Final Report on CTA027

(Project merged into a new collaborative project with the BSES, 1/7/1999)

Project Title: Transformation of sugarcane using *Agrobacterium tumefaciens*.

Project Reference Number: CTA027

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(b) Summary.

A method for *Agrobacterium*-mediated transformation of sugarcane tissues has been developed in this project. This method offers an alternative technique for the introduction of transgenes into sugarcane. In general, *Agrobacterium* is viewed as the method of choice for the range of plant species where both *Agrobacterium* and microprojectile-mediated techniques have been applied successfully.

A number of transgenic sugarcane plants have been produced in this project using different *Agrobacterium* vectors, different selection strategies, different techniques and the protocol has been published in an international journal. The applicability of the technique to sugarcane cvs Q117, NCo-310, Q124 and Q155 has also been demonstrated. Many experiments were performed to optimise the *Agrobacterium* transformation methodology for sugarcane with parameters having a significant effect on transformation efficiency being identified.

A selection system based on herbicide resistance was implemented to select for the growth of transgenic cells. The GFP system was further developed and facilitated the rapid production of transgenic cell lines and the scoring of transformation experiments.

Collaborative work with the BSES has led to the development of new techniques and opportunities, which should result in a very useful industry outcome.

Although the development of an *Agrobacterium*-mediated transformation technique for sugarcane is described here, the real value of the technique will not be realized until the integration patterns, level of gene expression and level of somaclonal variation are determined in the transgenic plants produced.

(c) Background to the Research Project including technical information concerning the problem or research need.

Commercial plantings of some genetically-engineered major crops in the US are now exceeding their non-genetically engineered counterparts. This methodology is highly advantageous because superior seedlings or elite clones can be further improved by the insertion of a single, or few genes, that increase the agronomic value of the original elite line. Novel traits available for engineering into elite cultivars may originate from within the sugarcane gene pool or from any other organism. A desirable strategy may be the manipulation or translocation of genes (or pathways) identified within the sugarcane gene pool, either by improving expression, increasing activity or down-regulation. Alternatively, traits may be sourced from other organisms outside the sugarcane gene pool.

A technique to introduce transgenes into sugarcane based on microprojectiles (Molecular Breeding 2:239-249) has established that genetic engineering can compliment traditional breeding strategies for sugarcane improvement. Following this advance, it is necessary that related technologies be developed to ensure that genetic engineering strategies will be

effective. Advanced genetic engineering strategies in other crops have identified that for sugarcane, priorities existed in:

- providing consistent and high level expression of introduced genes in sugarcane,
- preventing or minimizing any modification of the basic traits of the original elite cultivar during the transformation process, and,
- providing a system which can uniquely characterise any genetic introduction prior to commercial release.

The gene delivery technique based upon *Agrobacterium tumefaciens* offers some specific advantages over microprojectile bombardment for producing genetically engineered crops, which are described below.

- **Integration of transferred DNA is less complex.** The transferred DNA is defined between 2 border sequences and is transferred to a recipient plant cell facilitated by proteins produced by *Agrobacterium.* This results in less complex gene integration patterns (fewer rearrangements, truncations, inverted repeats etc). This is clearly an advantage for the genetic characterisation of any highly desirable transgenic plant, which must occur prior to any commercial release, particularly as GMAC requirements are likely to become more stringent.

- **Fewer copies are inserted** in recipient plant cells. This is a preference for most plant transformation systems, although this does not seem to be a specific problem in the highly polyploid species, sugarcane. However, it remains to be tested whether the insertion of fewer genes is preferable for the sustained expression of transgenes over a longer time interval, such as over a number of ratoon generations or throughout a breeding program.

- **An ability to transfer longer single DNA molecules into plant cells**. Increasingly, as our understanding of plant genetic systems increases, a requirement for the insertion of longer genes, fusions or regulating elements in close proximity may eventually require that genes are co-transferred together and remain together in the recipient genetic material. Reports of the proximal clustering of plant defense genes or the requirement for proximal positioning of gene regulating elements are two examples of this. New BIBAC vectors, for use in *Agrobacterium*-mediated transformation, have been demonstrated to transfer extremely long T-DNAs.

- **Insertion into transcriptionally active regions.** *Agrobacterium* facilitates the insertion of T-DNA into transcriptionally active regions of a plant's genome, an ability that is thought to lead to higher and sustained expression of introduced genes.

- **A 2 T-DNA system allows the separate integration of the gene driving the selection agent and the gene of interest.** The forced separation of the antibiotic resistance gene and the gene of interest, enables the removal of the antibiotic resistance gene as part of a breeding program.

Previously *Agrobacterium* was not envisaged to be a suitable vector system for sugarcane because monocots in general, were thought to be outside the *Agrobacterium* host range. Recent success with this technique in other monocots such as barley and rice, indicated that the technique may also be applicable to sugarcane. The development of an *Agrobacterium*-mediated transformation technique for sugarcane is described herein.

(d) Project objectives and the extent to which the research has achieved them.

The primary objectives of this project were:

1. To test the potential for transformation of sugarcane using established *Agrobacterium*-mediated transformation techniques currently being successfully applied to other monocot species.
2. To compare the copy number and expression of marker transgenes from *Agrobacterium*-mediated transformation with that of microprojectile-mediated transformation.

Objective 1 was exceeded when an *Agrobacterium*-mediated transformation protocol was developed and definitive proof of the gene delivery demonstrated and finalised through to publication. The research demonstrated the following:

- A number of transgenic sugarcane plants have been produced using different *Agrobacterium* vectors and the technique has been published in an international journal.
- Over fifty transgenic callus lines with some regenerated into plants were produced during the research project, which more than adequately demonstrated the applicability of this methodology for sugarcane.
- The applicability of the technique to different cultivars was demonstrated. Cells of four cultivars (Q117, NCo-310, Q124 and Q155) were found to be susceptible to *Agrobacterium*-mediated gene transfer.
- Many experiments were performed to optimise the *Agrobacterium* methodology for sugarcane over the course of this project and the results are discussed in further detail below.

Objective 2. The incorporation of this project into a new BSES project, has shortened the length of this project by one third. Extensive characterisation of the transgenic plants was planned for the final year of the project as detailed in CTA027 milestones. Some analyses will be conducted as part of the new transformation project. If current transgenic cultures are regenerated and maintained, then it is envisaged that the following investigations can be performed:

- An evaluation of copy number between *Agrobacterium*-transformed lines generated using two different vector systems.
- A comparison of copy number for *Agrobacterium*-mediated and microprojectile-mediated transgenic plants with the same vector, and,
- A comparison of marker gene expression levels in plants introduced using either *Agrobacterium* or microprojectiles.

Other Research Findings:

A new herbicide selection system was implemented during the course of this research. The *bar* gene which encodes resistance to bialaphos, the active constituent of the herbicide Basta, was used to select the first transgenic sugarcane cells transformed using *Agrobacterium*.

A comparison of bombardment of the same chimeric gene (Ubi-*gfp*) in a small plasmid vector (~6.5kb) and a larger plasmid (~15.5kb) resulted in greatly reduced transient transformation frequencies with the larger plasmid. Difficulties have also been observed during transformation of gene fusions, which also require larger vectors. These data support a notion that the efficiency of transfer decreases as larger genes are bombarded into plant cells.

(e) Methodology and justification

Agrobacterium-mediated transformation techniques were primarily applied to regenerable sugarcane tissues to enable transgenic plant recovery. Although leaf and seed explants were initially tested, embryogenic callus was the primary explant used, because of its demonstrated regenerable properties, and since introduced genes (including selectable marker genes and reporter genes) are highly expressed in these tissues. With time, work with other tissues was discontinued in favour of embryogenic callus because:

- Seed tissues were not considered appropriate targets since a clone's agronomic integrity is not maintained in the seedling generation.
- Meristem transformation was proposed in the original project, but work with this target tissue was incorporated into work in the collaborative BSES project on meristem transformation (BSS209).
- Cell suspension cultures and protoplasts were not considered suitable targets because of a potential for increased somaclonal variation in resultant transgenic plants.
- Pollen tube transformation and other novel techniques were not investigated due to inadequate prior development of these techniques and for the reason already described for seed tissues above.

Evidence also existed in previous *Agrobacterium* studies of rice, that callus may be a highly susceptible explant for *Agrobacterium*-mediated transformation and thus preferable over other sugarcane tissues for testing.

Agrobacterium transfection testing was determined primarily by assessing transient expression using reporter genes. Two reporter gene systems (GUS and GFP) were utilised:

- GUS: A 35S:intron-*gus* binary vector which contains an intron-interrupted *gus* gene which only expresses in plant cells (not *Agrobacterium*) was beneficial in showing the initial transformation results. However, use of this vector system was later discontinued

in favour of GFP because GUS assays kill the transgenic cells, preventing any further regeneration.

- GFP-pTO134: This binary vector contains a 35S promoter driving a synthetic GFP gene and a herbicide resistance gene (35S-bar-ocs3') to select transgenic cells. With this vector system, the generation of GFP positive cells on herbicide-containing media could be monitored. The first transgenic sugarcane plants produced using *Agrobacterium* were created using this vector system. However, the 35S promoter was not considered appropriate for further experimentation and the development of an improved vector for further transformation studies was initiated.
- GFP-pBIN.Ubi: This binary vector was created with (1) the GFP gene as above, but driven by the stronger maize ubiquitin promoter, Ubi1, (2) a more defined and researched binary vector system based on pBIN19, and (3) the geneticin selection system which works particularly well for selecting transgenic sugarcane cells.

Transgenic sugarcane plants were produced very rapidly following initial successes in *Agrobacterium* testing. This was mainly due to the use of GFP and regenerable callus. This rapid development enabled some optimisation of the transformation protocol to be pursued. This was not initially proposed in the project description but was considered highly worthwhile in view of the rapid early success.

Production of transgenic sugarcane plants for an analysis of copy number and expression levels was the primary aim during the second year of the project. Their production progressed during the optimisation of *Agrobacterium* parameters, experimentation which itself led to the generation of transgenic sugarcane callus for regeneration into plants. The optimisation has led to some significant increases in transformation frequency and a number of transgenic plants have now been produced for further analysis.

(f) Detailed results

Objective 1: To test the potential for transformation of sugarcane using established Agrobacterium-mediated transformation techniques currently being successfully applied to other monocot species.

This objective was reached and exceeded when an *Agrobacterium*-meditated protocol produced transgenic sugarcane plants. Definitive proof of transformation was obtained by Southern analysis and by the characterisation of the expressed genes in sugarcane tissues. These research results are presented in the paper published in Australian Journal of Plant Physiology 25: 739-743, a copy of which is attached to this report.

Further optimisation of the transformation protocol was performed following the initial successful demonstration of the methodology, and has so far established:

- A comparison of different sugarcane cultivars: 4 cultivars (Q117, NCo-310, Q124 and Q155) were shown to be amenable to *Agrobacterium* transformation as shown by transient expression studies conducted on callus tissue.

- A Comparison of different binary vectors and selection regimes: Relatively little difference was found between transformation frequencies for the binary vectors pTO134 (bialaphos selection) and pBin.Ubi (*npt*II selection). Work initially proceeded with pTO134 but switched to *npt*II selection so that comparisons to the conventional microprojectile-mediated system could be made.

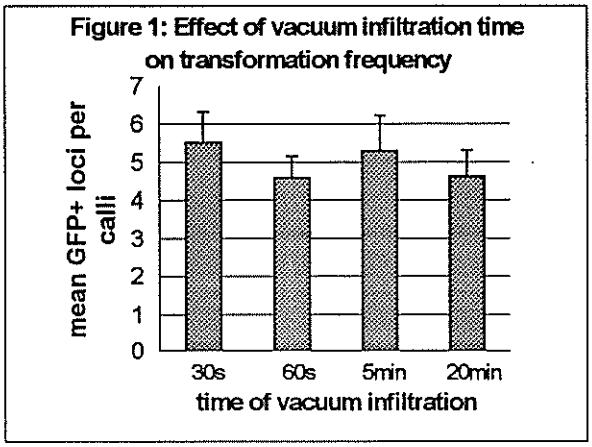
- An optimization of transformation parameters: Optimization was initiated to increase the frequencies for *Agrobacterium*-meditated gene transfer to sugarcane cells. Physical parameters affecting the interaction between *Agrobacterium* and sugarcane cells were investigated in large scale experiments requiring large amounts of sugarcane callus (upwards of 1000 callus explants per experiment).

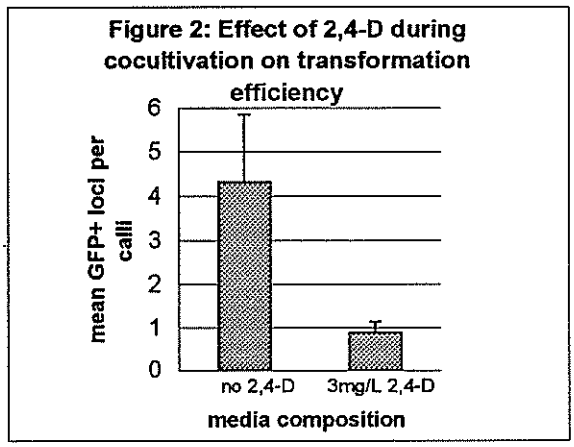
  Parameters investigated were those found to be most beneficial for improving transformation frequencies in similar monocot transformation studies. These included:

  - temperature during cocultivation
  - presence of inducing factors, such as acetosyringone and glucose
  - the absence of 2,4-D and coconut water
  - painting of explant surfaces for inoculation.
  - bacterium inoculation density
  - desiccation length before cocultivation
  - sonication pretreatment: type and duration
  - vacuum infiltration during inoculation
  - cocultivation length
  - presence of antioxidants in the media
  - bombardment of calli prior to cocultivation
  - addition of cytokinin during cocultivation, to stimulate cell division.

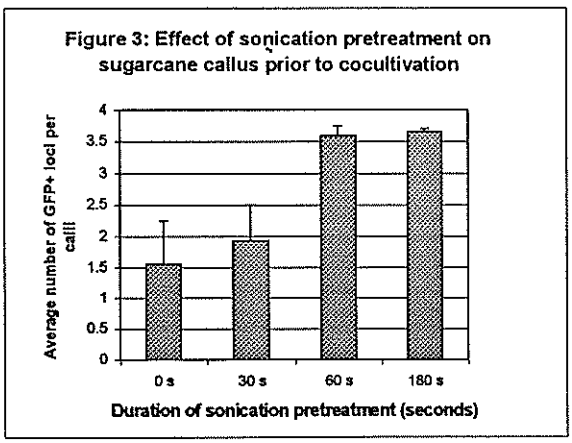
The testing of the above parameters was carried out in *Agrobacterium* transformation experiments aimed at producing a number of transgenic sugarcane plants of cv Q117. All optimal conditions for sugarcane were required to be empirically determined, as no data for *Agrobacterium*-mediated transformation of sugarcane was available in the literature. The effect of each parameter on transformation efficiency was measured by the changes in the frequency of transient transformation events (GFP+ loci) in direct comparisons. Some treatments did not significantly effect the transformation frequency, while some produced only minor increases, and some were detrimental. Vacuum infiltration is thought to promote efficient *Agrobacterium*-mediated transformation by assisting *Agrobacterium* into intracellular spaces but no effect was seen by varying the duration applied to calli (Figure 1), probably because there is little intracellular space in embryogenic sugarcane calli. In comparison, the presence of 2,4-D which is present to induce callus formation had a significant negative effect on

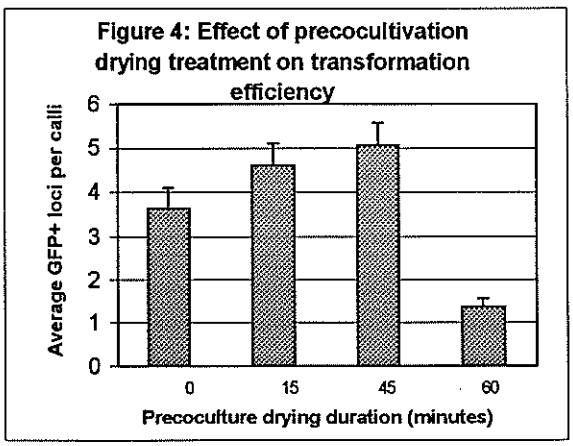
transformation frequency (Figure 2) and was therefore omitted during the cocultivation period in all subsequent experiments.





Wounding was implicated in initial reports of *Agrobacterium*-mediated transformation of plants and has often been incorporated to increase transformation frequencies. Physical wounding has been routinely used, but less rigorous pretreatments are now more commonly applied, such as drying, bombardment with tungsten particles and sonication. Of these, a technique now termed "SAAT", sonication-assisted *Agrobacterium*-mediated transformation has been presented as the most beneficial in the literature. Although there are a number of glowing reports utilising this technique (e.g. Transgenic Research 6:329-336; Plant Cell Reports 17:482-499, 17:752-759; Agricell Report 29:1) for recalcitrant species such as soybean and cereals, all such reports have originated from a single laboratory in the USA. Sonication was applied to sugarcane callus using either a sonicating water bath or a sonication probe but no large increase in transformation efficiency was observed with either method (e.g. Figure 3). The technique may be difficult to apply to sugarcane, because the intensity and frequency of sonication and the specific conditions of the culture containers reported in the literature are difficult to reproduce. A drying pretreatment which produces plasmolysis of the recipient callus cells was shown to have an even smaller effect, whilst a pretreatment time of 60 minutes was found to have a negative effect on transformation frequency (Figure 4).





Summarizing the optimisation experiments has identified the crucial parameters from those less critical for *Agrobacterium*-mediated transformation of sugarcane.
Critical parameters were:

- the inclusion of 200µM acetosyringone
- presence of antioxidants in the tissue culture media
- the omission of 2,4-D from the cocultivation conditions
- temperature: 22-25°C during cocultivation

Other parameters had little or no significant effect. An increase in the transformation frequency was achieved through this work but the resultant frequency is still less than 10% (proportion of calli showing transient expression following cocultivation with *Agrobacterium*). It is hoped that the *Agrobacterium*-mediated transformation technique may now be applied in the new collaborative project with the BSES.

- Effect on regenerative capacity of the transformed callus: *Agrobacterium* protocols involve the bathing of callus tissue in *Agrobacterium* broth for inoculation and then a 2-3 day incubation period. This treatment, although essential for *Agrobacterium*-mediated gene transfer, has a negative effect on the regenerative potential of the callus which often would not regenerate. This negative effect has resulted in some limitation to our ability to increase the frequency for obtaining transgenic sugarcane plants.

Objective 2: To compare the copy number and expression of marker transgenes from Agrobacterium-mediated transformation with that of microprojectile-mediated transformation.

Discussion of Objective 2 is provided in Section (d) and was not completed due to the merger of this project into the new BSES project. Transgenic plants will hopefully be analysed during the course of the new project.

(g) Discussion of results including analysis of outcomes compared with objectives.

The successful establishment of an *Agrobacterium*-mediated transformation technique for sugarcane is a significant output for the project. Although previously attempted in other laboratories in Australia and Hawaii, a successful outcome had not been achieved. A technique was established in minimal time, due to the use of GFP, which expedited the development and facilitated the early scoring of transformation experiments. Objective one, to transform sugarcane with *Agrobacterium* was essentially over-achieved, as it was achieved in a minimal time, which then enabled optimisation of the protocol to be initiated.

Collaborative research with the BSES in project BSS209: "Meristem transformation for sugarcane genetic engineering" has lead to significant research findings, which have progressed to the formulation of a new combined project, beginning in July 1999.

The production of transgenic plants produced by both *Agrobacterium* and microprojectile techniques, was to enable the direct comparison of the plants in the final year. Analysis of

transgene copy number, expression levels of introduced genes for the plants obtained via either technique will hopefully be possible within the new transformation project.

(h) An assessment of the likely impact for the Sugar Industry in Australia and elsewhere and where possible the cost and potential of the Project Technology, such as commercially significant developments, patents, licenses etc.

- The developed *Agrobacterium*-mediated transformation protocol in its present form is not patentable.

- The *Agrobacterium* transformation methodology has been published and presented at local meetings for the benefit of other sugarcane research laboratories.

(j) A technical summary of any other information developed including discoveries in methodology, equipment design etc.

Other significant research results and concurrent activities include:

- The 35S promoter has been shown to provide only poor expression of introduced genes in monocots, yet it has been widely used in rice transformation research. More importantly, the 35S promoter has been recently used to drive agronomically important genes in sugarcane by Cuban researchers. The level of expression provided by this promoter has never been clearly established in transgenic sugarcane plants. Transgenic plants containing a GFP gene driven by a 35S promoter were produced during this project and preliminary data has shown that the level of expression appears to be nil to very weak.

- Ongoing development of GFP technologies for use in transgenic plant research has continued during the course of this project. These technologies have been particularly useful for:
  - Screening for low frequency transformation events whilst providing an early estimation of transformation efficiency.
  - Regenerating transgenic cell clusters/calli with, or without antibiotic or herbicide selection.
  - Comparing the strength of promoter elements. This can be pursued through rapid visual screening of various plant organs and recording via visual assessment, or through more quantitative techniques such as fluorometry. A fluorometric assay for GFP quantification was developed during this research.
  - Studying the patterns of emergence of transgenic cell sections and tissues.
- Collaborative research with the BSES in project BSS209 has produced some promising results. The combining of technologies from CTA027 into the new BSES project should accelerate progress towards a very useful industry outcome.

(k) Recommendations on the activities or other steps to further develop, disseminate or exploit the Project Technology.

The technologies developed as part of this project will be instrumental in the success of the new joint CSIRO/BSES transformation project.

(l) A list of publications arising from the Project.

(a) Refereed Journal Articles

Elliott, A.R., Campbell, J.A., Brettell, R.I.S. and Grof, C.P.L. (1998) *Agrobacterium*-mediated transformation of sugarcane using the vital marker GFP. *Australian Journal of Plant Physiology* 25: 739-743.

Elliott, A.R., Campbell, J.A., Dugdale, B., Brettell, R.I.S. and Grof, C.P.L. (1999) GFP facilitates rapid, *in vivo* detection of genetically transformed plant cells. *Plant Cell Reports* 18:707-714.

Elliott, A.R., Schenk, P.M. and Grof, C.P.L. (1999) Quantitative analysis of GFP in plant extracts using the VersaFluor fluorometer. BioRad Technical Note 2434.

(b) Conference Proceedings

Elliott, A.R., Campbell, J.A., Brettell, R.I.S. and Grof, C.P.L. (1998) GFP is a vital marker for detecting genetically modified plant cells following microprojectile bombardment or *Agrobacterium*-mediated transformation techniques. Proceedings of the 4th Asia-Pacific Conference on Agriculture Biotechnology, 13-16th July, Darwin, Australia

(c) Patent Applications

Schenk, P.M., Geering, A., Elliott, A.R., McMichael, L., Grof, C.P.L., Thomas, J. and Dietzsen, R.G. (1997) Provisional patent application entitled "Plant and Viral Promoters" June 26, 1997 filed; Full patent entitled "Plant and Viral Promoters" June 26, 1998 with the Commissioner of Patents, Australia.