Studies on *Subterranean clover mottle virus* towards development of a gene silencing vector

This thesis is submitted to Murdoch University for the degree of Doctor of Philosophy

by

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary education institute.

John Fosu-Nyarko
Abstract

Subterranean clover mottle virus (SCMoV) is a positive sense, single-stranded RNA virus that infects subterranean clover (Trifolium subterraneum) and a number of related legume species. The ultimate aim of this research was to investigate aspects of SCMoV that would support its use as a gene silencing vector for legume species, since RNA (gene) silencing is now a potential tool for studying gene function. The ability of viruses to induce an antiviral defense system is being explored by virus-induced gene silencing (VIGS), in which engineered viral genomes are used as vectors to introduce genes or gene fragments to understand the function of endogenous genes by silencing them. To develop a gene silencing vector, a number of aspects of SCMoV host range and molecular biology needed to be studied.

A requirement for a useful viral vector is a suitably wide host range. Hence the first part of this work involved study of the host range and symptom development of SCMoV in a range of leguminous and non-leguminous plants. The aim of this work was to identify new and most suitable hosts among economically important crop and model legumes for functional genomic studies, and also to study symptom development in these hosts for comparison with host responses to any SCMoV-based viral vectors that might be used in later infection studies. A total of 61 plant genotypes representing 52 species from 25 different genera belonging to 7 families were examined for their response to SCMoV infection, including established and new crop legumes, established pasture, and novel pasture and forage legumes, and 12 host indicator plants belonging to the families Amaranthaceae, Apiaceae, Chenopodiaceae, Cruciferae, Cucurbitaceae and Solanaceae. Following mechanical inoculation, plants were examined for symptoms and tested for primary and secondary infection by RT-PCR and/or ELISA after 2-3 weeks and 3-9 weeks, respectively. Thirty-six legume hosts belonging to eight different genera of legumes were identified as suitable hosts of SCMoV, 22 of them systemic hosts and 15 were infected locally. Only two non-legumes were infected with SCMoV-P23, one systemically and one as a local host, so confirming that SCMoV is essentially a legume-infecting virus. This work considerably expanded knowledge of the host range of SCMoV.
Abstract

To provide the information needed to modify the SCMoV genome to develop gene vectors, the virus was characterized in detail. The complete genomes of four isolates, SCMoV-AL, SCMoV-MB, SCMoV-MJ and SCMoV-pFL, were sequenced using high fidelity RT-PCR and molecular cloning, and compared to the first sequenced isolate (SCMoV-P23) to give a complete picture of the genome organisation of the virus. The 4,258 nucleotide (nt) sequence of SCMoV RNA is not polyadenylated. The 5’ non-coding region (NCR) is 68 nt in length and the 3’ NCR is 174 nt. The coding region contains four overlapping open reading frames (ORFs). The first, ORF1 (nt 68-608), encodes a putative protein containing 179 amino acids with a calculated molecular mass (M_w) of 20.3 kDa. It overlaps with the next ORF, ORF2a, by four bases. ORF2a (nt 605-2347) encodes a putative protein of 580 amino acids with a M_w of 63.7 kDa and contains a motif characteristic of chymotrypsin-like serine proteases. The ORF2b is probably translated as part of a polyprotein by -1 ribosomal frameshifting in ORF2a. The transframe product (M_w = 107.5 kDa) is made up of 966 amino acids. A GDD motif typical of RNA virus polymerases is present in ORF2b. The 3’ terminal ORF3 (nt 3323-4084) encodes the 27.3 kDa coat protein (CP).

Nucleotide variation between the complete sequences of the isolates was two to three orders of magnitude larger than base misincorporation rates of the polymerases used in RT-PCR. Molecular relationship analysis between all five isolates, undertaken with the complete nucleotide sequences, clearly separated them into three groups. These groups reflect similar significantly diverse groupings based on the symptoms and their severity in subterranean clover. Intra-isolate sequence variability is therefore a possible cause of the differences in symptom severity. The analysis also showed that there were more nucleotide substitutions at the 5’ terminal half of SCMoV than at the 3’ end. This observation was confirmed by the higher value of nucleotide diversities at nonsynonymous versus synonymous sites (d_n/d_s ratio) estimated for the ORF1, compared to the near conservation of sequences of the other ORFs. These results, together with functional and comparative sequence analysis with other sobemoviruses, implicate the ORF1 gene product in pathogenicity of SCMoV, possibly as a severity determinant or as a viral suppressor of RNA silencing in plants.
Because more information on SCMoV genome function was required, the possible involvement of the ORF1 gene product (P1) and the CP in movement of SCMoV was studied in cells of grasspea (*Lathyrus clymenum* L) and chickpea as C-terminal fusion constructs with jellyfish (*Aequorea victoriae*) green fluorescent protein (GFP). A transient expression vector, pTEV, for *in planta* synthesis of reporter gene constructs was developed. The vector was based on pGEM-T with 35S RNA transcriptional promoter of *Cauliflower Mosaic virus* (CaMV) and nopaline synthase gene transcription terminator signal (T-Nos) separated by a multiple subcloning site. A custom-made particle inflow gun was used to introduce the constructs into plant cells. The bombardment conditions were first optimised for efficient delivery of DNA-coated particles. Transient gene expression of GFP was monitored 24-96 hours after particle bombardment. Fluorescence from GFP alone, GFP:CP and GFP:P1 constructs was observed in the nucleus of single cells, cytoplasm and cell periphery of neighbouring cells. There was limited spread of these fusion proteins from one cell to another 36-48 hours after transformation. These results indicate that the P1 and CP cannot move independently from cell to cell. Other viral/cellular components might be needed to form a complex with these proteins to transport the viral genome. Putative nuclear export signals in the P1 and CP sequences of SCMoV were identified by sequence comparison. These could be tested by mutagenesis using full-length infectious clones.

To determine the possibility of gene expression of vectors based on SCMoV, three forms of a full-length cDNA clone of SCMoV-pFL were developed: one with no heterologous transcriptional factors (pFL), a second under the control of only 35S (p35SFL) and a third with 35S and T-Nos (pTEVFL). Fifteen day-old *in vitro*-cultured chickpea, grasspea and subterranean clover seedlings were inoculated by particle bombardment using gold particles coated with plasmid pTEVFL. *In vivo*-transcribed RNA transcripts were detected by RT-PCR after two weeks in grasspea but not in subterranean clover and chickpea.
Abstract

Experiments were undertaken towards developing the SCMoV genome into a VIGS vector. Three forms each of five major GFP chimeric constructs of pFL (the full length SCMoV cDNA clone) were generated from which *in vitro-* and *in vivo-*transcribed RNA transcripts could be derived. The rationale used in developing these constructs was gene insertion and/or replacement with *gfp*, and duplication of the putative subgenomic RNA promoter (sgPro) of SCMoV. The major constructs were as follows:

- pFLCPgfp, pFL with the *gfp* gene fused to the 3’ end of the CP gene,
- pFLP1gfp, pFL with *gfp* gene fused to the 3’ end of the ORF1,
- pFLCPsgprogfp, pFL with a putative sgPro sequence and a translatable *gfp* gene cloned in tandem between the CP gene and the 3’ NCR of SCMoV,
- pFLCPVsgprogfp, pFL with a putative sgPro sequence and a translatable *gfp* gene cloned in tandem between a truncated CP gene and the 3’ NCR and
- pFLREPsgprogfp, pFL with the ORF2b, a putative sgPro sequence and a translatable *gfp* gene cloned in tandem between a truncated CP gene and the 3’ NCR

These constructs were all made, but a detailed assessment of their vector potential could not be done because there was a delay of about one year whilst the Office of the Gene Technology Regulator processed the application for permission for glasshouse testing. Although additional work needs to be undertaken to complete development of a final RNA silencing vector, this study has contributed to new knowledge in terms of extending understanding of SCMoV host range, symptoms, sequence variation and control of gene expression. The constructs made have also laid the groundwork for development of a legume gene silencing vector based on SCMoV.
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Dedicated to

The One I Loved And Wished I Could Know More, My Dear Sister, Abigail Serwaa

Publications and Presentations

Refeered Publications


Poster Presentations


Submitted Nucleic Acid Sequences

Fosu-Nyarko, J., Jones, R. A. C., Dwyer, G.I. and Jones, M. G. K. (2003). Complete genomic sequence of four Subterranean clover mottle Sobemovirus isolates: SCMoV-AL (Genbank Accession No. AY376451); SCMoV-MB (Genbank Accession No. AY376452); SCMoV-MJ (Genbank Accession No. AY376453) and SCMoV-pFL (Genbank Accession No. AY376454).
List of frequently used abbreviations

3’ hydroxyl terminus of DNA molecule
35S 35S RNA transcriptional promoter of CaMV
5’ phosphate terminus of DNA molecule
bp base pair
cDNA complementary DNA
CIP Calf intestinal alkaline phosphatase
CP coat protein
C-terminus carboxy terminus
cv cultivar
DNA deoxyribonucleic acid
dNTP deoxynucleoside triphosphate
dsRNA double stranded RNA
E. coli Eschericia coli
EDTA ethylenediaminetetra-acetate acid disodium salt
ELISA Enzyme-Linked Immunosorbent Assay
GFP green fluorescent protein
gRNA genomic RNA
GUS β-glucoronidase gene
kb kilobases
Mₐr average calculated molecular mass
MCR multiple cloning region
MP movement protein
NCR non-coding region
NES Nuclear export signal
N-terminus amino terminus
ORF open reading frame
PCR Polymerase chain reaction
PDS Phytoene desaturase
PTGS Post-transcriptional gene silencing
RdRp RNA dependent RNA polymerase
RNA ribonucleic acid
RNAi RNA interference
RNase ribonuclease
RT reverse transcription
SatRNA satellite RNA
SEL size exclusion limit
sgPro subgenomic promoter
sgRNA subgenomic RNA
siRNA short interfering RNA
TAE Tris-acetate-EDTA
TE Tris-EDTA
T-Nos Nopaline synthase gene transcription terminator signal
U Unit
VIGS Virus-induced gene silencing
Vol volumes
vRNA viral RNA

Viruses and scientific names
ACMV African cassava mosaic virus
AMV Alfalfa mosaic virus
BaMV Bamboo mosaic virus
BDMV Bean dwarf mosaic virus
BMV Brome mosaic virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Virus Name</th>
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<tbody>
<tr>
<td>BNYVV</td>
<td>Beet necrotic yellow vein virus</td>
</tr>
<tr>
<td>BSMV</td>
<td>Barley stripe mosaic virus</td>
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<tr>
<td>BSSV</td>
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