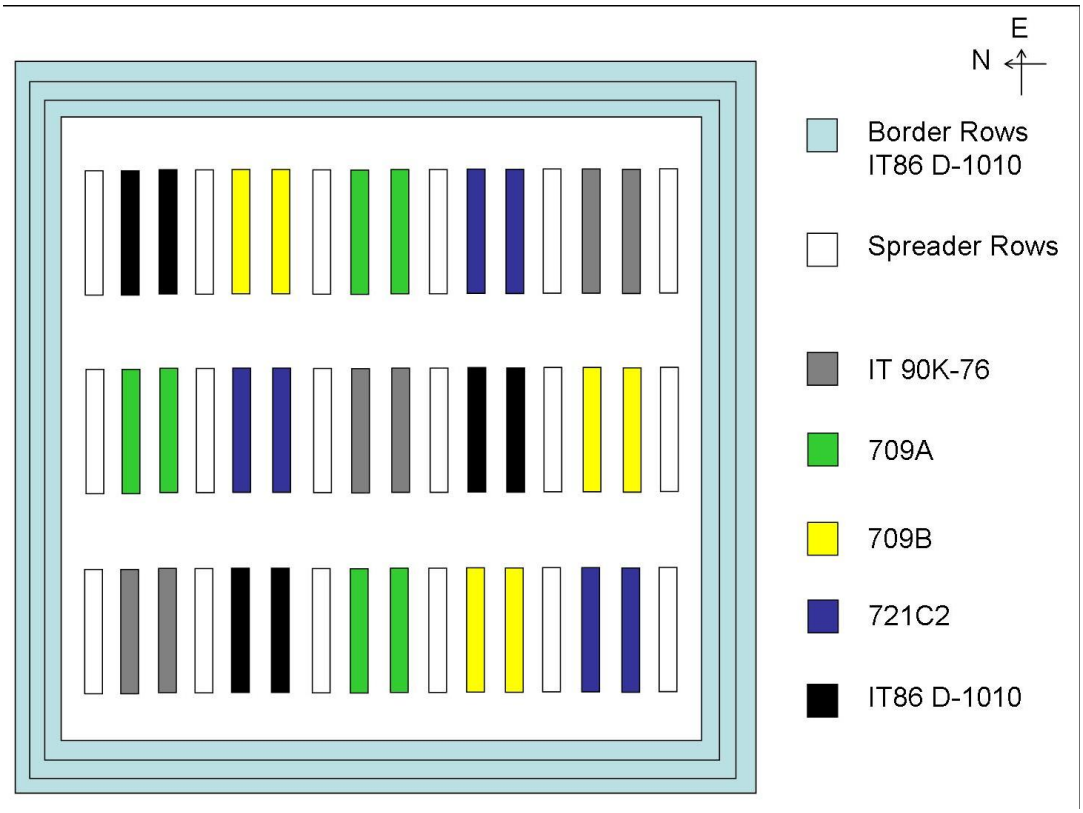

Fig 9. Layout of CFT in Nigeria in 2010

The field trial was sown on July 27th which is the most appropriate season and much better than was achieved in 2009 when seed were sown more than one month late. The plot was netted on Sept 1st (Fig 10).

Fig 10. The CFT enclosed in netting to exclude non target insects.

We anticipated getting better data on efficacy of the Cry 1Ab than we had in the earlier trials. This data was collected by the entomologist Dr S Misari who acted upon the input of Drs Margam and Huesing. The seed yield at crop maturity in November 2010 was determined to ascertain plant performance in combination with efficacy in the field (Table 2).

Table 2. Pod and Grain Yield from CFT 2010

Lines	Pod Yield (g/five plants)	Grain Yield (g/five plants)
IT86D-1010	6.5	4.6
709A	51.0	36.3
709B	47.2	33.7
721C2	23.2	16.5
Mean	32.0	22.8
SE	21.0	15.0

The CFT in 2010 was a marked improvement over 2009. We obtained excellent agronomic data and good evidence for efficacy of the Bt gene against *Maruca* in the field. For instance, line 709A had over seven–fold greater grain yield than the non-transgenic parent IT86D-1010. Line 709B performed nearly as well while line 721C had a near four-fold increase (Table 2).

Completion of an application to conduct contained and confined trials in Ghana and Burkina Faso in 2011

Applications to conduct field trials in Burkina Faso and Ghana, as well as a new trial in Nigeria in 2011 and 2012, have been lodged with the appropriate authorities. Approval for the first cowpea confined field trial has been obtained in Burkina Faso for 2011 and 2012 and negotiations are at an advanced stage in Ghana. I expect that we will obtain approval for a CFT in Ghana in time for the 2012 sowing season.

Construct a Cry 1 Ab gene with chloroplast targeting of the protein

As a result of a PAC and wider stakeholder meeting in W.Lafayette in 2009 we canvassed the idea of having a backup version of the 1Ab gene which targets the protein to the chloroplast. The rationale for this back-up gene was that it may help avoid the reduced vigor associated with high Bt expression. This was based on the fact that Monsanto have used such a construct in Bt soybean which is being commercialized in Brazil. A comment in patent WO2009/064652 A1 noted that high levels of Bt caused stunting of soybeans and that targeting the protein to the chloroplast eliminated the problem. There was general agreement that we should consider the construction of such a gene while continuing to characterize the existing lines with the cytoplasmic version of the protein, here called version 1.0 to distinguish it from the new version called v2.0 (see the diagrams of the two genes in Fig 11).

Fig 11. Diagrams of the original chimeric 1Ab gene (v1.0) constructed for cytoplasmic accumulation and the new version (v2.0) constructed for deposition in the chloroplast.

Based on the results obtained with v1.0 and the data in Miklos et al 2007 we designed the new version of a gene for Cry 1Ab which is shown in diagramatic form in Fig 11. The first change was to make a gene for the full-length version of the 1Ab protein ie 1155 amino acids instead of the 615 amino acids in the truncated (tryptic) fragment as used in v1.0. Thus the new gene (v2.0) consists of the Arabidopsis RbcS 1A promoter plus the RbcS 5' UT plus an extended part of the RbcS chloroplast targeting

peptide of the SSU protein (which we abbreviate here to CTP_e) plus the full open reading frame of the Cry 1Ab protein (1155 amino acids) followed by the 3' poly A signal from Tobacco RbcS 23. Note that the CTP is similar to that used in Miklos et al and was described earlier [6] . This reconstructed CTP-Cry 1Ab gene (v2.0) was transferred to an Agrobacterium binary vector and used to transform IT86D-1010 using the improved transformation methodology developed as part of the earlier phases of this project. Several tens of lines have been produced with this new construct and we anticipate that several will be available for a CFT in 2012.

Suitable lines are now (since the W Lafayette 2009 meeting) defined as those that are homozygous and have a vigorous phenotype but moderate to high level of Bt toxin. This has greatly increased the selection pressure on the transgenic lines and has been a major activity for us this year. We have used several screens to help us select vigorous lines as fast as possible so that we do not have to wait for field results to estimate plant agronomic performance. We used the advances made in 2009 to speed up the process of getting lines through to T_3 but still leaving us with sufficient seed for a replicated field trial by T_3 generation. We expect to have 4-6 new lines of the v 1.0 type ready for CFT3 in Nigeria in 2011.

Obtain access to a gene for a second line of defense against *Maruca*

At a meeting of the cowpea community in Aug 2009 in West Lafayette it was agreed that we should seek access to a second gene for *Maruca* resistant cowpea. Consideration was given to seeking access to VIP3 or Cry 2Ab. With considerable help from Bill Moar it was ascertained that access to VIP3 was very unlikely but Mpumi Obokoh and Joe Huesing have pursued discussions with Monsanto. It now seems possible that we can gain access to Cry 2Ab and AATF has made an approach to Monsanto seeking similar conditions to those attached to Cry 1Ab. There are still several hurdles to overcome but progress is most promising.

Conclusion

We have produced several hundred transgenic cowpeas expressing the Cry 1Ab gene. These lines have been screened for single copy insertion, zygosity, vector backbone sequences, level of expression, plant phenotype and efficacy against *Maruca* in the lab and in the field. At least one line has now been selected by the cowpea breeder in Nigeria for introgression into his advanced germplasm for potential varietal development. This line shows excellent agronomic performance and has demonstrated protection against pod borer in the field.

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