FINAL REPORT

SUGAR RESEARCH COUNCIL PROJECT BS9S

THE PRODUCTION OF GENETIC CONSTRUCTS FOR EXPRESSION OF SUGARCANE VIRUS COAT PROTEIN IN SUGARCANE PROTOPLASTS

AIM: To clone the coat protein gene of sugarcane mosaic virus (SCMV) and develop constructs for expression of this gene in sugarcane protoplasts.

FINAL PROJECT STATUS: The coat protein of SCMV has been detected at low levels in sugarcane protoplasts transformed with a plasmid construct containing the coat protein gene. Thus, the project achieved its objective. The level of expression of the coat protein gene in protoplasts is being quantified.

SIGNIFICANCE OF PROJECT RESULT TO THE SUGAR INDUSTRY: This project was one phase of research required for production of transgenic sugarcane. Through this project the sugar industry now has a synthetic resistance gene against SCMV that expresses in sugarcane plant cells. When sugarcane plants are regenerated from protoplasts or the biolistic gene is adopted for use in sugarcane, this gene may be used to produce transgenic cane plants combining their original agronomic characteristics with SCMV resistance.

DETAILED PROJECT ANALYSIS

The four original major objectives of this project were:-

1. Molecular cloning of SCMV RNA (ribonucleic acid)
2. Characterisation of cDNA clones (complementary deoxyribonucleic acid)
3. Expression of SCMV coat protein in tobacco plants
4. Expression of SCMV coat protein in sugarcane protoplasts

During the course of the project the objectives were modified to take advantage of recently developed methods. As outlined at the SRC review of this project in 1989, the availability of the CSIRO-cloned gene and the optimisation of the BSES electroporation system made parts of objectives (1) and (2) unnecessary and allowed replacement of objective (3) with:-

3a. Expression of SCMV coat protein in *E. coli*

MOLECULAR CLONING AND CLONE CHARACTERISATION

The virus had to be isolated from infected plant tissue before its viral RNA could be cloned. This process proved more difficult than predicted. Several published methods exist for the purification of SCMV and other potyviruses from plant tissue. None gave the expected results, the usual problem being that the viral particles would aggregate and fall though the density gradient and become contaminated with plant constituents. Discussions with other potyvirus researchers revealed this was a common problem and that potyvirus purification was often laboratory dependent. We modified the techniques to produce viral preparations that were pure according to analyses using SDS-PAGE (polyacrylamide gel electrophoresis in the presence of the detergent sodium dodecyl sulphate). The viral RNA isolated from these preparations often appeared as a single band after agarose gel electrophoresis, suggesting the presence of full length RNA.

Complementary DNA to the purified RNA was produced by standard molecular biology cloning techniques. The cDNA was inserted into a standard cloning plasmid (pUC19) and the recombinant plasmids so produced were screened for the presence of viral cDNA inserts by nucleic acid hybridisation. It became apparent during this phase of the project that the viral RNA preparation was contaminated with plant RNA. Thus many of the cDNA inserts were of plant rather than viral origin.

At this time, Dr Shukla from CSIRO suggested we collaborate on this research as his group had recently cloned the coat protein region from the 'Brisbane' isolate of SCMV, as part of Dr Shukla's overall investigation into taxonomy and classification of the potyvirus group. Dr Shukla supplied the coat protein gene as a 1.37 kilo base pair cDNA insert in the plasmid pKS#SCMV64 (Figure 1). The insert included 171 nucleotides 5' to the CP gene, the CP gene of 939 nucleotides, the untranslated 3' region of 235 nucleotides and 13 adenosine residues from the poly-A tail. The CP gene is part of a larger viral gene, and as such it requires the addition of a start codon (ATG) to provide the correct signals for transcription and translation. This was achieved by a DNA oligomer containing the start codon in the consensus of a NcoI restriction enzyme site and with an 5' XbaI site for cloning into the modified plant expression vector. This adaptor was joined to the rest of the gene at the HaeII site. This construction was cloned into the plasmid pUC19 to produce pN3UT.

This plasmid served as the basis for further work as it contained the CP gene in a biologically functional form.

EXPRESSION OF THE COAT PROTEIN GENE IN *E. coli*

The plasmid pKKNP-2 (Figure 1) was constructed for expression of the coat protein gene in *E. coli*, by cloning the gene construct from pN3UT into the appropriate restriction sites of the commercially available expression vector pKK233-2. Induction of the gene promoter by addition of the chemical inducer IPTG to the bacterial growth medium resulted in expression of the coat protein. Expression was evaluated by 'western blotting'

in which denatured proteins were transferred to a nitrocellulose membrane after size separation by SDS-PAGE, probed with antisera and developed with the light generating substrate 'luminol'. Some of this technology was developed as part of the SRC funded gene probe project (BS10S).

The Western blot (Figure 2) clearly indicates induction of an increased level of expression, however the promoter in this system is very strong and difficult to shut down completely. Expression of the coat protein gene construct in the easily manipulated *E. coli* system was important - this established that the genetic construction was correct, that the construct was recognised as a gene in a biological system, and that the gene produced an authentic coat protein of the correct size and antisera specificity.

EXPRESSION IN SUGARCANE PROTOPLASTS

Expression of foreign genes in monocotyledons requires a promoter such as the cauliflower mosaic virus 35S promoter and gene termination signals like those from the nopaline synthesase gene (NOS-ter). Plant expression plasmids with this combination (35S and NOS-ter) are used in commercially available reporter plasmids like pBI221. This plasmid was modified to produce a plant gene expression system to express the SCMV coat protein gene. This modification involved removal of the reporter (GUS) gene from pBI221 and inserting a 0.11kbp multiple cloning site fragment from the plasmid pBluescript II KS to produce pGS1 (Figure 1). The genetic construct from pN3UT was cloned into the appropriate restriction enzyme sites in pGS1 to produce the plasmid pSCMVCP/2. The important features of this plasmid construct as they relate to expression of the SCMV gene in sugarcane protoplasts are:-

1. The 35S promoter and NOS-ter signals.

2. A consensus sequence surrounding the ATG start codon that is as similar as possible to a plant start codon consensus sequence, given the constraints of the NcoI and XbaI restriction site recognition sequences.

3. The 3' untranslated region and part of the poly A tail before the NOS-ter signal. This may help stabilise the mRNA transcribed from the gene before translation into protein.

This plasmid (pSCMVCP/2) was electroporated into sugarcane protoplasts using the conditions and techniques developed at BSES by Carl Rathus. The aim of this step was to demonstrate transient expression before attempting stable incorporation and expression. Transient expression of the gene construct was demonstrated by western blotting (Figure 3). However the expression level is low and has not been quantified. Also the polyclonal antisera has some cross reactivity against sugarcane proteins making detection of small amounts of SCMV coat protein difficult. This result fulfils the overall aim of the project, although some of the original objectives became unnecessary as a result of new developments during the course of the project.

FURTHER RESEARCH

The information and genetic construct from this project will be used in the SRC project 'The Production of Transgenic Plants' (BS44S). The expertise and skills developed by BSES staff during this project should allow for the easy integration of other defined genes into the program.

PUBLICATIONS

A manuscript tilted 'Expression of the Coat Protein of Sugarcane Mosaic Virus in *Escherichia coli* and Sugarcane Protoplasts' is being prepared for submission to a journal such as 'Journal of General Virology'.

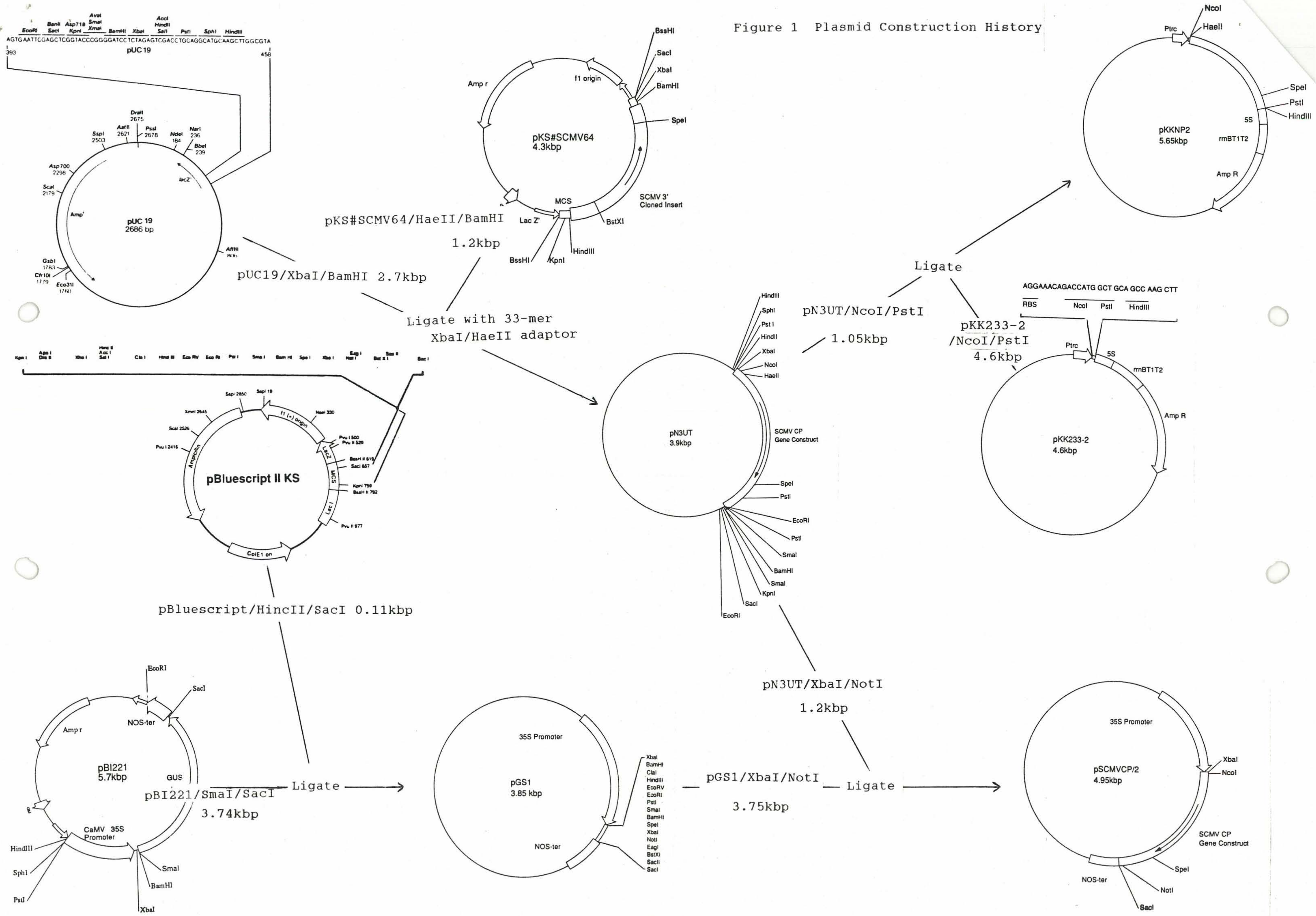
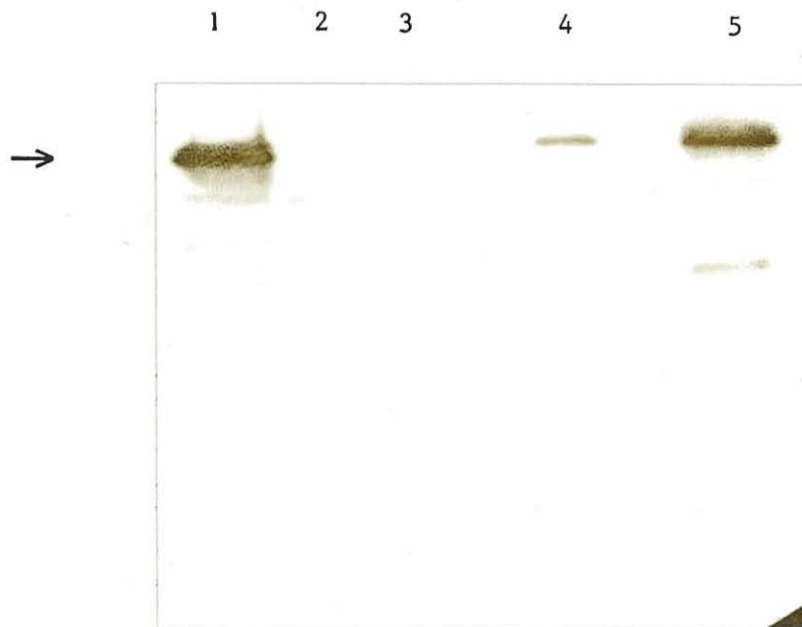


Figure 1 Plasmid Construction History

Figure 2 **Expression of SCMV coat protein in *E. Coli***

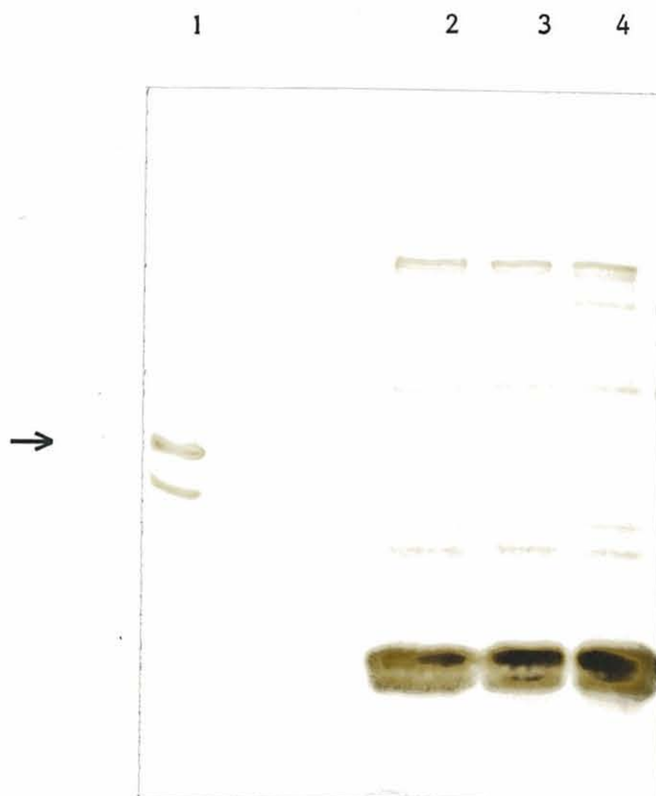


Western blot analysis of total cellular protein two hours after plasmid protein synthesis induction by addition of IPTG to 1mM. Proteins were separated by SDS-PAGE before electroblotting onto nitrocellulose membrane and probing with polyclonal primary antibody (supplied by Dr Dharma Shulka), goat anti-rabbit secondary antibody (Biorad) and detection with ECL luminol (Amersham). Results were recorded on Kodak X-Omat film. The coat protein marker (arrow) in lane 1 is SCMV coat protein produced in a non-regulated *E. coli* strain and been proved earlier to be identical to authentic coat protein (supplied by Dr Shulka).

Lane key

1. Coat protein marker
2. *E. coli*/pKK233-2 uninduced
3. *E. coli*/pKK233-2 induced
4. *E. coli*/pKKNP-2 uninduced
5. *E. coli*/pKKNP-2 induced

Figure 3 **Expression of SCMV coat protein in protoplasts**



Western blot analysis of soluble proteins after electroporation and transient expression for 24h. Blots were probed as described for Figure 2. The coat protein band in the control is arrowed (the other band is a antisera reactive *E. coli* protein). The coat protein from the protoplast preparations is the same size as the control. Some non-specific reaction with sugarcane proteins (especially a low molecular weight protein) is apparent.

Lane key

1. Coat protein marker (*E. coli* recombinant protein)
2. Protoplast/pSCMVCP/2.1
3. Protoplast/pSCMVCP/2.2
4. Protoplast