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FINAL REPORT – SRDC PROJECT QT002

DEVELOPMENT OF TRANSFORMATION CASSETTES FOR SUGARCANE

by

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1.0 SUMMARY

There are currently few gene regulation sequences (promoters/terminators) which have been shown to be effective in sugarcane. At present, the industry standard gene regulatory sequences used for genetic engineering of sugarcane are the maize polyubiquitin (ubi) promoter and the nopaline synthase (nos) terminator. The overall aim of this project was to develop further promoter and terminator sequences for use in the generation of transgenic sugarcane by testing whether the regulatory sequences derived from banana bunchy top nanovirus (BBTV) would function in sugarcane.

Initially, a number of different native BBTV promoters were assessed for expression of the green fluorescent protein (GFP) reporter gene in transient assays, and several of the BBTV promoters were found to express GFP as well as the ubi control. However, when stably transformed mature sugarcane plants were analysed, expression of GFP was almost always abolished. In an attempt to enhance expression, the BBTV promoter sequences were modified by the inclusion of the intron from the ubi promoter. In transient assays, the BBTV promoters performed as well as, or better than, the ubi control. When stably transformed plants were analysed by western blot, GFP expression levels equal to that of the ubi control were observed. These results indicated that (i) the inclusion of the ubi intron enhanced the activity of the BBTV-derived promoters and (ii) the promoter sequences derived from BBTV could be used to drive transgene expression in sugarcane.

The BBTV promoters were assessed for their ability to drive the selectable marker gene, neomycin phosphotransferase (nptII). Initially, several native BBTV promoters were tested but none of the transformed sugarcane callus survived selection, indicating that these promoters were unable to drive sufficient levels of nptII expression. When several intron-enhanced promoters were tested, transformed callus was able to survive the selection process, but plantlets could not be regenerated due to problems with the callus. Although preliminary, these results indicated that intron-enhanced promoter sequences from BBTV could be used to drive the nptII selectable marker gene in sugarcane.

Several BBTV terminator sequences were assessed for their ability to terminate transcription in sugarcane using nos as a control. These studies revealed that the BBTV termination sequences were as effective as nos in terminating transcription in both transiently and stably transformed sugarcane, thus providing alternative termination sequences for the industry.

Constructs containing the promoters from BBTV DNA-4, 5 or 6, the ubi intron and the terminator from BBTV DNA-6 have been prepared. These constructs also contain a multiple cloning site to facilitate use in a variety of systems in sugarcane. Further, two cassettes for selection of transformed tissue have also been prepared which contain the promoter from either BBTV DNA-6 or -4, the ubi intron, the NPTII selectable marker gene and the terminator from BBTV DNA-3.

2.0 BACKGROUND

One of the major constraints to plant genetic engineering is the lack of suitable regulatory sequences (known as promoters and terminators) needed to control the expression of transgenes. Further, most of the promoters and terminators currently used for sugarcane transformation are either patented or are not freely available, thereby restricting their practical application.

Plant promoters and terminators have been derived from a variety of sources including plants (maize polyubiquitin, rice actin) and plant pathogens (*Agrobacterium* nos, cauliflower mosaic virus (CaMV) 35S). The CaMV 35S is probably the most extensively used promoter, however, the activity of this promoter is generally restricted to dicotyledonous plants, with little expression in monocotyledonous plants such as sugarcane.

Banana bunchy top nanovirus (BBTV) is a single-stranded (ss) DNA virus which infects the monocotyledonous plant, banana. The genome of BBTV comprises at least six circular ssDNA components, approximately 1 kb. Each BBTV component consists of a coding region and a non-coding (or intergenic) region. The intergenic regions of the BBTV DNA components have been shown to possess varying levels of promoter activity in banana embryogenic callus. Further, the level of promoter activity was increased by the inclusion of intron sequences. The promoter from BBTV DNA-6 is able to drive a selectable marker gene to a sufficiently high level in banana to enable antibiotic selection of stably transformed tissue. Preliminary studies with sugarcane embryogenic callus have shown that the promoter derived from BBTV DNA-6 has activity in transient assays. Therefore, it is likely that the BBTV-derived promoters will be suitable to drive transgene expression in sugarcane. The intergenic regions of BBTV also function as termination signals for the viral genes and it is also highly likely that these sequences can be exploited for use in genetic engineering of sugarcane.

QUT has submitted patent specifications internationally covering the DNA sequences of BBTV. Thus, cassettes incorporating BBTV promoters and terminators will be owned within Australia and will be available for commercial exploitation in Australian sugarcane crops.

The aim of this project was to determine the activity of the BBTV-derived promoter and termination sequences in sugarcane, and ultimately to use these sequences to develop transformation cassettes which would be useful for the expression of any transgene in sugarcane.

3.0 OBJECTIVES

- Bombard sugarcane with reporter genes under the control of viral promoters (banana bunchy top virus (BBTV)) and assess the levels and patterns of expression
- Develop and test a selectable marker construct driven by viral promoters
- Develop and test constructs containing BBTV termination signals
- Develop a sugarcane transformation cassette containing BBTV-derived promoters and terminators

4.0 TECHNICAL INFORMATION AND METHODOLOGY

4.1 Promoters tested

The genome of BBTV consists of at least six ssDNA components, BBTV DNAs 1-6. BBTV DNAs 2-6 each encode a single large gene while BBTV DNA-1 encodes one large gene and an additional smaller gene, and thus contains two potential promoter regions. An additional two ssDNA molecules, named DNA-S1 and S2, have been associated with some BBTV infections.The promoters derived from BBTV DNA 1-6 were named BT1-6, that derived from the internal ORF of BBTV DNA-1 was named BT-intorf while those derived from DNA-S1 and S2 were named BTS1 and BTS2, respectively.

The control promoter for this study was the maize polyubiquitin promoter (ubi) which is comprised of a 1 kbp promoter region followed by a 1 kbp intron. Since the presence of the intron is believed to enhance the expression of the ubi promoter, several of the native BBTV promoters were modified to include this intron. Promoters containing the ubi intron were denoted as BT*-int.

4.2 Development of constructs

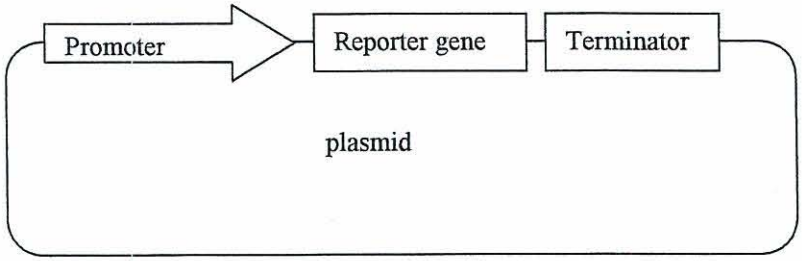

Figure 1. Basic construct design consisting of a promoter, reporter gene and terminator cassette cloned into a bacterial plasmid.

The reporter gene selected for this project was the green fluorescent protein (GFP) gene. The form of GFP used in this study was mGFP (with a mutation S65T) since this particular form of GFP was previously shown by our group to express well in banana.

A cassette containing the ubi promoter, the GFP reporter gene and the nos terminator was subcloned into the plasmid, pGEM 3ZF. The ubi promoter was then replaced with the various BBTV promoters by subcloning with two restriction endonucleases, *Hin*dIII and *Bam*HI. Similarly, the terminators were interchangeable by subcloning using *Not*I and *Eco*RI restriction endonucleases.

The selectable marker cassette used in this project contained the neomycin phosphotransferase (nptII) gene controlled by the Emu promoter and nos terminator. To test the ability of the BBTV promoters to drive the nptII selectable marker gene, the nptII/nos fragment from this cassette was amplified by PCR using primers containing *Bam*HI and *Eco*RI sites to facilitate subcloning. The BBTV promoters cassettes prepared previously were digested with *Bam*HI/*Eco*RI to remove the GFP/nos fragment and this was replaced with the nptII/nos fragment.

4.3 Transformation/tissue culture

Tissue culture was performed using C3 media. One litre of basic media contained 25 g sucrose, 0.5 g casein hydrolysate, 4.4 g MS basal medium (SIGMA), 200 ml coconut water, 3 mg 2,4-dichlorophenoxyacetic acid (2,4-D) and distilled water to make up the volume. This media was used to prepare and maintain sugarcane embryogenic callus derived from Q117. For selection 20mg l^{-1} geneticin was added to the media, and the selection pressure was continued through the regeneration steps, however the regeneration media differed from selection media by omission of 2,4-D.

Embryogenic callus of Q117 was prepared from sugarcane tissue using standard protocols and was subcultured on C3 media. The callus was bombarded with the various transformation cassettes which had been coated onto tungsten particles using standard protocols. Callus was examined under a fluorescence microscope two days post-bombardment to determine the transient activity of the various constructs. The material was then assessed seven days post-bombardment and only callus pieces still expressing GFP were subcultured. The callus was then subcultured every two weeks for a total of eight to ten weeks on selection medium then transferred to regeneration medium. The plants were subcultured on regeneration medium every three weeks until the plantlets were large enough to be planted in soil (approximately 5-10 cm tall). Small regenerated plantlets were periodically examined using a fluorescent microscope to determine whether they were still expressing GFP. Finally, the plants were acclimatised in soil and transferred to a PC2 glasshouse.

4.4 Assessing levels of GFP expression

GFP is an excellent non-destructive marker and can be used to monitor progress throughout the tissue culture process, however, quantifying the level of GFP expression has not yet been optimised. In the course of this project, we have developed a semi-quantitative western blot assay which can be used to compare GFP expression levels in total leaf extracts.

Total protein was extracted from 100 mg of leaf tissue by first freezing the tissue with liquid nitrogen and grinding with an approximately equal volume of acid-washed sand. The protein extraction method used was one mini-protease tablet (Roche) resuspended in 7 ml of 0.1 M phosphate buffer. A 300 µl aliquot of this extraction buffer was added to the ground tissue, and tissue debris removed by a low speed spin for five minutes. The protein concentration of the supernatant was determined using a Bradford assay and 10 µg of each sample was denatured and analysed by SDS-PAGE. The gel was blotted and then probed with a mixture of two anti-GFP monoclonal antibodies (Roche). Two standards were run on each gel to assist with quantifying the GFP. Imagequant was used to analyse scanned images of the blots.

4.5 Genomic analysis of stably transformed plants

Total nucleic acid was extracted using the CTAB method. Initially, PCR was used to determine whether the transformed plants contained the GFP gene. Southern blots were performed to demonstrate that the plants occurred from different transformation events (i.e. that they were not clones of each other) and to give an indication of copy number. Genomic DNA was digested with *Hind*III, electrophoresed through agarose and then blotted onto positively charged membranes. The blots were probed with DIG-labelled GFP and developed according to the Roche protocol.

5.0 RESULTS

Objective 1: Bombard sugarcane with reporter genes under the control of viral promoters and assess the levels and patterns of expression

(i) Native BBTV Promoters

Initially, sugarcane callus was bombarded using constructs containing the nine BT promoter regions, BT1-6, BT1-intorf and BTS1/S2, fused to the GFP reporter gene and the nos terminator. Transient promoter activity was assessed by examining callus with a fluorescent microscope two days post-bombardment. The BT4, BT5, BTS1 and BTS2 promoters all produced levels of transient expression similar to that observed with the ubi control. Promoters BT1, BT2 and BT6 produced slightly lower levels of expression than the ubi control, while BT3 produced very low levels of expression. No GFP expression was observed using BT1-intorf, therefore, no further studies were performed using this promoter.

Stably transformed plants were produced from callus bombarded with the BT2-6 and BTS1/S2 promoter constructs. However, despite repeated attempts, no stable transformants containing the BT1 promoter could be generated. The activity of the native BT promoters diminished through the selection process. For example, when the bombarded callus was ready for transfer from selection medium onto regeneration

medium, no GFP expression was visible in callus transformed with the BT2 and BT3 promoters. Plantlets transformed with the BT4-6 and BTS1/S2 promoters generally showed weak GFP expression which was primarily localised at the base of the newly emerging leaves. The plantlets were eventually potted in soil and allowed to grow in the glasshouse. When these glasshouse acclimatised plants were analysed, no GFP expression was observed in any of the plants. Therefore, the presence/absence of GFP was assessed using the more sensitive western blot analysis. By the time the plants reached approximately 1 metre in height, expression of GFP was no longer detected in any plants containing BT2-6 and BTS1-derived promoters. One plant transformed with the BTS2 promoter was found to express GFP, although seven other BTS2-transformed lines showed no expression. These results are summarised in Table 1.

Table 1. GFP expression in leaves of mature plants transformed with BT native promoter-GFP-Nos cassette

Promoter	Number of lines	Number of lines expressing GFP in mature plants
BT2	6	0
BT3	6	0
BT4	7	0
BT5	3	0
BT6	5	0
BTS1	3	0
BTS2	8	1

(ii) Intron-enhanced BBTV promoters

In an attempt to increase the promoter activity of the native BBTV promoters, the intron from the maize polyubiquitin promoter was added to the BT2, BT4-6 and BTS1/S2 promoters. In transient assays, the BT2-int and BT4-int constructs produced levels of expression similar to that of the ubi control, while the remaining constructs expressed levels of GFP significantly greater than the control.

Stably transformed plants were generated containing the BT4-6-int and BTS1/S2-int promoters. The intron-enhanced promoters were able to drive strong GFP expression throughout the selection process, with strongest expression occurring in newly emerging leaves of the regenerating plants. GFP expression in plantlets transformed with BT6-int showed a phloem-associated dot-dash pattern on the leaves, while the remaining promoters appeared to drive constitutive expression throughout the entire leaf. Attempts were made to determine more precise GFP expression patterns using confocal microscopy, but these were unsuccessful. As the plants grew larger (80-100 cm), the detection of GFP using the fluorescent microscope became increasing difficult. A filter was used to block fluorescence due to chlorophyll, however, it was still difficult to detect GFP in the

samples, possibly due to low levels of expression of GFP. In mature plants it was impossible to detect expression of GFP using the fluorescent microscope, therefore, the GFP western blotting technique was again used to determine if the mature plants were still expressing GFP, and to allow comparison of the expression levels in the leaves. The results of these analyses are shown in Table 2 and Figure 2.

Table 2. GFP expression in leaves of mature plants transformed with BT-int promoter-GFP-Nos cassette

Promoter	Number of lines	Number of lines expressing GFP in mature plants
BT4-int	9	6
BT5-int	11	7
BT6-int	9	6
BTS1-int	2	2
BTS2-int	5	1
Ubi	7	7

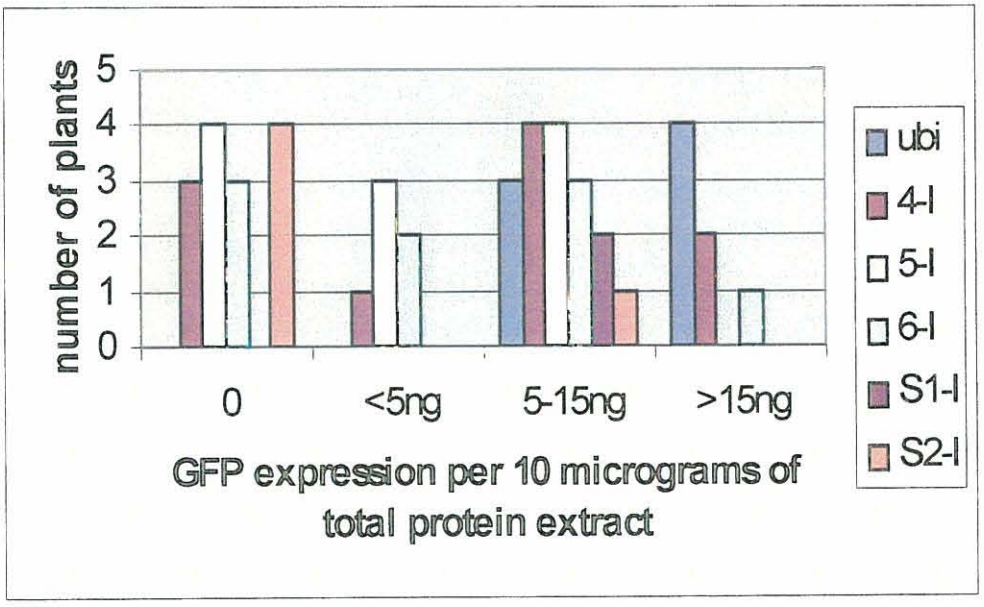

Figure 2. Levels of GFP expression in leaves of plants transformed with BT-int promoter-GFP-Nos cassette

In summary, the plants transformed with the current industry standard, ubi, all expressed GFP at levels of greater than 5 ng per 10 µg total protein extract. Two of the BT-int promoters, BT4-int and BT5-int, were capable of driving expression at levels as high as the ubi control.

Attempts to determine the activity of the promoters in roots using Western analysis were unsuccessful due to difficulties in extracting protein from this source. However, when root samples were examined by fluorescent microscopy, none of the samples examined, including the ubi control, showed any fluorescence.

Objective 2: Develop and test a selectable marker construct driven by the BBTV promoters

Initial observations using plants stably transformed with the native BT promoters indicated that the activity of the majority of the native BT promoters either decreased or was abolished upon regeneration of the callus into plantlets. As such, these promoters were considered potentially useful to drive selectable marker genes for sugarcane transformation since regenerated plants wouldn't express detoxifying chemicals(ie. nptII).

The promoters from BT5, BT6, BTS1 and BTS2 were chosen to drive the selectable marker gene, nptII. BT5, BTS1 and BTS2 were used to replace the emu promoter in the nptII cassette and used to bombard sugarcane with the ubi-GFP-nos control plasmid. The BT6 promoter had previously been cloned into a plasmid containing NPT II-nos and 35S-GFP-nos. This cassette had been shown to work well for selection in banana therefore was tested in sugarcane.

Sugarcane callus was bombarded with the four BT-nptII constructs, and with emu-nptII-nos as a control. Within three months, all tissue bombarded with the BT promoters had died, while the control tissue was healthy and ready to regenerate. This indicated that the BBTV promoters used in these constructs were not capable of driving the expression of the selectable marker genes and thus were unsuitable for use in transformation cassettes.

Three new constructs were subsequently prepared which contained intron-enhanced BBTV promoters (BT4-int, BT5-int, BT6-int). These promoters were chosen because they had previously been shown to drive strong reporter gene expression in sugarcane embryogenic callus. The constructs were used to bombard embryogenic callus, and the callus was then placed on selection medium. The callus survived on selection medium for 2-3 months, suggesting that these promoters were capable of driving the expression of a selectable marker gene. The callus was then placed on regeneration medium, however, regeneration did not occur with either the BT transformed callus or callus transformed with the Emu control. This may have been due to the fact that the callus had been cultured for over three months prior to bombardment hence was altered by the tissue culture process and no longer able to regenerate. Time constraints prevented a repeat of this experiment, however, the BT-int constructs performed as well as the Emu promoter through selection and appear to be a suitable alternative promoter.

Objective 3: Develop and test constructs containing BBTV termination signals

The termination signals from BBTV DNAs 2, 3, 4 and 6 were amplified from a BBTV viral preparation and subcloned into a plasmid containing the BT6-int promoter and the GFP reporter gene. Embryogenic callus was bombarded with these constructs using a BT6-int-GFP-nos as control.

(i) Transient analysis

The bombarded callus was analysed for green foci of GFP two days post-bombardment. The BT6 terminator appeared to function as well as the nos control, with a similar number of green foci observed per plate (nos: average of ~1100 foci per plate; BT6: average of ~1000 foci per plate). In contrast, the BT2, 3 and 4 termination signals did not appear to function as well as the nos control, with approximately 50% of the expression exhibited by BT6 and the nos control.

(ii) Stable transformation

Stable independent transformants were regenerated and GFP expression levels analysed by western blot. The levels of GFP expression in the transgenic plants varied considerably (Figure 3) and all sets of plants contained at least one line with undetectable levels of GFP expression. This variability is not uncommon and has been attributed to positional effects and differences in transgene copy number (which we found varied from 8-16).

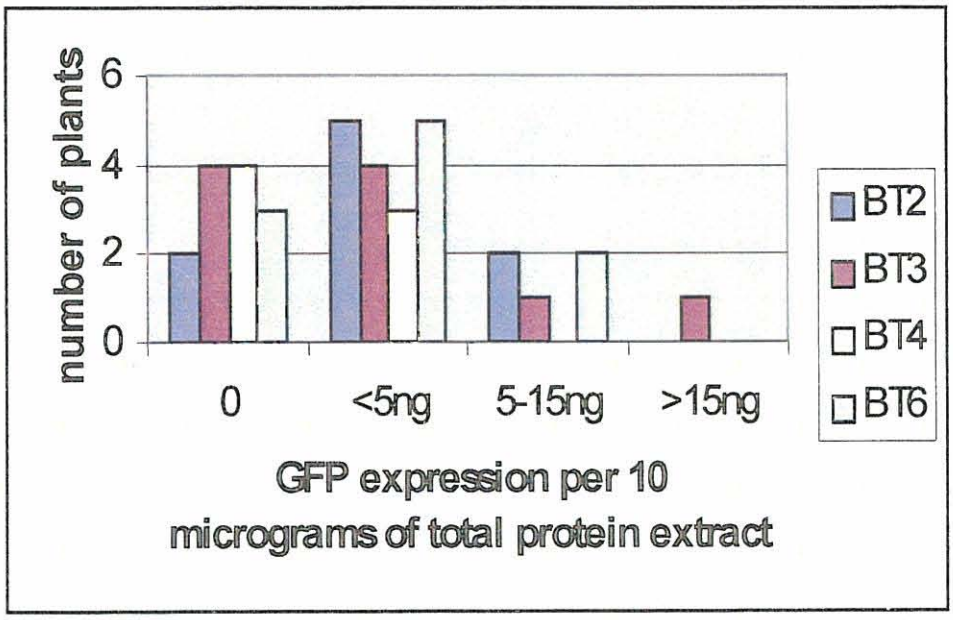

Figure 3. Levels of GFP expression in plants stably transformed with BT6-int-GFP-BT terminator cassette

The set of plants transformed with the BT3 terminator contained the highest expressing individual plant and had the highest average expression levels. However, statistical analysis of the results indicated that the GFP expression levels associated with plants transformed with the BBTV terminators were not significantly different to each other or the nos control. This implied that any of the BBTV terminators would be an adequate replacement for nos, the current industry standard. The BT6 terminator was chosen as the terminator of choice in the development of a transformation cassette because it showed the least variability in GFP expression from the plants analysed.

Objective 4. Develop a sugarcane transformation cassette containing BBTV-derived promoters and terminators

The BT6-int promoter was initially selected as an alternative to ubi, and was used with the BT6 terminator to prepare a generic plasmid with a multiple cloning site (MCS). A map of the construct is shown in Figure 4.

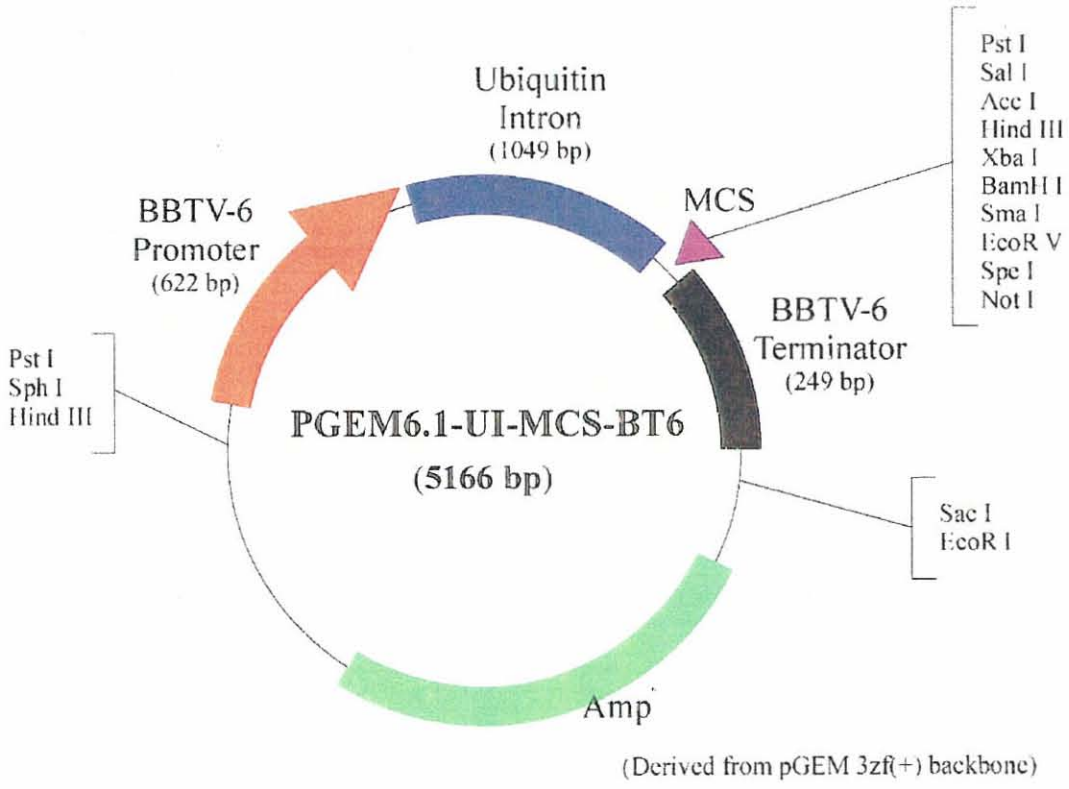

Figure 4. Map of sugarcane transformation construct

Two further constructs have also been made which are identical to that shown above except that they contain either the BT4-int or BT5-int promoters.

In addition, two cassette for selection of transformed tissue have also been prepared which contain either the BT6-int or BT4-int promoter, the NPTII selectable marker gene and the terminator from BBTV DNA-3.

6.0 OUTCOMES

- Transgenic sugarcane transformed with BBTV promoters BT4, BT5 and BT6, fused to the intron from the maize polyubiquitin gene (BT4-int, BT5-int and BT6-int), contained reporter gene expression levels as high as the ubiquitin control. These promoters provide an alternative to the current industry standard, ubiquitin.
- The BT4-int, BT5-int and BT6-int promoters were able to drive the expression of the NPT-II selectable marker gene to sufficient levels to allow selection of transformed sugarcane callus.
- BBTV terminators BT2, 3, 4 & 6 all function as well as nos, and thus provide an alternative source of terminators for use in sugarcane transformation.
- Several transformation constructs, as well as a selectable marker constructs, have been prepared.

7.0 IMPLICATIONS AND RECOMMENDATIONS

We have produced several generic sugarcane transformation constructs, using BBTV regulatory sequences, for use in transformation of sugarcane. This investigation has revealed that the BBTV sequences appear to perform as well as the industry standard sequences and are adequate replacements. However, because of the difficulties associated with quantitation of reporter gene activity and the determination of expression patterns, further characterisation of this construct, using a suitable "gene of interest" such as the GUS reporter gene, is considered necessary. The constructs produced in this study are available for use by the Australian sugar industry subject to negotiation with QUT who holds the patent licenses for the BBTV sequence. It is envisaged that an agreement will need to be drafted to protect the QUT IP and ensure any plant material containing the constructs is kept within Australia.

8.0 ACKNOWLEDGEMENTS

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9.0 PUBLICATIONS

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