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

Tipburn and cigar burn can cause major losses in UK storage cabbage. There are currently no effective control measures.

Genetic transformation of commercial brassica varieties conferring resistance to the causal viruses could provide a non-chemical control measure.

Resistance genes were inserted into brassicas during the project, however, insufficient material was obtained to take forward for virus testing.

The transformed plants contained too many copies of the resistance genes to be of commercial interest to the seed house involved with this project. For these reasons the project was terminated at a pre-determined decision point within the agreement.

Action points for growers

There are no action points for growers resulting from this project.

Headline

A large amount of preparatory work on molecular tools to facilitate effective 'clean' transformation of brassicas without transferring antibiotic (or other) marker genes to plants has been carried out. Transformation of White Rock and one additional rapid cycling line using this approach has been achieved. During the project new rapid cycling lines that responded well to transformation and microspore culture were identified and hence deployed. Four turnip mosaic virus (TuMV) and two cauliflower mosaic (CaMV) constructs were used in transformation work on brassicas. Transgenic plants of one of the rapid cycling lines have been produced for one CaMV constructs and microspore cultured to produce double haploid lines. Transgenic White Rock plants have also been produced for two TuMV constructs and one CaMV construct. The project has provided data on the frequency of insertions of transgenes in brassicas and on the competence of different brassica lines for transformation using *Agrobacterium rhizogenes*. As so few transgenic lines with single transgene insertion events were produced (the commercial partners required single insertion events in order to facilitate subsequent breeding), no virus resistance testing of transformed lines was carried out and hence no transgenic (pathogen-derived) resistance to TuMV or CaMV has been identified. A number of transgenic lines (12) containing viral sequences are available to the commercial partners and may be available to others subject to agreement by the consortium.

Commercial benefits of the project

This project was aimed at developing new, non-chemical control measures for TuMV and CaMV in brassicas. This was to be achieved through 'pathogen-derived resistance' where integrating (transforming) part of the genome of TuMV and CaMV into *Brassica oleracea* would have provided resistance to these viruses. Such resistance would have reduced losses to growers and reduced dependence on and hence costs of pesticides.

Background and expected deliverables

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. The HortLINK project 15 (FV 160a) has recently demonstrated the involvement of TuMV in the internal disorder of white cabbage known by growers as 'cigar burn'. This HortLINK project (Hort 32; FV 214) aimed to produce genetically improved *B. oleracea* plants by transgenic means that are resistant to TuMV and CaMV. Nickerson - Zwaan would have subsequently incorporated the resistance into their breeding programmes to produce virus resistant brassica cultivars.

The strategy pursued to produce transgenic resistance is known as 'pathogen-derived resistance'. This involves incorporating regions of the viruses' genes in to the genes of plants. This then interferes with the replication of any of these viruses that attempt to infect the transgenic plants. The approach taken was that of 'gene-silencing'. This approach avoids the risk of viral transencapsidation that accompanies the most commonly used 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.

The commercial objective was to produce transgenic rapid cycling *B. oleracea* plants with resistance to both TuMV and CaMV which could then have been incorporated in to a range of cultivated brassica types by the commercial partners. As so few transgenic lines with single transgene insertion events were produced (the commercial partners required single insertion events in order to facilitate subsequent breeding), no virus resistance testing of transformed lines was carried out and hence no transgenic (pathogen-derived) resistance to TuMV or CaMV has been identified. A number of transgenic lines containing viral sequences are available to the commercial partners and may be available to others subject to agreement by the consortium.

Summary of the project and main conclusions

The marker genes *gus* and *gfp* (GFP = green fluorescent protein) necessary for identifying transformed plants were incorporated into the bacterium used for transforming plants (*Agrobacterium rhizogenes*). These markers allowed us to achieve two objectives, firstly the identification of plants containing the genes that were intended to confer resistance to the viruses. Subsequently the markers were used to eliminate unwanted DNA sequences (genes) from the *A. rhizogenes* that had been necessary during the transformation process. The only additional gene in the resultant brassica plants were those that it was hoped would confer viral resistance. The strains of *A. rhizogenes* possessing these marker genes and most effective in transforming the rapid cycling *B. oleracea* line Senna, cauliflower cv. White Rock and three additional rapid cycling breeding lines were identified.

Molecular tools that efficiently copied regions of the TuMV isolate UK 2 and the CaMV isolate UK 4, were produced. They were also modified to incorporate extra sequences necessary for their correct utilisation. These were used to copy the viral sequences that were then cloned and checked to confirm the correct arrangement of the regulatory sequences necessary for their expression and the viral sequences themselves. There were 4 TuMV clones and 2 CaMV clones.

Transformation of White Rock and one additional rapid cycling line by the co-infection approach has been achieved. Due to the identification of new rapid cycling lines that responded well to transformation and microspore culture, these rapid cycling lines were also deployed. Seedling explants were inoculated with *Agrobacterium* carrying the four TuMV and two CaMV sequences. Transgenic plants of one of the rapid cycling lines have also been produced for one CaMV construct and microspore cultured to produce double haploid lines. Transgenic White Rock plants have also been produced for two TuMV constructs and the one CaMV constructs. As so few transgenic lines with single transgene insertion events were produced (the commercial partners required single insertion events in order to facilitate subsequent breeding), no virus resistance testing of transformed lines was carried out and hence no transgenic (pathogen-derived) resistance to TuMV or CaMV has been identified. The transgenic lines (12) containing viral sequences are available to the commercial partners and may be available to others subject to agreement by the consortium.

Milestones

Milestone		Completed	
		Yes	No
Objective 1			
1.1	October 1999: produce the three constructs containing the TuMV sequences and incorporate the *gfp* and *gus* into the virulence plasmid pRiA4 or pRi1855.	completed	
1.2	Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with the above constructs.	transformants produced for two TuMV constructs in White Rock	
1.3	Regenerate five shoots from root clones produced for each TuMV construct.	completed	
1.4	Test To plants from each line for resistance to the UK 1 isolate of TuMV.	available lines tested	
1.5	Produce homozygous marker-free T_1 plants by microspore culture from the above transformed lines.	no marker-free plants produced	
1.6	Produce hemizygous and homozygous T_2 seed populations.	T_2 seed populations not produced.	
Objective 3			
3.1	October 1999: produce the two constructs containing the CaMV sequences.	completed.	
3.2	Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with the above constructs.	transformants produced for White Rock and alternative rapid cycling line for one construct.	
3.3	Regenerate five shoots from root clones produced for each CaMV construct.	completed.	
3.4	Test T_0 plants from each line for resistance to the UK isolate of CaMV.	plants not available for testing.	

3.5	Produce homozygous marker-free T_1 plants by microspore culture from the above transformed lines.	homozygous plants produced for construct CaMV2.
3.6	Produce hemizygous and homozygous T_2 seed populations.	T_2 seed populations not produced.

Science Section

i) Introduction

The problem. Turnip mosaic virus (TuMV) and cauliflower mosaic virus (CaMV) are major problems for brassica producers in the U.K. There are currently no effective control measures; no effective 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 aimed to produce virus resistance in transgenic *B. oleracea* plants, which Nickerson - Zwaan would subsequently incorporate into their breeding programmes to produce virus resistant brassica cultivars.

Both viruses have been reported causing serious losses in cauliflower (*B. oleracea*; Pink & Walkey, 1988), Brussels sprout (*B. oleracea*; Tomlinson & Ward, 1981), cabbage (*B. oleracea*; Walkey & Webb, 1978), swede (*B. napus*; Tomlinson & Ward, 1982) and oilseed rape (*B. napus*; Hardwick *et al.*, 1994). In recent years HRI have been involved in diagnosing TuMV and CaMV infections in a number of different brassica crops in the U.K. and have seen examples of total crop loss. Swede crops have been ploughed in due to the massive loss in yield and poor quality of roots. Serious reductions in yield and quality of cauliflowers, which led to all harvested heads being downgraded as only suitable for freezing, and yield reduction and cosmetic damage to Brussels sprout, which made the majority of the crop unmarketable have also been seen. Both viruses have been implicated in internal disorders causing storage problems in Dutch white cabbage in recent years; a conservative estimate of losses caused by these disorders in white cabbage has put these at £2.1 million per crop year.

TuMV naturally infects all horticultural and arable *Brassica* crops as well as edible horticultural non-brassica crops (artichoke, peas, watercress, rhubarb, chicory, radish, courgettes, onion and lettuce), ornamentals (*Abutilon*, stocks and wall flowers) and weed plants belonging to 14 different families. It occurs worldwide, and in certain regions including Canada (Stobbs *et al*., 1991), China (Liu *et al*., 1996), Taiwan (Yoon *et al*., 1993), Korea (Choi *et al.,* 1992), Japan (Sako, 1981) and the U.K. (Hardwick *et al*., 1994) where horticultural and arable brassica crops are grown all year round, it is particularly damaging. In an extensive survey of economically important field vegetable viruses present in 28 countries (Tomlinson, 1987), it was found to be one of the two most important viruses.

CaMV is the type member of the *Caulimovirus* group. It has a much more restricted host range than TuMV and is limited almost exclusively to members of the *Cruciferae*. It naturally infects all the horticultural and arable *Brassica* crops. It occurs worldwide but has not been reported causing major losses in Asia or North America. However, in a number of countries including the U.K., Ireland, Italy, Poland and New Zealand it is considered to be one of the most important viruses affecting field vegetables (Tomlinson, 1987).

There are currently no effective means of controlling either TuMV or CaMV in horticultural brassica crops. TuMV is spread and transmitted in the non-persistent manner by 40-50 aphid species (Edwardson & Christie, 1986) whereas CaMV is

spread and transmitted in the semi-persistent manner. Attempts to control the vector and TuMV spread, by insecticidal sprays have proven ineffective (Evans & MacNeil, 1983; Niu *et al*., 1983). Despite this, growers still spray brassica crops in an attempt to control both viruses and effective, environmentally friendly control measures are still required. The advent of pathogen-derived transgenic resistance has provided the opportunity to confer novel resistance to *B. oleracea* types to protect them against TuMV and CaMV.

Transformation of *B. oleracea* has been achieved at HRI Wellesbourne (Riggs *et al*., 1996; Puddephat *et al*., 2001) and transgenic resistance to TuMV has been demonstrated for brassicas (Lehmann *et al*., 1996), tobacco (Lam *et al*., 1996) and *Nicotiana benthamiana* (Jan *et al*., 1999).

ii) Results and Discussion

Milestones 1.1 and 3.1. Produce the three constructs containing the TuMV sequences and the two constructs containing the CaMV sequences.

Revised milestone date: October 1999

The genomic sequences of UK 2 (the chosen TuMV isolate) and UK 4 (the chosen CaMV isolate) were not known, so PCR primers that efficiently amplify the required regions from these viruses were designed. The primers were 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 resulting in 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 was incorporated into the project. Eliminating the *gus* gene and closing the blunt-ended linearised plasmid produced a construct (pMSCV3/control) to be used as the 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. The constructs were transformed into *Agrobacterium rhizogenes* by electroporation.

Milestones 1.2 and 3.2 Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with three TuMV constructs and two CaMV constructs.

Revised milestone date: July 2000

Construction of intermediate vector with reporter genes, *gus* and *gfp*: The incorporation of *gus* and *gfp* genes into the virulence plasmid was to facilitate 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 was 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 were analysed and their sequences determined confirming that they were as expected. They are also moved into *Agrobacterium rhizogenes* and test plant transformations confirmed the reporter genes were still functional.

Incorporation of *gfp* and *gus* into virulence plasmids: 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 underwent 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 were 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 were chosen for the introduction of binary vectors carrying the constructs for TuMV and CaMV resistance.

Plant transformation**:** Seed stock of the target rapid cycling genotype was bulked up and stock plants for selfing were raised. As part of HRI's BBSRC programme on plant transformation, an alternative transformation strategy was studied. Experimental material was 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 were 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 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 was a decrease in transformation efficiency but roots selected as GFP-positive all carried the *gus* reporter indicating that the strategy was effective. Different co-infection systems were used to determine whether the system could 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 four TuMV and two CaMV constructs were been introduced into LBA9402 by electroporation. These strains were used in co-infection transformations with LBA4404 harbouring a binary vector carrying the *gfp* gene to introduce constructs into the rapid cycling genotype Senna. A series of experiments to establish the effectiveness of the co-infection approach were carried out. Explants were 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 were conducted with each strain carrying each reporter gene (Table 2). In a further series of experiments, explants were initially 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 in place of the viral construct in these preliminary 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 cauliflower cultivar White Rock that 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 a number of co-transformation strategies were studied (see Tables 2 and 3). Results with Senna were not as successful as the preliminary work with White Rock. Whilst the data was not conclusive, a number of transgenic root lines were isolated .

For transformation of viral constructs into Senna the most efficient approach identified from our preliminary experiments with White Rock was used.

Working with the plant line Senna and alternative target plant genotypes with superior transformation characteristics a series of transformations were carried out in which engineered strains of *A. rhizogenes* were used to produce transgenic roots for each of the five viral constructs. Sufficient root clones that were thought to be transgenic at the time were produced (Table 4). The transgenic roots identified were isolated and established as proliferating root clones for PCR analysis and subsequent use in shoot regeneration. Direct PCR on each root clone selected by GFP fluorescence was used to identify transgenic roots that were predicted to also contain the viral construct. The total number of GFP-positive roots in which the viral constructs were detected was 416 (Table 4).

Table 4. Production of GFP-fluorescent root clones and root clones transformed with viral constructs. Inoculations were made to explants of three genotypes of *B. oleracea*.

Viral construct	Inoculated explants (no.)	Explants with GFP-positive roots		GFP-positive roots produced (no.)	No. of transgenic root clones produced possessing viral construct
		(no.)	(%)		
TuMV1	222	50	22.5	94	42
TuMV2	351	135	37.8	221	92
TuMV3	193	77	40	141	66
TuMV4	219	62	28.3	105	76
CaMV1	345	123	35.7	201	59
CaMV2	345	140	40.6	232	81
Total	**1675**	**587**	**34.2**	**994**	**416**

In excess of the required 20 root clones for each viral construct (mean no. of root clones per construct is 69.3) was produced and this allowed specific clones to be selected for ease of maintenance in culture prior to shoot regeneration.

Milestones 1.3 and 3.3. Regenerate shoots from root clones for each TuMV and CaMV construct.

Revised milestone date: June 2001

For each construct, we selected a sub-set of GFP-positive root clones, on the basis of ease of maintenance in culture and genotypic background (see Table 5).

Table 5. Regeneration of established transgenic root clones identified by direct PCR to contain TuMV or CaMV constructs (but see later for comments on PCR).

Viral Construct	No. of Transgenic root clones produced	No. of Transgenic root clones regenerated	No. of Transgenic root clones producing T_0 Plants	Efficiency of T_0 Plant production %
TuMV1	42	17	4	23.5
TuMV2	92	39	10	25.6
TuMV3	66	24	20	83.3
TuMV4	76	33	22	66.7
CaMV1	59	26	4	15.4
CaMV2	81	34	15	44.1
Total	416	173	75	43.4

The target was to attempt regeneration from a minimum of 20 root clones for each construct. As root clones often deteriorate rapidly in quality, it was not always possible to take through this number. TuMV1 illustrated this point. Also due to the lengthy process of regeneration it was not possible to keep bringing further transformed root clones into the system, even if they were available. Consequently, the final number of root clones producing established T_0 plants was lower than the 10 anticipated for some constructs (TuMV1 and CaMV1).

The number of root clones that produced developed shoots that acclimatised in the glasshouse to produce transgenic (T_0) plants was variable. This efficiency was construct dependent. Table 5 illustrates this efficiency as a percentage of selected root clones that resulted in T_0 plants. The efficiency ranged from 15.4% to 83.3%.

Milestones 1.4 and 3.4. Testing T_0 plants for resistance to TuMV UK 2 and CaMV UK 4.

Revised milestone date: October 2001

T_0 plants were acclimatised to glasshouse conditions. Plants of three T_0 lines were tested for resistance to the UK 2 isolate of TuMV from which the viral sequences used to transform the plants were derived. One of the lines contained the viral construct B (~900nt of the TuMV coat protein region without start codon and 'out of frame') and two contained construct D (~500nt of the TuMV nuclear inclusion protein b + ~500nt of the coat protein without start codon and 'out of frame').

Control non-transformed and transformed Senna plants at the 3-7 true-leaf stage were mechanically inoculated (all leaves) with the UK 2 isolate of TuMV (1g infected leaf / 2 ml of inoculation buffer). Plants were visually assessed at regular intervals and tested by enzyme-linked immunosorbent assay (ELISA) four weeks after inoculation to determine the presence or absence of TuMV and to quantify any virus present.

The plant lines thought to be transformed with the ~900nt of the TuMV coat protein without start codon (construct B) and those transformed with the ~500nt of the TuMV nuclear inclusion protein b (NIb) + ~500nt of the coat protein without start codon (construct D) were all susceptible to the UK 2 isolate of TuMV. Virus was detected by ELISA in the youngest uninoculated leaves of all plants (Tables 6-8), demonstrating that the virus was moving systemically in the plants. Clear virus symptoms were seen in the leaves of most transgenic and control plants. Mean levels of TuMV in the inoculated and uninoculated leaves of plants transformed with construct B (as detected by ELISA) were lower than those in non-transgenic control Senna plants (Table 6). This may have been due to the physiological age of the transformed plants derived from tissue culture, relative to the young control plants grown from seed. Mean levels of TuMV in the inoculated and uninoculated leaves of plants transformed with construct D (also as detected by ELISA) suggested the transgenic plants were as susceptible as the control non-transgenic plants (Tables 7 and 8).

Table 6. Detection of turnip mosaic virus (TuMV) in Senna plants transformed with construct B (TuMV coat protein region without start codon and 'out of frame').

Plant no.		**Inoculated leaf** [a]		**Uninoculated leaf** [b]	
Control	1[c]	1.22[e]	1.59	1.24	1.04
	2	1.33	1.53	0.19	0.20
	3	0.67	0.81	0.06	0.04
	4	0.56	0.61	0.25	0.44
	5	0.81	0.95	-0.02	-0.02
	6	1.46	1.31	-0.01	-0.04
Mean absorbance		**1.07**		**0.28**	
Transformed	7[d]	0.01	0.04	0.22	0.19
	8	0.14	0.11	0.00	-0.01
	9	0.05	0.06	0.02	0.00
	10	0.13	0.28	0.04	0.01
	11	0.07	0.09	0.27	0.31
	12	0.00	0.08	0.05	0.07
Mean absorbance		**0.09**		**0.09**	

[a] Inoculated leaf tested was the last (youngest) inoculated leaf.
[b] Uninoculated leaf tested was the last (youngest) uninoculated leaf.
[c] Plants 1-6 are control Senna plants grown from seed.
[d] Plants 7-12 are transgenic T_0 Senna plants.
[e] There are 2 ELISA plate wells for each sample, hence the 2 figures for each sample.

Table 7. Detection of turnip mosaic virus (TuMV) in Senna plants transformed with construct D (~500nt of the TuMV nuclear inclusion protein b + ~500nt of the coat protein without start codon and 'out of frame').

Plant no.		A_{405}					
		Inoculated leaf [a]		Uninoculated leaf [b]		Last leaf [c]	
Control	1[d]	-		1.61	1.53	0.92	0.85
	2	1.04	0.98[g]	1.05	0.86	2.28	2.29
	3	-		0.02	0.03	0.34	0.27
	4	1.37	1.43	1.19	1.24	1.59	1.77
	Mean	**1.21**		**0.94**		**1.29**	
	5[e]	-		0.02	0.03	-	
Transgenic	6[f]	0.31	0.27	0.01	0.01	0.97	0.85
	7	0.27	0.29	0.85	0.86	0.39	0.39
	8	1.08	1.28	0.10	0.17	3.00	3.00
	9	0.24	0.23	0.30	0.28	2.66	3.00
	Mean	**0.50**		**0.32**		**1.78**	
	10[e]	-		0.02	0.02	-	

[a] Inoculated leaf tested was the last (youngest) inoculated leaf.
[b] Uninoculated leaf tested was the first (oldest) uninoculated leaf.
[c] Last leaf was the youngest leaf on the plant.
[d] Plants 1-5 are control Senna plants grown from seed.
[e] Plants 5 and 10 were not inoculated.
[f] Plants 6-10 are transgenic T_0 Senna plants.
[g] There are 2 ELISA plate wells for each sample, hence the 2 figures for each sample.

Table 8. Detection of turnip mosaic virus (TuMV) in Senna plants transformed with construct D (~500nt of the TuMV nuclear inclusion protein b + ~500nt of the coat protein without start codon and 'out of frame').

Plant line		Inoculated leaf [a]	Uninoculated leaf [b]
Transgenic	1[c]	0.42	0.23
	2	0.27	0.33
	3	0.09	0.37
	4	0.12	0.31
	5	0.24	0.21
Control	7[d]	0.19	0.30

[a] Inoculated leaf tested was the first (oldest) inoculated leaf.
[b] Uninoculated leaf tested was the last (youngest) uninoculated leaf.
[c] Lines 1-5 are transgenic T_0 Senna plants
[d] Line 7 is control Senna grown from seed.

No resistance to TuMV was found in any of the Senna plant lines thought at the time to be transformed. The timing of these tests was dictated by the availability of the plants and hence were carried out before it was discovered that none of the Senna lines were transgenic.

Milestones 1.5 and 3.5. Production of homozygous T_1 plants.

Revised milestone date: *December 2001*

Two strategies were deployed to produce homozygous seed of transgenic lines.

The first strategy was via a series of selfing and sowing. This strategy is ideal for rapid cycling lines such as Senna and was carried out for all established Senna lines (4 lines for TuMV2 and 13 lines for TuMV4 (Table 9). For each line, 30 seeds were sown to produce T_1 plants. These plants were screened, by PCR, for presence of the construct and absence of GFP/Rol. Each T_1 plant with these criteria was selfed. The T_2 seed derived from these individual plants would then have been sown to create T_2 populations, depending on their copy number (i.e. only low copy numbers). These populations would have been screened for construct presence by PCR. Those populations in which all individuals contained the transgene (i.e. not segregating for transgene presence) would have been derived from homozygous parents.

The second strategy was microspore culture of flower buds. Due to the long growth cycle of White Rock cauliflower lines (12 months), this was the preferred route taken for such plants. One TuMV1, two TuMV3 and four CaMV2 lines were microspored on several occasions. Various bud sizes (2.5-2.99 mm, 3-3.99 mm, 4-5 mm) were tested. Despite much effort there was limited success. The CaMV2 White Rock line 12 eventually produced one embryo which was acclimatised to the glasshouse. Further to this the only successfully acclimatised AG line also had a positive response, producing 12 putative diploid plants. Both of these microspore lines will be tested for the presence of the CaMV2 construct and GFP/Rol. Only Construct positive and GFP/Rol negative individuals will be retained. The poor response of White Rock to microspore meant that this unfortunately was not a viable technique and further attempts were abandoned. For various reasons including ploidy, plants positive for GFP and plants being negative for the viral construct, all microspored plants were discarded. Thus there is no seed from these plants.

Table 9. Progress with transformed plants at October 2002

Viral Construct	Transgenic Line[2]	Copy Number[1]	T_1 Seed obtained	T_2 Seed Constructs that were PCR +ve GFP -ve
TuMV1	WR 8	12	■	
TuMV2	SE 1	0	■	3
	SE 2	0	■	
	SE 4	0	■	16
	SE 5	0	■	8
TuMV3	WR 22	3	■	
	WR 27	8	■	
	WR 32	-	■	
	WR 35	2	■	
	WR 37	1	■	
	WR 43	5	■	
TuMV4	SE 2	0	■	1
	SE 8	0	■	5
	SE 11	0	■	11
	SE 12	0	■	11
	SE 14	0	■	9
	SE 17	0	■	7
	SE 24	0	■	15
	SE 25	0	■	11
	SE 27	0	■	14
	SE 28	0	■	
	SE 34	0	■	20
	SE 39	0	■	
	SE 43	0	■	28
CaMV2	WR 2	6	■	
	WR 5	3	■	
	WR 9	5	■	
	WR 12	6	■	
	AG 6	4	■	

[1]Copy number of the lines as determined by Southern analysis.
[2]WR = White Rock, SE = Senna, AG = AG 4199.

Analysis of transgene copy number in lines

Southern Blotting. Leaf material from T_0 generation plants produced within the project were processed for Southern blot analysis using standard procedures. DNA extraction was by commercial kit (Qiagen DNeasy), digestion with restriction endonuclease followed the enzyme manufacturer's procedures, blotting was by alkaline transfer to Hybond N+ membrane and probing used ^{32}P labelled probe. The probe used was a fragment of the pea plastocyanin promoter amplified from the original construct by PCR. This probe was used as this promoter was used for all six virus constructs and obviated the need to use separate probes for each construct.

White Rock. Twenty eight lines were tested and the numbers of plants in each category are shown below in Table 10.

Table 10. Transgene copy numbers for the different viral constructs in White Rock

No. of Transgenes	Construct					
	TuMV1	TuMV2	TuMV3	TuMV4	CaMV1	CaMV2
0	-	-	1	-	-	1
1	-	-	2 (1)[1]	(1)	-	-
2	-	-	2	-	-	(1)
3	-	-	2	(1)	-	1
4	-	-	-	(1)	-	-
5	-	-	2	-	-	1
6	-	-	2	-	1	4
7	1	-	-	-	-	-
8	-	-	1	(1)	-	-
12	1	-	-	-	-	-

[1]Dead plants indicated in brackets.

The overall distribution of copy number is in line with the current accumulated experience for *Agrobacterium rhizogenes* transformation based on Southern blot analysis for other projects (with 25% single or double inserts for this project compared with 21% overall) (Table 11).

Table 11. Transgene copy numbers in brassicas from this project and previous work

Copy Number	***1***	***2***	***3***	***4***	***5+***
This project (%)	14	11	18	7	50
Other projects (%)	8	13	17	16	46

Senna. Seventeen Senna lines from lines TuMV2 and TuMV4 were similarly tested and all gave no hybridisation *i.e.* they were apparently untransformed. This result appears robust as all the lines (Senna, White Rock and AGs) were tested at the same time and all membranes had at least some positives (ruling out technical faults at the Southern analysis stage). However, the result was also unexpected as all lines had been selected initially by PCR with the same primers.
Other lines. One plant each of AG4025 and AG4199 were also tested and showed 3 and 4 inserts respectively of the CaMV2 construct.

PCR analysis. All the lines tested above had been selected initially as root clones by PCR using the same primers as used to make the probe used in Southern analysis. To try and resolve the unexpected finding of no inserts in Senna lines, the DNA prepared by Qiagen DNeasy (i.e. that used for the Southerns) was also tested by PCR. There was complete agreement between the Southern results and these PCRs. All the Senna lines gave no amplicon whilst the White Rock (positive from Southern analysis) gave clear positive results in PCR tests.

Seed of the T_1 plants was sown to give a T_2 generation. DNA from this material prepared by the Qiagen method was tested by PCR, again using the pea plastocyanin promoters. All Senna material, be it transformed or untransformed, gave weak amplicons as did untransformed White Rock. The transformed White Rock positive controls gave much stronger signals. More detailed electrophoretic analysis showed that the weak bands from both Senna and untransformed White Rock are actually slightly smaller than the band from the positive controls, strongly suggesting that they do not originate from the same sequence (i.e. not from an inserted construct but some *Brassica* sequence with primers sites). PCR products have now been sequenced. This sequencing revealed that the DNA from the positive White Rock transformed plants that gave rise to the larger bands is clearly pea plastocyanin promoter. DNA from untransformed White Rock control plants and from Senna plants that had been thought to be transformed (which both gave rise to the slightly smaller band) is clearly not pea plastocyanin. A database search revealed that the DNA from the latter had no homology to the pea plastocyanin promoter.

The recent PCR tests and sequencing seem to show that no transformed Senna plants were produced, confirming the Southern blotting results. The initial selection of these plants by PCR appears to have been erroneous. The primers designed to amplify the pea plastocyanin promoter do amplify the target sequence but in addition, are amplifying from some related sequence in the *Brassica* plants (White Rock and Senna). However, why variable PCR results were obtained is not clear. It may be

related to age of the plants and/or the specificity of the primers due to imperfect homology between the primers and the region of the brassica genome they amplify.

iii) Conclusions

A large amount of preparatory work on molecular tools to facilitate effective 'clean' transformation of brassicas without transferring antibiotic (or other) marker genes to plants has been carried out. Transformation of White Rock and one additional rapid cycling line using this approach has been achieved. No transformation of the rapid cycling line Senna has been achieved. During the project new rapid cycling lines that responded well to transformation and microspore culture were discovered and hence deployed. Four turnip mosaic virus (TuMV) and two cauliflower mosaic (CaMV) sequences were used in transformation work on brassicas. Transgenic plants of one of the rapid cycling lines have been produced for one CaMV construct and microspore cultured to produce double haploid lines. This line appears to have four copies of the transgene. Transgenic White Rock plants have also been produced for two TuMV constructs and one CaMV construct. Transgene copy number for construct TuMV1 was 12 and for TuMV3 was, 1, 2 (2 lines), 3, 5, 8 and unknown. For CaMV2, it was 3, 5 and 6 (2lines). Surviving transformed White Rock plants with single (one line, WR37) or double (one line, WR35) copy inserts were limited to the one TuMV construct (TuMV3).

The project has provided data on the frequency of insertions of transgenes in brassicas and on the competence of different brassica lines for transformation using *Agrobacterium rhizogenes*. A number of transgenic lines (8) containing viral sequences are available to the commercial partners and may be available to others subject to agreement by the consortium. No transgenic (pathogen-derived) resistance to TuMV or CaMV has been identified.

Following discussion, the consortium agreed that the criteria for progression to the next phase of the project (resistance to TuMV and CaMV) had not been fulfilled and hence the second phase of the project would not take place. It was also agreed that due to the very slim chance of obtaining resistance from line WR 37, the requirement of the commercial partners for single transgene insertion – mediated resistance, the long multiplication time for White Rock (1 year / generation) and lack of funds in the project account, that the project should end.

Technology transfer

Due to the sensitive nature of this "G.M." project presentations and posters have not been made. The project has provided data on the frequency of insertions of transgenes in brassicas and on the competence of different brassica lines for transformation using *Agrobacterium rhizogenes*. For future work where commercially single transgene copy material was required, it would be necessary to have a system in place that identified single copy lines at an early stage i.e. in the primary T_0 transformants. A number of transgenic lines (8) containing viral sequences are available to the commercial partners Nickerson – Zwaan and may be available to others subject to agreement by the consortium.

References

Choi, C. W., Ryu, K. H., Choi, S. R., Park, W. M. & Yoon, K. E. (1992). Mixed infection of turnip mosaic virus and cucumber mosaic virus identified from vegetables in Daekwallyeong area. Korean Journal of Plant Pathology Newsletter **3**: 85-86.

Edwardson, J. R. & Christie, R. G. (1986). Turnip mosaic virus. p438 - 453. In: Viruses infecting forage legumes. Vol. II. Agr. Exp. Inst. Food. Agr. Sci. Monograph 14, University of Florida., Gainesville.

Evans, I. R. & MacNeil, B. H. (1983). Virus disease of Rutabagas (Turnips). Ontario Ministry of Agriculture and Food Factsheet 73-061.

Hardwick, N. V., Davies, J. M. L. & Wright, D. M. (1994). The incidence of three virus diseases of winter oilseed rape in England and Wales in the 1991/92 and 1992/93 growing seasons. Plant Pathology **43:** 1045-1049.

Jan, F.-J., Pang, S.-H., Fagoaga, C. & Gonsalves, D. (1999). Turnip mosaic virus resistance in *Nicotiana benthamiana* derived by post-transcriptional gene silencing. Transgenic Research **8:** 203-213.

Lam, Y.-H., Wong, Y.-S., Wang, B., Wong, R.N.-S., Yeung, H.-W. and Shaw, P.-C. (1996) Use of trichosanthin to reduce infection by turnip mosaic virus. Plant Science **114:** 111-117.

Lehmann, P., Walsh, J., Jenner, C., Kozubek, E. & Greenland, A. (1996). Genetically engineered protection against turnip mosiac virus infection in transgenic oilseed rape (*Brassica napus* var. *oleifera*). Journal of Applied Genetics **37A:** 118-121.

Liu, X. P., Lu, W. C., Liu, Y. K., Wei, S. Q., Xu, J. B., Liu, J. B., Liu, Z. R., Zhang, H. J., Li, J. L., Ke, G. L., Yao, W. Y., Cai, Y. S., Wu, F. Y., Cao, S. C., Li, Y. H., Xie, S. D., Lin, B. X. & Zhang, C. L. (1996). Occurrence and strain differentiation of turnip mosaic potyvirus and sources of resistance in Chinese cabbage in China. Acta Horticulturae **407:** 431-440.

Niu, X., Leung, H. & Williams, P. H. (1983). Sources and nature of resistance to downy mildew and turnip mosaic virus in Chinese cabbage. Journal of the American Society for Horticultural Science **108:** 775 - 778.

Pink, D. A. C. & Walkey, D. G. A. (1988). The reaction of summer- and autumn-maturing cauliflowers to infection by cauliflower mosaic and turnip mosaic viruses. Journal of Horticultural Science **63:** 95-102.

Puddephat I. J., Robinson H. T., Fenning T. M., Barbara D. J., Morton A. and Pink D. A. C. (2001) Recovery of phenotypically normal transgenic plants of *Brassica oleracea* following *Agrobacterium rhizogenes*-mediated co-transformation and selection of transformed hairy roots by GUS assay. Molecular Breeding **7:** 229-242.

Riggs, T., Puddephat, I., Robinson, H., Fenning, T. & Tymens, S. (1996). *Agrobacterium rhizogenes*-mediated transformation of *Brassica oleracea* L. HRI Annual Report 1994-95, 74.

Sako, N. (1981). Virus diseases of Chinese cabbage in Japan. In: Chinese cabbage. Proceedings of the First International Symposium. N. S. Talekar & T. D. Griggs Eds. AVRDC Shanhua, Taiwan. 489pp.

Stobbs, L. W., Shattuck, V. I. & Shelp, B. J. (1991). Effect of turnip mosaic virus infection on the development, virus titer, glucosinolate concentrations and storability of rutabaga roots. Plant Disease **75:** 575-579.

Tomlinson, J. A. (1987). Epidemiology and control of virus diseases of vegetables. Annals of Applied Biology **110:** 661-681.

Tomlinson, J. A. & Ward, C. M. (1981). The reactions of some Brussel sprout F_1 hybrids and inbreds to cauliflower mosaic and turnip mosaic viruses. Annals of Applied Biology **97:** 205-212.

Tomlinson, J. A. & Ward C. M. (1982). Selection for immunity in swede (*Brassica napus*) to infection by turnip mosaic virus. Annals of Applied Biology **101:** 43-50.

Walkey, D. G. A. & Webb, M. J. W. (1978). Internal necrosis in stored white cabbage caused by turnip mosaic virus. Annals of Applied Biology **89:** 435-441.

Yoon, J. Y., Green, S. K. & Opena, R. T. (1993). Inheritance of resistance to turnip mosaic virus in Chinese cabbage. Euphytica **69:** 103-108.