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

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Key words: *Brassica oleracea*, turnip mosaic virus (TuMV), cauliflower mosaic virus (CaMV), transgenic resistance, marker-free.

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This project is aimed at developing new, non-chemical control measures for TuMV and CaMV in brassicas. This is to be achieved through 'pathogen-derived resistance'.

Background and objectives

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'. HortLINK project (FV 214) aims to produce genetically improved *B. oleracea* plants by transgenic means that are resistant to TuMV and CaMV. Nickerson Zwaan will subsequently incorporate this resistance into their breeding programmes to produce virus resistant brassica cultivars.

The strategy we are pursuing 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 commercial objective is to produce transgenic rapid cycling *B. oleracea* plants with resistance to both TuMV and CaMV which can then be incorporated in to a range of cultivated brassica types by the commercial partners.

Summary of results and conclusions

The marker genes *gus* and *gfp* (GFP = green fluorescent protein) necessary for identifying transformed plants have been incorporated into the bacterium used for transforming plants (*Agrobacterium rhizogenes*). These markers will allow us to achieve two objectives. Firstly they will allow us to identify plants containing the genes that are intended to confer resistance to the viruses. Subsequently the markers will be used in the elimination of unwanted DNA sequences (genes) from the *A. rhizogenes* that were necessary during the transformation process. The only additional gene in the resultant brassica plants will be that conferring 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 have been identified.

Molecular tools that will efficiently copy regions of the TuMV isolate UK 2 and the CaMV isolate UK 4, have been produced. They have also been 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 sequence itself. There are 4 TuMV clones and 2 CaMV clones.

Transformation of Senna and three additional rapid cycling lines by the co-infection approach has been achieved. Due to difficulties in transforming Senna, the alternative rapid cycling lines have been deployed. Seedling explants have been inoculated with *Agrobacterium* carrying the four TuMV and two CaMV sequences. Transgenic root clones have been produced for the four TuMV constructs and the two CaMV constructs and plants are now being regenerated from these root clones.

Anticipated practical and financial benefits

A one percent increase in U.K. productivity from growing broccoli and cauliflower (value of these two crops in 1995 was £96 million; Anon., 1996) with pathogen-derived resistance to TuMV and CaMV (financial value of increase £0.96 million) would equate to almost three times the total costs of the project (£346,645) in a single year. Losses from TuMV and CaMV in severe years exceed 1%, consequently, savings are likely to be massive in relation to the costs of the project. Additional cost and environmental benefits will be derived from the reduction in insecticide usage. In 1991, over 69,000 kg of active ingredient of insecticide was used on nearly 190,000 ha of vegetable brassica production in the U.K. (Anon., 1992) - 98% of broccoli crops received insecticide treatment mainly for aphid control. The calculation below assumes a very modest reduction (10%) in insecticide spraying for aphids and recognises that growers will continue to spray against aphids, albeit less frequently, even when virus resistant cultivars are available. Increases in productivity (quality and yield) derived, particularly at the beginning and end of seasons would not only boost profits, but would also reduce imports; in 1995 an estimated 72,300 tonnes of cauliflower and broccoli were imported with a value of £48.2 million (Anon., 1996).

Projected benefits/annum:

From the reduction in losses from TuMV and CaMV[1]	>£960,000
From the reduction in pesticide usage[2]	>£112,486
From the reduction in imports[3]	~£480,000
Total benefit/annum	**>£1,552,486**
Total cost of project:	£346,645

[1] This conservative estimate assumes only a 1% increase in productivity of broccoli and cauliflower.
[2] Calculated from a modest 10% reduction in insecticide spraying for aphids (based on recorded usage on brassicas in 1991; Anon., 1992), and only includes cost of insecticides, savings on fuel, labour etc. would be over and above this estimate.
[3] Based on only half of the increased productivity replacing imports.

Science Section

Progress against milestones

Milestone	Completed Yes	No
Objective 1		
Year 2: 1.2 Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with the above constructs.	complete for all three TuMV constructs	
1.3 Regenerate five shoots from root clones produced for each TuMV construct.	in progress	
1.4 Test T_0 plants from each line for resistance to the UK 1 isolate of TuMV.	in progress	
1.5 Produce homozygous marker-free T_1 plants by microspore culture from the above transformed lines.	dependent on 1.3	
Objective 3		
3.2 Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with the above constructs.	complete for both CaMV constructs	
3.3 Regenerate five shoots from root clones produced for each CaMV construct.	in progress	
3.4 Test T_0 plants from each line for resistance to the UK isolate of CaMV.	T_0 lines with 2 constructs tested for resistance to UK 2 isolate of TuMV	
3.5 Produce homozygous marker-free T_1 plants by microspore culture from the above transformed lines.	dependent on 3.3	

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 aims to produce virus resistance in transgenic *B. oleracea* plants, which Nickerson Zwaan will 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. Such treatments kill beneficial insects including natural enemies and predators of aphids and, of course, introduce pesticide residues into the human and wildlife food chains. Effective, environmentally friendly control measures are urgently 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).

Results and Discussion

Milestones 1.2 and 3.2. Production of transgenic root clones of *B. oleracea* with four TuMV constructs and two CaMV constructs.
Revised milestone date: July 2000

Previously we reported that difficulties in transforming the original target genotype 'Senna' had delayed progress and restricted the number of transgenic roots identified. In the annual report for 2000 we indicated that alternative target plant genotypes with superior transformation characteristics had been identified in other research projects. These alternative plant genotypes have continued to be used. A series of transformations have taken place since September '00 in which engineered strains of *A. rhizogenes* have been used to produce transgenic roots for each of the five viral constructs. Transformation into these genotypes are now complete and sufficient root clones have been produced (Table 1).

We inoculated a further 551 explants of rapid cycling genotypes of *B. oleracea* using the engineered strain of *A. rhizogenes*. Transgenic root clones were produced for each of the six viral constructs although emphasis was given to the constructs TuMV2, CaMV1 and CaMV2 where root clone numbers had been lowest. The transgenic roots identified have been isolated and established as proliferating root clones for PCR analysis and subsequent use in shoot regeneration. The number of GFP-positive roots containing viral constructs, as identified via PCR analysis, has increased markedly as compared to those figures given in the March 2001 report: The total number of GFP-positive roots with viral construct now identified stands at 356, as compared to 170 in March (Table 1).

Table 1. Production of GFP-fluorescent root clones for viral constructs for resistance to TuMV and CaMV. 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.)	GFP-positive roots with viral construct (no.)
		(no.)	(%)		
TuMV1	222	50	22.5	94	43
TuMV2	351	135	37.8	221	97
TuMV3	193	77	40	141	71
TuMV4	219	62	28.3	105	37
CaMV1	345	123	35.7	201	59
CaMV2	345	140	40.6	232	86
Total	**1675**	**587**	**34.2**	**994**	**393**

We have now produced an excess of the required 20 root clones for each viral construct (mean no. of root clones per construct is 71.2) and this has allowed us to select specific clones 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 have selected a sub-set of GFP-positive roots, on the basis of ease of maintenance in culture and genotypic backgound (i.e. the brassica genotype into which the construct has been inserted) (see Table 1). For each construct, transformation events into rapid cycling lines, including Senna, and a cauliflower background have been selected. Shoot regeneration from established root clones is now well underway. Previously, we reported on the material from the first regeneration cultures having reached the stage of shoot initiation. This material produced well-developed shoots that have been excised from the parent material, and subsequently transgenic (T_0) plants were successfully acclimatised to the containment glasshouses. To date, T_0 plant production has been obtained from 29 (21%) of the root clones under regeneration. Additional transgenic plants will be obtained in due course. At the present time many of the cultures undergoing regeneration have developed to the point at which shoot initials are forming, this includes a further 27 (19%) of the root clones under regeneration. Consequently, in overall terms of the status of the regeneration work, 40% of the required root clones have either produced transgenic plants or are currently regenerating shoots.

Regeneration is currently the major priority in the project and will continue until December ‘01. We are attempting regeneration from 140 of the established root clones (Table 2). The aim of this work is to attempt regeneration from a minimum of

20 root clones for each construct. We anticipate that we will successfully regenerate from at least 10 root clones of each construct and this has already been achieved for transgenic lines containing the CaMV2 construct (see Table 2). Where this proves problematic we will select additional root clones from those we are not currently regenerating from.

Table 2. Regeneration of established transgenic root clones identified by direct PCR to contain TuMV or CaMV constructs.

Viral construct	No. of transgenic root clones produced	No. of transgenic root clones under regeneration	No. of transgenic lines with shoots produced (as at 8th Oct '01)	No. of transgenic lines regenerating shoots (as at 8th Oct '01)
TuMV1	42	17	4	2
TuMV2	92	39	7	3
TuMV3	66	24	6	4
TuMV4	76	33	7	6
CaMV1	59	26	2	12
CaMV2	81	34	10	6
Total	**416**	**173**	**36**	**36**

Milestones 1.4 and 3.4. Testing T_0 plants from each transgenic line for resistance to TuMV UK 1 and CaMV UK 4.
Revised milestone date: October 2001

Some T_0 plants were acclimatised to glasshouse conditions. Plants of two T_0 lines were tested for resistance to the UK 2 isolate of TuMV from which the viral sequences used to transform the plant were derived. One of the lines possessed the viral construct TuMV2 (TuMV coat protein without start codon and 'out of frame') and the other possessed construct TuMV4 (TuMV NIb without start codon and 'out of frame').

Control non-transformed Senna plants 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 were tested by enzyme-linked immunosorbent assay four weeks after inoculation to determine the presence or absence of TuMV and to quantify any virus present.

The plants transformed with the TuMV coat protein without start codon and 'out of frame' were all susceptible to the UK 2 isolate of TuMV. Virus was detected by ELISA in the youngest leaves of all plants (Table 3), demonstrating that the virus was moving systemically in the plants. No clear virus symptoms were seen in one of the

plants (number 7 in Table 3). This plant had the lowest level of virus in the youngest leaf and the highest level of virus in the oldest uninoculted leaf relative to other transgenic plants. This might indicate that the systemic infection was delayed. This plant was the biggest at the time of inoculation having 7 leaves, so the delay in virus spread may have been due to the size / physiological age of the plant. Although there was an indication of lower levels of virus in the older leaves of transgenic plants, the mean levels in the youngest leaves suggested the transgenic plants were as susceptible as the control non-transgenic plants.

Table 3. Detection of turnip mosaic virus (TuMV) in Senna plants transformed with the TuMV coat protein without start codon and 'out of frame'.

Plant no.	**A_{405}**					
	Inoculated leaf [a]		**Uninoculated leaf [b]**		**Last leaf [c]**	
1[d]	-		1.61	1.53	0.92	0.85
	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	-	
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] Uninoculated leaf tested was the first (oldest) uninoculated leaf.
[b] Inoculated leaf tested was the last (youngest) inoculated 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.

The plants transformed with the TuMV NIb coding region without start codon and 'out of frame' were all susceptible to the UK 2 isolate of TuMV and showed clear symptoms of virus infection. Virus was detected by ELISA in the inoculated and uninoculated leaves of all plants (Table 4), again demonstrating that the virus was moving systemically in the plants. The levels of virus detected in transgenic plants was much lower than in non-transgenic plants, suggesting that silencing was taking place.

Table 4. Detection of turnip mosaic virus (TuMV) in Senna plants transformed with the TuMV NIb coding region without start codon and 'out of frame'.

Plant no.	Inoculated leaf [a]		Uninoculated leaf [b]	
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**	
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] Uninoculated leaf tested was the first (oldest) uninoculated leaf.
[b] Inoculated leaf tested was the last (youngest) inoculated 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.

Milestones 1.5 and 3.5. Production of homozygous T_1 plants.
Revised milestone date: *December 2001*

We will use two strategies to produce homozygous seed of transgenic lines.

For Senna it will prove faster to identify homozygous lines by growing on selfed progeny of the T_0 plants. Of the acclimatised T_0 plants, 6 individual clones containing TuMV constructs have produced seed to date. For this rapid cycling line we will start sowing T_0 seed in November to produce T_1 plants to determine segregation for the constuct and *rol* phenotype. Plants with *rol* phenotype will be discarded. T_1 plants containing only the construct will be selfed. Selfed T_1 seed will then be sown out to identify seed lines that were homozygous at the T_1 stage.

We will produce homozygous T_1 plants from transgenic cauliflower lines by microspore culture. Currently, we have successfully acclimatised plants of 12 transgenic lines (Table 5) and microspore cultures will be established when this material forms flower buds.

Table 5. Transgenic lines (root clones) of cauliflower with acclimatised T_0 plants for microspore culture.

Viral construct	No. of transgenic root clones with T_0 plants
TuMV1	2
TuMV2	0
TuMV3	7
TuMV4	5
CaMV1	1
CaMV2	5
Total	**20**

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