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Executive Summary

The current project aims to develop a reliable transformation procedure for African cassava lines and to express "defective interfering" (DI) viral molecules derived from two major cassava viruses in order to engineer virus protection in the transgenic plants. A series of experiments were carried out to optimise the tissue culture and regeneration parameters from friable embryogenic cultures (FEC) and embryogenic suspensions of cassava. Bombardment of FEC with DI DNA, and regeneration via tissue culture led to the recovery of four transgenic plants. The latter stages of the project were hampered by the resignation of both the principle scientist, and the post doctoral worker. Despite this, the plants have been bulked-up by vegetative propagation and when large enough, will be transported to Dr John Stanley's laboratory at JIC for further analysis.

Background

Cassava is a major staple in the humid tropics, but in most areas cassava production is significantly affected by virus infections and by mealybug infestations. Conventional breeding approaches are being applied to these problems, but there is also great potential for the publication of biotechnology in these areas to deliver genetically engineered cassava lines with virus or insect resistance. We intend to use genetic engineering technology to produce transgenic cassava plants protected against ACMV and ICMV infection by the expression of "defective interfering" (DI) DNA molecules. In addition, to provide technology for the genetic manipulation of other pest and pathogen resistance and quality traits in cassava.

Project Purpose

The improvement of cassava production has been identified as a priority for the Forest/Agriculture Interface System of the ODA Plant Sciences Programme. This aims to increase production by 10% by 1995, with a geographical focus on major constraint to cassava production in such countries is the lack of effective strategies for crop protection and yield losses due to viral infection are significant in most production areas.

The purpose of the project is to develop and apply genetic engineering technology to the improvement and protection of cassava yields. The specific purpose of the project is to engineer resistance to major cassava viruses, while the general purpose is the provision of generic cassava transformation technology which will have potential for application in the removal of various constraints on cassava production such as mealybug and mite infestations, in the reduction of cyanogenic glucoside levels, or in the engineering of traits such as tuber protein level or starch composition.

Research Activities

1 Refinement of cassava culture systems

1a To provide optimal target tissue for bombardments, immature leaflet culture procedures will be further improved. In particular, hormonal, nutritional and physical factors will be examined to optimise embryogenesis, to accelerate the regeneration process and to further improve the frequency of conversion of somatic embryos to plants. The aim will be to produce a "robust" protocol, maintaining high productivity under selection pressure.

1b Selected African cassava germplasm supplied by IITA will be assessed in the RES culture system. Adaptations will be made as needed and two or three responsive genotypes will be selected for use in DI DNA transformations (in addition to the standard genotype, Mcol 1505).

2. Improvement of selection procedures
The two selection systems currently in use (*bar* gene/PPT selection, *neo* gene/G418 selection) will modified to maximise efficiency for direct leaflet bombardment (see 3 below). Specifically, the effect of the timing of application of selection pressure will be examined (selection too early reduces regeneration from sensitive explants, while late selection of larger, resistant cultures leads to the production of chimaeric transformants).

3. Optimisation of leaflet bombardment.
A whole-tissue Feulgen staining procedure will be used in conjunction with transient GUS assays to correlate immature leaflet cell types targeted by bombardment with those proliferating and subsequently regenerating. This study has already started and will allow us to monitor the early stages of transformation and improve efficiency. Direct leaflet bombardment has advantages over the usual approach of bombarding embryogenic cultures in that the relatively fe3w source cells which give rise to the tissue culture can be targeted, which will r4educe the frequency of chimaerism and that early leaflet cultures are much more sensitive to selection than compact embryogenic cultures or somatic embryos.

4. Production of D DNA transformants.

4a Leaflet cultures will be bombarded with an ACMV DI DNA construct, in co-transformations with plasmids containing selectable (*bar*, pDE110 or *neo*; pDE108) and scorable (GUS; pDE4) marker genes. In each pDE plasmid a CaMV35S promoter known to express at high level in cassava expresses the marker gene. From our experience, co-transformation with three constructs will give frequencies of co-integration of the non-selected genes of >70%. Gene delivery by direct gene transfer usually results in the integration of

multiple copies of the transgenes which may well be advantageous in this system as more copies of the DI DNAs should be available for amplification and interference with virus replication. Plantlets will be regenerated from bombarded cultures under PPT or G418 selection and confirmed as transformants by GUS assays and PCR screening for the marker gene.

4b Initial transformations will be made with cassava line Mcol 1505, but when culture procedures for African lines are defined these lines will be used for bombardment experiments.

4c Transgenic plants will be transferred to a containment glasshouse and analysed by Southern blotting to ascertain transgene copy numbers and integration patterns. During this period individual transformants will be cloned by stem segment propagation.

5. ACMV inoculation by particle bombardment
Methods for transferring infectious recombinant ACMV DNA clones into cassava by particle bombardment of small plants or *in vitro*-grown plantlets will be developed. This will allow transgenic lines containing DI DNAs to be infected without the use of whitefly vectors (other methods of mechanical infection are not efficient in cassava). This technique could also facilitate an early assessment of the function of DI DNA-mediated protection if it is possible to transfer amplifiable DI DNAs into cassava plantlets.

6. Analysis of response of DI DNA transformants to ACMV infection
DI DNA-containing transformants will be transferred to JIC, where they will be infected with ACMV (alternatively, if a protocol for bombardment-mediated infection has been developed then transformants will be infected at RES and then transferred to JIC). Levels of DI DNA replication on infection will be assessed, as will the effects of the DI DNAs on the replication of the A and B virus components and on virus multiplication, spread and symptom development.
DI DNA amplification relies on the ability of incoming virus for trans-replication. The interaction between the replicase-associated protein (AC1) and the viral genome is very specific and geminiviruses are generally unable to trans-replicate the genome components of other viruses (Frischmuth *et al* 1993, Virology 196: 666-773) but Stanley and colleagues have shown that ACMV isolates from widely separated locations (ie Nigeria and Kenya) can produce viable pseudorecombinants (Stanley *et al*, 1985, *J Gen. Virol.* **66**: 105-1061). Therefore, it is anticipated that the Kenyan isolate DI DNA to be used in the project will be mobilised and amplified by most ACMV isolates.
If possible, symptom development in different transgenic lines will be correlated with the number of DI DNA copies integrated. The response of transformants to repeated challenges with ACMV will be examined as will the growth and development of these and control plants and the stability of DI DNA-mediated effects over cycles of vegetative propagation.

7. DI DNA transgenic lines, which show amelioration of ACMV symptoms, will be multiplied *in vitro* and transferred to IITA to be assessed for their performance under field conditions.

8. Transformation of cassava with ICMV DI DNAs
Dependent on initial results and progress with ACMV DI DNA experiments, a similar approach will be adopted for ICMV. DI DNA clones for ICMV will be constructed and transgenic cassava lines produced. As for ACMV, initial

analyses of the effects of the inserted DI DNAs on ICMV replication and symptom development will be performed under controlled conditions at JIC and lines showing protection against ICMV will be propagated and made available for field assessment to JIC's collaborators in India and to IITA.

9. Identification of promoters for cassava genetic engineering
As a secondary priority to DI DNA transformation experiments and dependent on their progress, the studies on the activity of different constitutive and tissue-specific promoters initiated as part of ODA Plant Sciences |Project Reef No R4876 (gene transfer and expression in cassava) will be continued. Constructs containing patatin, β-amylase, sporamin and CaMV35S promoter variants fused to the GUS gene have been examined for their activity in various tissues in transient expression assays and these constructs would be used in stable transformation experiments to examine the utility of these promoters for expressing transgenes in cassava. It is of particular interest to find promoters giving good expression in tuber storage tissues and also promoters highly expressed in tuber rind tissues, where the higher concentrations of cyanogenic glucosides are located.

Outputs

Regeneration from somatic embryo of model cassava genotype Mcol1505 was more than doubled from 30% to 75% by the inclusion of embryo desiccation step and the improvement of the regeneration media components, including the addition of $CuSO_4$, maltose and an increase in BAP concentration.

A plantlet development medium was developed which increased survival rate of somatic embryo-derived plantlets up to 92%.

Reproducible particle bombardment and tissue electroporation transformation systems were developed for cassava immature leaves and/or somatic embryos, respectively.

The application of particle bombardment to study the promoter strength and specificity in different cassava tissues.

Transformation and regeneration of cassava tissues and plantlets expressing the GUS gene in a chimeric pattern.

Conditions for induction of somatic embryos from immature leaves under low levels of selection pressure were determined to permit early selection of transformed cells.

In addition to the model genotype (Mcol1505), somatic embryogenesis from immature leaves was obtained in three African genotypes (TMS60444, TMS60142 and TMS30572). Friable embryogenic cultures (FEC) and embryogenic suspensions have also been established in these genotypes.

Bombardment of embryogenic suspensions and assessment of marker gene expression (GUS and NPTII) under selection by the antibiotics geneticin or paramycin.

120 FEC lines were subjected to selection pressure, of which seven survived selection and were transferred to soil. Four lines tested positive in PCR for the DI transgene.

These four lines were vegetatively propagated.

Contribution to Outputs to Project Goal

We have developed a transformation and regeneration protocol and have succeeded in generating a number of transformed lines. The latter stages of the project were hampered by the resignation of both the principle scientist, and the post doctoral worker. Despite this, the plants have been bulked-up and will be transported to Dr John Stanley's laboratory at JIC for further analysis.

If DI DNA transgenic lines show amelioration of ACMV symptoms, they will be multiplied and transferred to IITA to be assessed for their performance under field conditions. Also, this will provide proof of concept, and a similar approach could be applied to Indian Cassava Mosaic Virus.