1. Project title: Genetic modification of *Brassica oleracea* for resistance to turnip and cauliflower mosaic viruses.

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Project Liaison Officer:	Dr JA Walsh
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Key workers:	Dr JA Walsh, Plant Virologist Dr IJ Puddephat, Plant Biotechnologist Dr DJ Barbara, Molecular Biologist Miss HT Robinson, Plant Biotechnologist Dr S Muthumeenakshi, Molecular Biologist Miss LC Griffiths, Plant Biotechnologist
Location:	HRI, Wellesbourne
Project Co-ordinator:	Mr R Montgomery
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Key words: *Brassica oleracea*, turnip mosaic virus (TuMV), cauliflower mosaic virus (CaMV), transgenic resistance, marker free.

2. Contents

3. Practical section for Growers

Turnip mosaic virus (TuMV) and cauliflower mosaic virus (CaMV) are major problems for brassica producers in the UK. There are currently no effective control measures; no potent forms of resistance to either virus exist in *B. oleracea* types (cabbage, cauliflower, broccoli, sprouts etc.) and insecticide sprays do not stop virus spread. This project aims to produce transgenic *B. oleracea* plants which Nickerson Zwaan will subsequently incorporate into their breeding programmes to produce virus resistant brassica cultivars.

The marker genes *gus* and *gfp* have been incorporated into the virulence plasmids of *Agrobacterium rhizogenes* strains. These markers will allow the efficient identification and elimination of brassica plants possessing undesirable DNA following transformation. The strains of *A. rhizogenes* possessing these marker genes and most effective in transforming the rapid cycling *B. oleracea* line Senna and cauliflower cv. Lateman are being identified.

PCR primers that will efficiently amplify the required regions of the genome of the TuMV isolate UK 2 and the CaMV isolate UK 4 were identified and modified to incorporate extra sequences necessary for their correct utilisation. These were used to amplify the viral sequences which were then cloned and sequenced to confirm the correct arrangement of promoter, virus sequence and terminator.

Attempts to transform Senna by the co-infection approach have commenced.

4. Progress against milestones

Milestone	Completed Yes	No
Objective 1		
Year 1: 1.1 Produce the three constructs containing the TuMV sequences and incorporate the *gfp* and *gus* into the virulence plasmids	□	-
1.2 Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with the above constructs	in progress	-
3.1 Produce the two constructs containing the CaMV sequences	□	-

Objective 1: To transform rapid cycling *B. oleracea* plants with constructs containing 3 different sequences from TuMV

Introduction

The aim of the project is to induce resistance to TuMV (and CaMV) through 'gene-silencing' thereby avoiding the risk of viral transencapsidation that accompanies the alternative type of transgenic resistance ('coat-protein induced' resistance). In 'gene-silencing' the sequences used are not expressed in the transformed plant; this somewhat simplifies the construct production but does require some modification of the sequences to ensure that protein expression is entirely eliminated.

Virus construct production (S Muthumeenakshi, DJ Barbara, JA Walsh)

'Meena' Muthumeenakshi was appointed at the beginning of October 1998 to carry out this component of the project. The genomic sequences of UK 2 (the chosen TuMV isolate) and UK 4 (the chosen CaMV isolate) are not known, so the initial task was to identify PCR primers that would efficiently amplify the required regions from these viruses. This has been achieved. These primers have been modified to incorporate the necessary extra features into the PCR product (*e.g.* stop signals to prevent any translation and the restriction sites required for efficient cloning). Examination of the genome sequences of related isolates of TuMV showed that the presence of certain restriction endonuclease sites would make direct cloning difficult. As a result it was decided to alter the multiple cloning site (MCS) present in pSCV1 (the vector to be used in the transformation) to simplify the later steps in the construction process. pSCV1 has now been modified to contain several convenient restriction sites to form the derivative plasmid, pMSCV1. A *gus* gene with flanking pea plastocyanin promoter (PPCP) and *nos* terminator was taken from pWP364 and inserted into pMSCV1 to give the construct pMSCV2.

The regions of the TuMV (isolate UK 2) and CaMV (isolate UK 4) genomes that had previously been identified as potentially suitable were amplified by PCR. This was done using primers designed to incorporate multiple stop signals at the beginning of the sequence (to prevent any translation in the plants) and the restriction sites required for cloning. The *gus* gene in pMSCV2 was then replaced in turn with each of these five amplified TuMV and CaMV viral sequences resulting in a series of constructs labelled pMSCV3/Tcp, pMSCV3/Tnibcp, pMSCV3/Tcputr, pMSCV3/Ca19S and pMSCV3/Cacp. After further consideration, a fourth TuMV construct (pMSCV3/T5') originating from the 5' end of the virus was produced and may be incorporated into the project. Eliminating the gus gene and closing the blunt-ended linearised plasmid produced a construct (pMSCV3/control) to be used as negative control in transformation experiments.

Parts of all seven pMSCV3 constructs were sequenced to check that the arrangement of promoter, viral sequence (where present) and the terminator was as

expected. These constructs have been transformed into *Agrobacterium rhizogenes* by electroporation.

Construction of intermediate vector with reporter genes, *gus* and *gfp* (S Muthumeenakshi, DJ Barbara, IJ Puddephat)

The incorporation of *gus* and *gfp* genes into the virulence plasmid is intended to allow the easier selection of plant transformants. This incorporation was to be achieved using an intermediate vector unable to replicate in A*grobacterium* but which can cointegrate into the resident Ri (virulent) plasmid. The plasmid pMARCEL35, modified to contain the marker genes under the control of the cauliflower mosaic virus promoter and *nos* terminator, was to be used for this. However, pMARCEL35 was found to be unavailable and it has been necessary to construct a substitute plasmid with generally similar properties. The opportunity was taken to make alterations likely to make it more suitable for the project's aims and for more general use than the original would have been. This construction of a pMARCEL35 substitute has been a major task in its own right.

A two-kilobase segment from the virulence plasmid in *Agrobacterium rhizogenes* LBA9402 was amplified by PCR and cloned into pBR322, disrupting the ampicillin resistance gene, to produce pME36. A multiple cloning site was then introduced in pME36 by inserting a short linker forming pME36/linker.

Gus and *gfp* expression cassettes (CaMV35S/*gus*/*nos* and CaMV35S/*gfp*/*nos*) were derived from plasmids pMGI and pBIN/m-gfp5-ER respectively. These cassettes were assembled into pME36/linker to give pMBRE36/gus and pMBRE36/gfp. These intermediate vectors are now being analysed to confirm that their sequences are as expected. They are also being moved into *Agrobacterium rhizogenes.* When completed, test plant transformations will be used to check that the reporter genes are still functional.

Incorporation of *gfp* and *gus* into virulence plasmids (DJ Barbara, IJ Puddephat, S Muthumeenakshi)

For the *gus* and *gfp* genes, two versions each of an intermediate vector (pMBRE36) have been produced, GFP2, GFP5, GUS7 and GUS10. Triparental matings have been conducted using two strategies to incorporate the intermediate vector into the virulence plasmid (pRi1855) of *A. rhizogenes* strain LBA9402 *via* the *Eco*RI 36 region of homology. Triparental matings were conducted with the helper plasmid pRK2013 and separately with the helper plasmids pGJ28 and pR64drdIII. Both triparental mating strategies successfully produced putative transconjugant colonies (see Table 1).

Table 1. Number of putative transconjugant LBA9402 colonies produced from two triparental mating strategies for the introduction of intermediate vector pMBRE36 into pRi1855.

Intermediate vector pMBRE36/	Helper plasmid(s)	
	pRK2013	pGJ28 + pR64drdIII
GFP2	7	0
GFP5	1	2
GUS7	11	1
GUS10	1	5

Colonies arising from putative transconjugates have under gone serial subculture on YMB selection media containing rifampicin (100 mg/l) to eliminate *E. coli* harbouring intermediate vector and helper plasmids and tetracycline (10 mg/l) to select for transconjugant strains where the intermediate vector has been incorporated into pRi1855. These putative transconjugates are currently being evaluated for their ability to induce transformed GUS or GFP positive roots in explants of the rapid cycling *B. oleracea* 'Senna' and the cauliflower cultivar 'Lateman'. The most effective strains will be chosen for the introduction of binary vectors carrying the constructs for TuMV and CaMV resistance.

Plant transformation (IJ Puddephat, HT Robinson, LC Griffiths)

Preparation for the transformation work has commenced in advance of the May start date. Seed stock of the target rapid cycling genotype was bulked up last summer and stock plants for selfing this year are being raised. We appointed Lesley Griffiths to the band eight transformation post on the 1st March, she has been trained in the techniques required for completion of the transformation work that started at the end of May, ahead of the availability of the transconjugant LBA9402 strains.

As part of our BBSRC programme on plant transformation, we are studying an alternative strategy, which may be applied in the LINK project. Experimental material has been established to investigate the transformation efficiencies obtained using two infecting strains of *Agrobacterium*, in a process known as co-infection. Co-infection provides a means of physically separating marker genes from genes of interest and provides an alternative means of producing marker-free plants without the need for modification of the virulence plasmid of the *A. rhizogenes* strain. Co-infections have been conducted using *A. rhizogenes* strain LBA9402 harbouring a plasmid carrying a *gus* reporter gene as the gene of interest and with disarmed strains of *A. tumefaciens*, C58C1 MP90 and LBA4404 harbouring a plasmid carrying the *gfp* gene which is used for selection of transgenic roots. These experiments have established that the co-infecting strain has a significant bearing on the number of transformation events recovered, LBA4404 was more effective than C58C1 MP90.

There is a decrease in transformation efficiency but roots selected as GFP-positive all carried the *gus* reporter indicating that the strategy was effective. We are using different co-infection systems to determine whether the system can be optimised for production of co-transformed roots with integration of T-DNAs at unlinked sites to favour later segregation of genes-of-interest from marker sequences.

Introduction of the binary vector pMESCV3 carrying viral resistance constructs into LBA9402. Binary vectors harbouring the three TuMV and two CaMV contructs have been introduced into LBA9402 by electroporation. These strains are being used in co-infection transformations with LBA4404 harbouring a binary vector carrying the *gfp* gene to introduce constructs into the rapid cycling genotype Senna.

Since our previous report we have conducted a series of experiments to establish the effectiveness of the co-infection approach. Explants have been inoculated with two *Agrobacterium* strains (LBA4404 and LBA9402), one carries a *gfp* gene as the screenable marker and the other a *gus* gene to represent the gene of interest. Reciprocal experiments have been conducted with each strain carrying each reporter gene (Table 2). In a further series of experiments, explants were initally inoculated with an *A. tumefaciens* strain carrying either a *gfp* or a *gus* gene and then after 0, 1, 2 and 5 days, with an *A. rhizogenes* strain carrying either the *gus* gene if the *A. tumefaciens* strain carried *gfp*, or a *gfp* gene if the *A. tumefaciens* strain carried *gus* (Table 3).

Table 2. Co-infection of 'Senna' with (a) LBA9402 pRD400/GI and LBA4404 pGFP, (b) LBA9402 pGFP and LBA4404 pRD400/GI and (c) co-infection of 'White Rock' with LBA9402 pRD400/GI and LBA4404 pGFP. Roots were selected for GFP expression and then screened for GUS expression. GUS expression has been used to represent the viral construct in these experiments.

(a)

Treatment Ratio of LBA9402:LBA4404	No. of explants with GFP-expressing roots	No. of explants with GUS-expressing roots	No. of explants with GUS and GFP expressing roots	Total no. of explants
0:1 (LBA4404 alone)	14	0	0	123
1:1	7	7	0	131
10:1	3	7	1	124
1:10	12	4	0	126
Total	36	18	1	504

(b)

Treatment Ratio of LBA9402:LBA4404	No. of explants with GFP-expressing roots	No. of explants with GUS-expressing roots	No. of explants with GUS and GFP expressing roots	Total no. of explants
0:1 (LBA4404 alone)	2	11	0	130
1:1	11	3	0	133
10:1	16	2	0	133
1:10	12	0	0	137
Total	41	16	0	533

(c)

Treatment Ratio of LBA9402:LBA4404	No. of explants with GFP-expressing roots	No. of explants with GUS-expressing roots	No. of explants with GUS and GFP expressing roots	Total no. of explants
0:1 (LBA4404 alone)	-	-	-	-
1:1	**5**	**17**	**5**	24
10:1	3	19	1	24
1:10	-	-	-	-
Total	8	36	6	48

Table 3. Inoculation of 'Senna' explants with (a) LBA4404 pGFP followed by LBA9402 pRD400/GI and (b) LBA4404 pRD400/GI followed by LBA9402 pGFP at three time intervals, 0, 1, 2 and 3 days.

(a)

Time to inoculation with LBA9402 after initial inoculation with LBA4404 (days)	No. of explants with GFP-expressing roots	No. of explants with GUS-expressing roots	No. of explants with GUS and GFP expressing roots	Total no. of explants
0	18	1	0	140
1	13	6	2	135
2	15	1	0	138
5	5	0	0	137
Total	50	8	2	550

(b)

Time to inoculation with LBA9402 after initial inoculation with LBA4404 (days)	No. of explants with GFP-expressing roots	No. of explants with GUS-expressing roots	No. of explants with GUS and GFP expressing roots	Total no. of explants
0	11	10	0	119
1	20	9	4	143
2	22	4	1	143
5	15	3	1	133
Total	68	26	6	538

Preliminary experiments with the califlower cultivar White Rock which is easily transformed, produced promising results (Table 2(c)). From the 48 inoculated explants, six were detected that produced both GFP- and GUS-expressing roots (Table 2(c)). The results also suggest that an equal ratio of the two infecting strains produce better responses in terms of the number of explants with GFP- and GUS-expression.

We attempted to extend this approach to Senna in order to develop a workable transformation procedure whilst development of the transconjugant strains was progressing. For experiments with Senna we studied a number of co-transformation strategies (see Tables 2 and 3). Results with Senna have not been as successful as the preliminary work with White Rock. Data collected to date is not conclusive, a number of transgenic root lines have been isolated and we will produce molecular data by PCR to confirm the presence of *gfp* and *gus* reporter genes before reaching further conclusions.

For introduction of viral constructs into Senna we are using the most efficient approach identified from our preliminary experiments with White Rock. This will be modified once molecular data from current work with Senna is available, if the results indicate that is appropriate.