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HYPOTHETICAL EXPRESSION OF AN ENGINEERED CYSTEINE PROTEINASE INHIBITOR (OC-IDD86) FOR NEMATODE RESISTANCE IN TRANSGENIC PLANTAIN AND FIELD TESTING

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Abstract: Plantain yield loss associated with damage by plant parasitic nematodes is huge; yet farmers are often unaware of the damaging nematodes. The high cost and environmental implications of using nematicides make them quite unpopular with plantain often grown at subsistence level. Nonetheless resistant cultivars whose growth and yield characters are acceptable to consumers are not yet available in the market. Genetic modification would hasten up the process of developing cultivars resistant to these nematodes with yield characters that would meet market demands. However, the biosafety implications of this option needs to be established. This project looked into the possibility of inserting the proteinase inhibitor, cystatin, purified from rice into plantain, driven by the root specific promotor tubulin. This approach offers the ability to control several nematodes attacking plantain concurrently or at different locations without any need for awareness of nematode problems at the grower level.

Keywords: Plantain, tubulin, transgenic, *Pratylenchus coffeae*, *Radopholus similis*, *Helicotylenchus multicinctus*.

INTRODUCTION

Plant parasitic nematodes are recognized as the most important biotic constraint limiting the production of plantain in Nigeria (Speijer *et al.*, 2001). Plant parasitic nematodes destroy the roots and rhizome of plantain, subsequently hampering uptake of water and mineral nutrients, and reducing plant growth and yield (Gowen and Quénéhervé, 1990; Rotimi *et al.,* 2004a&b). Affected plants are highly susceptible to wilting. They cause on average, 50% production loss to plantain in southeastern Nigeria (Rotimi, 2003). Heavy losses in plantain usually result from toppling of fruit-bearing stems by wind or heavy rainfall, following destruction of the primary roots by plant parasitic nematodes (Gowen, 1995). In Nigeria, plant toppling could be up to 30 %, with a general average of 9 % (Speijer *et al*., 2001). Often, nematode species occur in concomitance under plantain and this constitutes a limitation in managing them under the crop. Species of nematodes most frequently found associated with plantain in Nigeria include *Helicotylenchus multicinctus*, *Hoplolaimus prarobustus*, *Meloidogyne* spp, *Pratylenchus coffeae* and *Radopholus similis* (Speijer *et al*., 2001). Resistance as a control intervention through classical breeding has a major limitation in that resistance is often bred for individual species, while nematode infection is concomitant. This makes managing plant parasitic nematodes on plantain through resistance breeding quite difficult.

At present there is no nematode-resistant plantain cultivar registered. It also takes a long time to breed for resistance, while hybrids often have some undesired traits. Plantain cultivar Agbagba, a False Horn, is the most preferred plantain

landrace in Nigeria because of its large fingers (Chukwu, 1997). Consumer acceptability of hybrid plantain is poor and no hybrid has been able to replace the preferred landraces. Therefore, nematode management on plantain has been mainly by improved cultural practices. Recombinant DNA technology offers feasible solution to the nematode problem of plantain and the consumer preference limitations of resistance breeding may be addressed by this technology. Additionally, the technology offers solution to effective management of concomitant nematode parasitism, a major factor that restricts the effectiveness of resistant cultivars. Nematode resistant transgenic plantain would overcome many of the risks associated with nematicide use and be safe for consumers.

Cystatins are naturally occurring proteinase inhibitors used by plants as a defense against insects and pests. They are found within many grain plants such as rice, sunflower and maize seeds. Cystatins naturally occur in human saliva and are swallowed at a rate of 40 µg ml^{-1} during mastication (Veerman *et al*., 1996). They work against nematodes by preventing them from digesting their food from plant roots properly and so they are unable to reach their egg laying size. Plants that contained a modified form of rice cystatin Oc-IDD86 have been produced with varying levels of resistance (www.biology.leeds.ac.uk). Results have indicated that combining partial natural resistance with a transgenically expressed cystatin does give an additive effect in protecting against plant parasitic nematodes.

Protease inhibitors have been used to engineer nematode resistance (Hepher and Atkinson, 1992; Urwin *et al*., 1995; 1997). Vain *et al.* (1998) reported a genetic modification strategy using cystatin, a proteinase inhibitor, which seeks to provide a basis for concomitant control for *M. incognita* and other root parasites of rice, which may be relevant for other crops. Cystatins are effective against a wide range of nematodes and thus can protect plantain from different combinations of pest species that can occur in plantations. The objective of this work is therefore, to propose suitable method to engineer nematode resistance into plantain against key nematode pests in Nigeria.

MATERIALS AND METHODS

Plantain Transformation (The construct, target plant and gene delivery)

The genes

The basic strategy for transforming a plant involves delivery of construct(s) containing the gene(s) to the target material, selection of the transformed cells, and then regeneration of the transformed plant lines. In this report, we propose the introduction of modified Cystatin gene from rice (OcIDD86) into plantain (www.biology.leeds.ac.uk). Nematodes attack the root of plantain but plant cystatins are not normally produced in the roots. The plant can therefore be modified by the use of root specific promoters so that the gene product can be confined to the plant roots by linking the gene to a root-specific promoter, tubulin. In an earlier work (www.biology.leeds.ac.uk), tubulin was reported to achieve 80% resistance in banana plants against developing root knot nematode (*Meloidogyne incognita*), which by comparison, was better than the constitutive promoter, CaMV35S. Root-specific promoter would ensure that the gene product is not present in the edible plant parts and will only be present in the plant roots where it can specifically target nematodes.

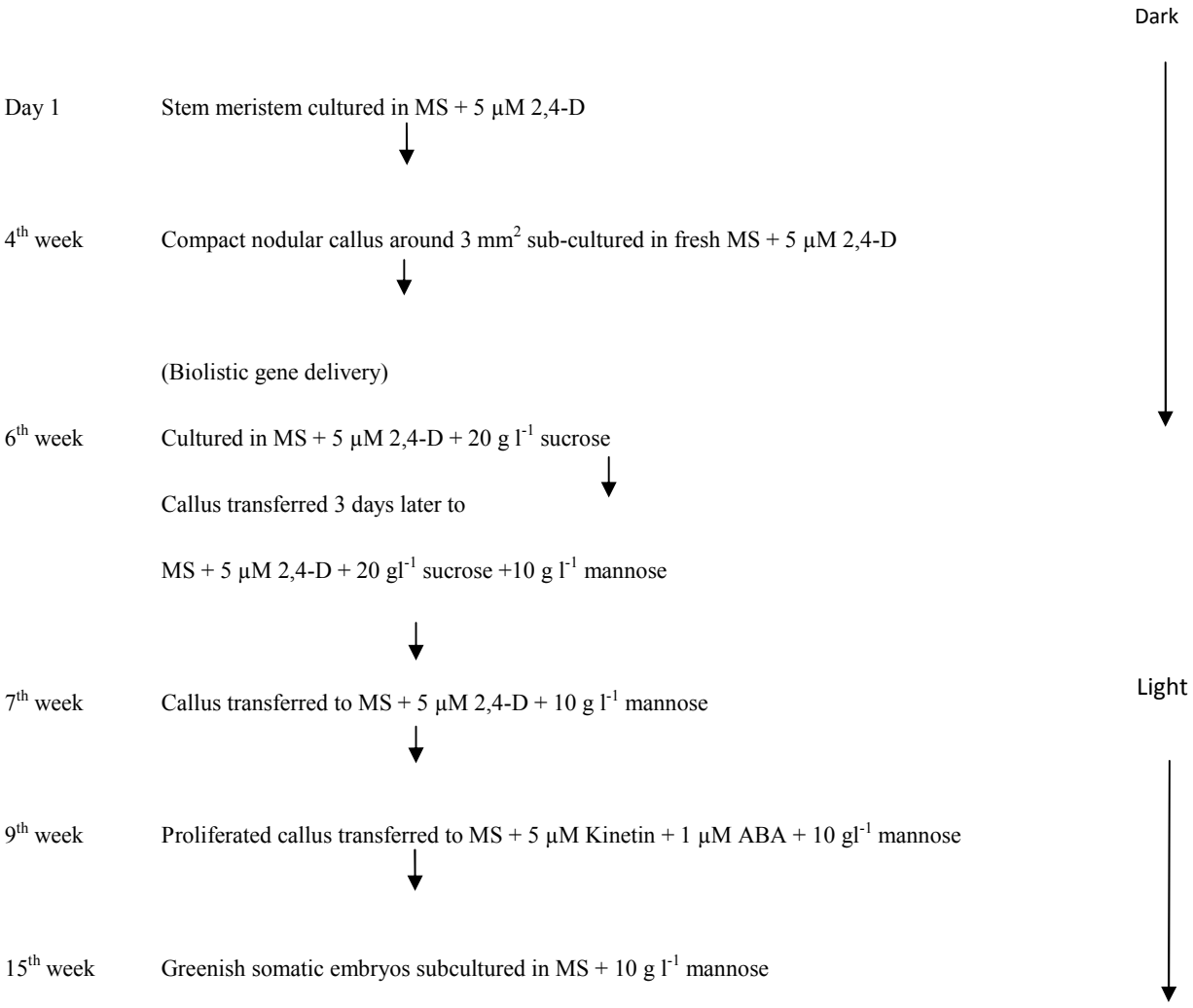
For the marker gene, a construct containing the *Escherichia coli* gene-derived *manA* gene, which encodes phosphomannose isomerase (*pmi*) driven by the Cestrum yellow leaf curling virus (CMPS) promoter and NOS terminator sequences (Aswath *et al*., 2006) would be used for transformation. Plant cells transformed with the *manA* gene can convert mannose-6-phosphate to easily metabolized fructose-6-phosphate, an intermediate of glycolisis (Aswath *et al*., 2006).

Plant Material

Stem meristem would be excised asceptically and cultured on Murashige and Skoog, (MS) basal medium (Murashige and Skoog, 1962) supplemented with 5µM 2, 4-dichlorophenoxy acetic acid (2, 4-D) (Zheng *et al*., (1998), to protect against microbial contaminants. As presented below in Figure 1 adapted from Aswath *et al.* (2006), the explants would be incubated at 25±1°C in the dark for 4 weeks, while the callus would be subcultured for another 2 weeks (Figure 1). Proliferated calli obtained by the 6th week would then be used for transformation.

Plasmids and Gene Delivery (particle bombardment)

Plasmids manA-OcIΔD86 (Tubulin Promoter::AMVleader::*OCID*D86::NOSpolyA + CMPS Promoter::*manA*::NOSpolyA) and pJIC200 (ubi-5' region::NOSpolyA) would be used for transformation. The *OC-I*ΔD86 gene was designed by protein engineering to improve the Ki and efficacy of native rice OC-I protein against nematodes; *OC-I*ΔD86 differs from *OC-I* by only one amino acid (Asp86 deleted, Urwin *et al*., 1995). Purified plant tubulin would be used as promoter. The *manA* gene has been cloned from several bacteria and yeast species, and from humans; and has also been purified from yeast, bacteria, pigs and humans (Prakash, 2000) at Novartis Agribusiness Biotechnology Research, Inc.



↓

19th week Plantlets transferred to half MS + 10 g l^{-1} mannose for rooting

↓

23rd week Bioassays

From day 1 to the 9th week, all cultures would be kept in the dark; and

From the end of the 9th week, all cultures would be transferred to light

Figure 1: Flow diagram showing the different steps that shall be followed for transformation (Adapted from Aswath *et al*., 2006).

The *OC*-IDD86 and *manA* genes would be delivered in co-transformation into callus culture of stem meristem of plantain (cvr Agbagba) by biolistics with the gene gun particle bombardment (Christou *et al*., 1991) on the 6th week (Figure 1). The gene constructs are shown in Figures 2 and 3.

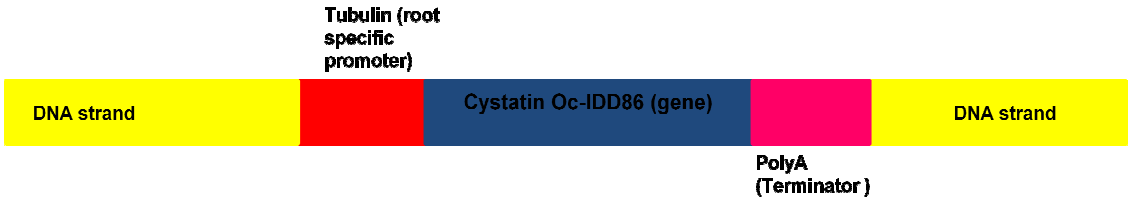


Figure 2. Cystatin gene construct

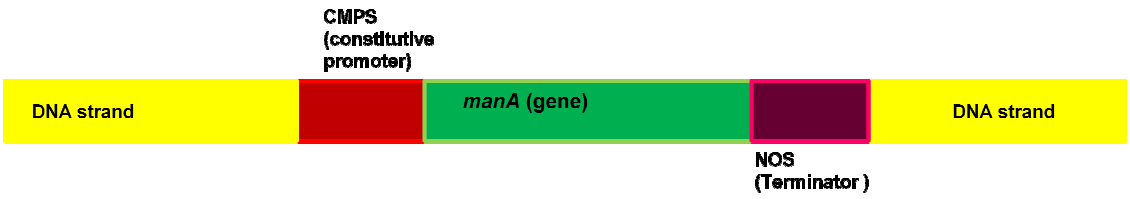


Figure 3: *manA* marker gene construct

The *E. coli*-derived *manA* gene is driven by the Cestrum yellow leaf curling virus (CmYLCV) constitutive promoter (CMPS) isolated from Cestrum yellow leaf curling virus (CmYLCV), a double-stranded DNA plant pararetrovirus belonging to the Caulimoviridae family. The CmYLCV promoter is highly active in callus, meristems and vegetative and reproductive tissues in *Arabidopsis thaliana*, *Nicotiana tabacum*, *Lycopersicon esculentum*, *Zea mays* and *Oryza sativa* (Stavolone *et al*., 2003).

Selection of Transformed Cells

After 3 days, callus would be transferred to MS basal medium supplemented with 5µM 2,4-D, 10g l^{-1} mannose and 10 g l^{-1} sucrose (Figure 1). The callus that survive without browning would be selected as transformed and transferred to only mannose medium without sucrose. This would reduce the number of untransformed callus that might escape and thereby improve the efficiency of selection (Aswath *et al*., 2006). Proliferation of callus clumps would further imply transformation.

Regeneration of Transgenic Events

The proliferated sectors from resistant callus would then be transferred to the regeneration medium containing MS medium with 5µM Kinetin, 1µM abscisic acid (ABA) and 10g l^{-1} mannose in order to induce somatic embryos (Aswath *et al*., 2006). Somatic embryos that develop would be transferred to MS basal medium supplemented with 10g l^{-1} mannose at the end of the 15^{th} week (Figure 1). Plantlets formed from well developed somatic embryos at the end of week 19 would be transferred to half-strength MS medium with 10g l^{-1} mannose. Exposure of the cells for 8 weeks and continuous exposure throughout rooting of plantlets would further help eliminate untransformed escapes.

Polymerase Chain Reaction (PCR)

PCR is carried out usually to verify that the inserted gene is intact. DNA would be prepared using a rapid extraction protocol. Leaf samples would be ground in a 1.5-ml Ependorf tube using hand pestle, sand and liquid nitrogen. 200 µl of extraction buffer (500 m*M* NaCl, 100 m*M* TRIS-HCl pH 8 and 50 m*M* EDTA; Ellis 1994 in Vain *et al*., 1998) and 20 µl 20% (w/v) SDS would be added to the powder before further grinding. After centifugation (5 min at 12 000 rpm) the supernatant would be removed and added to an equal volume of isopropanol for DNA precipitation. After centrifugation, the DNA pellets would be washed with 70% ethanol and suspended in TE buffer. PCR would be performed in a total volume of 25 µl containing 25 ng plantain genomic DNA, 10 m*M* TRIS-HCl pH 8.3, 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.05% Noridet p40, 200 l*M* dNTPs, 0.4 l*M* of each primer and 1 unit of *taq* polymerase. DNA would be denatured at 95 ^oC for 1 min followed by 30 cycles of amplification (30 s at 95 ^oC, 30 s at 60 ^oC, 1 min at 72 ^oC) and by 10 min at 72 ^oC.

Western Blot Hybridization for OC-I Activity

Total protein would be isolated from roots of plantain plants. 10 µg of total root protein and standard quantities of OC-I protein would be loaded on SDS PAGE gels using the BioRad mini system. The OC-I protein would be recovered from an *E-coli* expression system (Urwin *et al*., 1995). The gel would be blotted according to BioRad protocols and probed with a polyclonal antibody raised against OC-I (Urwin *et al*., 1995). The bands would be visualized using an image analyser system consisting of a monochrome CCD camera (Sony) plus a frame grabber and appropriate software (Quantimet 500, Leica).

RESULTS OF PLANT TRANSFORMATION & BIOASSAYS

Confirming the success of plant transformation

Southern blot analyses of regenerated plants would confirm the integration of *manA*-Oc1DD86 plasmid into the plantain genomic DNA (Vain *et al*., 1998). About 10 µg of genomic DNA would be restricted with *Eco*RL and separated on agarose gel. In line with manufacturer's instructions (Amersham Biosciences, wwwl.amershambiosciences.com), it shall then be transferred unto Hybond-N nylon membranes. The plasmids would be restricted with *Hin*dIII and *Kpn*1 and isolated using the QIAquick Gel Extraction Kit. Filters would be hybridized with probe as template using Alkphos Direct Labelling Reagent, as stipulated by Amersham Biosciences (www1.amershambiosciences.com). Hybridization, washing and detection would be performed according to the instructions of same manufacturer.

The expression of OC-IDD86 genes in transformed plants would be confirmed by enzymatic assays and western blot analysis.

Bioassays with Nematodes

In Vitro Culture

For each transformed event, micro-propagated plantlets would be separately inoculated with 50 juveniles each of *Pratylenchus coffeae*, *Radopholus similis* and *Helicotylenchus multicinctus*, which, listed in order of economic importance, are the most destructive on plantain (Speijer *et al*., 2001). Two months after inoculation, necrosis on the roots of plantlets would be assessed, and nematodes extracted from both roots and culture medium according to the methodology described by Speijer and De Waele (1997). Nematode densities would be counted and reproductive fitness of each species on the transgenic plants would be calculated. Transgenic materials supporting lower reproductive fitness of the nematode species compared to the control non-GM Agbagba (comparator) would be selected as exhibiting resistance to the nematodes. These would be multiplied for further testing in the green house.

Selected materials would further be screened for multiple resistance by inoculating with different mixtures of the three nematode species, following the procedure earlier described. Materials exhibiting resistance to more than one of the nematode species would be selected as priority, while those exhibiting resistance to single species would also be selected.

Root production is of paramount significance in the establishment of *Musa* plants and plays significant role in their tolerance and/or resistance to damage by plant parasitic nematodes (Elsen, 2002; Rotimi *et al*., 2005; Salau *et al*., 1992). Therefore, selected materials shall further be screened for rooting by growing them in rooting medium, which would contain a higher level of auxin than cytokinin. After 4 weeks, plants with good rooting (comparable with the standard comparator) shall be finally selected for Contained Environment Assessment. Finally, selected materials would be multiplied in readiness for further assessment in the screen house.

Contained Level Assessment (from test-tube to soil)

Selected plantlets would be multiplied and further tested under controlled green house conditions. The finally selected transgenic plants would be grown in sand/loam mix and hardened in the green house for about 8 weeks along with a substantial equivalence (the standard comparator) that has history of safe use. Rooted plantlets would be grown in sand/loam soil mix for 4 weeks after which they would be inoculated with 1,000 juveniles of the three nematode species mentioned above. On the 5^{th} week of transfer to the green house, plants would be singly inoculated with juveniles of the three nematode species as described below. Nematode suspension containing 1,000 juveniles of the required species would be introduced to a groove dug around the base of each plant and then covered with soil. Plants would be observed for 2 months and growth parameters measured every 2 weeks. Plants would be watered throughout the duration of the study. Root damage by nematodes and reproductive fitness of each of the species would be assessed as discussed below.

The root mass would be cleaned thoroughly in water and blended for 10 s in a Waring blender with sodium hypochlorite diluted to give 1% available chlorine. For each nematode species, nematodes would be counted as male, female and juveniles, while eggs would be washed from the blended roots into a large volume of water using a 1-mm sieve. The eggs would be collected on an open sieve, re-suspended and counted in 1-ml aliquots using a Peter's counting slide (Southey, 1986).

On comparing with the standard, promising lines whose roots show good resistance to nematodes, and exhibit good rooting and phenotypic properties would be selected for further testing. Since fruit production is the ultimate in plantain, then fruit production ability of the selected materials would be studied in a large screen house. It would be necessary at this stage to compile the results in a dossier and present to the government, a request for approval for confined field test because effects in controlled environment may be confounded in the field. It would be necessary therefore to verify greenhouse observations in the field.

Field Test

Field Testing Requirements

So far, all testing have been under containment where environmental conditions have been confounded. For plant selection however, field testing at multilocations is recommended in order to ensure stability of selected materials. Also, there has not been any report on the yield up till this moment; whereas the yield is of importance, since it is the produce of the plant. It would therefore be necessary to assess yield response of the selected materials in the field under natural environmental conditions.

Regulatory Requirements for Field Testing

At present in Nigeria, there is no existing biosafety law since what is available is a draft of the National Biosafety Bill, which has not been signed into law. Application shall be presented to the National Biosafety Council of the National Biosafety Authority (NBA) under the Federal Ministry of Environment, which is the competent national authority for biosafety in Nigeria, as proposed in the draft biosafety policy. To pursue approval for the field testing therefore, the Dossier to be presented shall be guided by the Cartagena protocol on Biosafety. Therefore, the

transgenic plants and plant products shall be subjected to risk assessment and management according to the specifications of articles 15, 16 and Annex III of the Cartagena protocol (CPB, 2000).

DISCUSSION

The anti-nematode defense in the proposed GM plantain offers major practical benefits for use in the developing world. It offers the ability to control several nematodes attacking crops like plantain concurrently or at different locations, without any need for awareness of nematode problems at the grower level.

Often times, farmers are unaware of the nematode problem because of the "hidden nature" of nematode damage due to its attack on the subterranean parts of plantain. Even when the farmer realises that there is a problem, incorrect diagnosis results in inappropriate control measures, thereby incurring additional production cost without efficient control of the problem. Where a farmer could identify plant parasitic nematodes as the problem, the huge financial cost of chemical nematicide makes that option not feasible. Additionally, use of these chemical pesticides is plagued with serious environmental and occupational hazards. The high mammalian toxicity of most nematicides is a bane to their use especially among the unskilled and resource poor subsistent farmers in most developing countries.

Transgenic nematode resistant plants will reduce or replace the use of toxic and environmentally damaging nematicides. It would also save the farmers the headache of struggling to identify nematode problem, which often receives wrong management practices. Plantain is widely grown by poor farmers for local consumption in Nigeria and West Africa at large. Scientific data available show cystatin to be effective and biosafe. The approach could benefit the very many rural communities that suffer plantain production losses to nematode.

Cystatins are naturally occurring proteinase inhibitors which are involved in plant defences against insects and pests. Cystatins are also found in a wide range of foods such as those found in rice seeds eaten daily by thousands of people. Other sources include maize and sunflower. Cystatins are not normally produced in the roots where the nematodes attack. Plants can be modified by the use of root-specific promoters driving the expression of cystatin in areas of the plants where the nematodes infest. This has the added advantage of ensuring that cystatin is not expressed in the edible plant parts (the fruits) or green tissue. Transgenic expression in plant roots would reduce nematode growth, development and fecundity, and hence enhancing sustainable plantain production.

This approach is unlikely to cause harm to man and animals when the fruit is used as food or for animal feed, and is not harmful in the environment. Safety assessment of the gene, gene product and crop product has established that except for the inserted gene, the crop product would be substantially equivalent to the landrace plantain cultivar Agbagba, which was the parent material used for transformation. Genetically modified plantain fruits were not toxic and expressed no allergenic potential to laboratory test animals. These make the GM plantain to be safe for human food and animal feed. Ecological risk assessment also established it as safe for use in the environment, not likely to result in horizontal or vertical gene flow, or result in weediness of the GM plantain crop.

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