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

Project number:	FV 214 (MAFF Project code: HL0110LFV)
Project Liaison Officer:	Dr JA Walsh
Progress Report:	Annual Report for 2000 (25.9.00)
Previous Reports:	6 month, 12 month
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Project Co-ordinator:	Mr R Montgomery
Date Project commenced:	October 1998
Date Project completed:	March 2003

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

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The following are members of the Consortium for LINK project HL0110LFV;

Horticultural Development Council
Horticultural Research International
Nickerson Zwaan Ltd

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 that were then cloned and sequenced to confirm the correct arrangement of promoter, virus sequence and terminator. There are 3 TuMV clones and 2 CaMV clones.

Attempts to transform Senna by the co-infection approach commenced this year. Seedling explants have been inoculated with *Agrobacterium* carrying vectors for the 3 TuMV and 2 CaMV constructs. Transgenic root clones have been produced for two TuMV constructs and two CaMV constructs.

4. 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 one TuMV construct, others in progress	
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	dependent on 1.2 and 1.3	
Objective 3		
3.1 Produce 20 transformed root clones of the rapid cycling *B. oleracea* line with the above constructs	in progress	
3.2 Regenerate five shoots from root clones produced for each CaMV construct	in progress	
3.3 Test T_0 plants from each line for resistance to the UK isolate of CaMV.	dependent on 3.1 and 3.2	

Objectives 1 and 3: To transform rapid cycling *B. oleracea* plants with constructs containing 3 different sequences from TuMV and 2 different sequences from CaMV

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.

Production of transgenic root clones of rapid cycling *B. oleracea* with three TuMV constructs and two CaMV constructs

At the last consortium meeting a revised timetable for the production of transgenic root clones was put forward. The objective was to produce transgenic root clones from inoculated explants of the rapid cycling line 'Senna', using the engineered strains of *A. rhizogenes* produced previously, by June and to have commenced shoot regeneration from these clones.

Inoculations

Seedling explants were inoculated with individual engineered strains carrying vectors for three TuMV constructs and two CaMV constructs. For each construct inoculations were made to between 804-906 explants (Table 1). Inoculations were completed by the end of February.

Selection of GFP-positive roots

Explants were screened 3-5 weeks after inoculation for production of GFP expressing roots. On average 56 GFP postive roots were obtained for inoculations made with each construct (Table 1), which is less than the anticipated 182 roots expected based on our previous data presented at the last consortium meeting. GFP-positive roots were excised and established on root culture medium and this was completed by April.

Screening of GFP-positive roots by PCR

PCR analysis has been conducted on all roots. We encountered problems with the development of a direct-PCR method used to analyse selected roots and this has delayed progress. Specifically, we encountered problems with reproducibility of

results that were overcome following a modification to sample preparation and analysis. We expected 95% of GFP-positive roots to also carry viral construct based on our experience with other genotypes in the same transformation system. However, of the 336 roots screened only 10% were found to carry viral construct and to be free of contaminating *Agrobacterium*. *Agrobacterium* contamination caused the loss of some root clones that were positive for viral construct but this did not account for all of the reduction in expected rates of co-transformation. Nevertheless, we have successfully produced transgenic root clones for two TuMV constructs. PCR work was completed at the end of October.

Shoot regeneration

Selected root clones that are postive for viral construct have been cultured onto shoot regeneration media. We expect the first transgenic shoots to be in the glasshouse in the next 4-6 months.

Table 1. Production of GFP-positive root clones for viral constructs for immunity to TuMV and CaMV. Inoculations were made to explants of 'Senna'. For each construct inoculations were made to approx 900 explants, the anticipated rate of explant transformation was 8% with an expected yield of 192 GFP-positive roots.

Viral construct	Inoculated explants (no.)	Explants with GFP-positive roots		GFP-positive roots produced (no.)	GFP-positive roots with viral construct (no.)
		(no.)	(%)		
T1	804	69	8.5	112	0
T2	906	42	4.6	59	7
T3	900	38	4.2	49	0
T4	900	53	5.9	70	26
C1	816	30	3.6	44	1*
C2	900	39	4.3	56	1*
Total	5226	271	5.2	390	35

Notes

* Root clones identified from earlier inoculations

Future work

As Table 1 shows we have yet to produce the full complement of transgenic root clones required. We estimated that a minimum of 40 transgenic root clones are required for each construct to ensure regeneration of 20 transgenic lines with at least

some lines being single copy insertions. We still have to produce transgenic roots for two constructs and increase the numbers obtained to date for four constructs. Form our research programme on the genetic analysis of transformation traits we have identified two rapid cycling genotypes with superior rates of transgenic root production (85% of explants with transgenic roots) and regeneration characteristics. Our current evidence is indicating that these lines will also be easier to work with subsequently during flowering in the glasshouse. In order to maintain progress we prpose to substitute these rapid cycling lines for Senna in additional inoculation work required. We anticipate that their use would allow us to continue to meet the revised deadlines agreed at the previous consortium meeting.