

SHORT-TERM SCIENTIFIC MISSION (STMS)

SCIENTIFIC REPORT

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Host's Name and Institution: Stuart A. MacFarlane, Scottish Crop Research Institute

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Introduction

The Scottish Crop Research Institute (SCRI) is situated in Invergowrie –Dundee. The is a major international centre from research in plant and microbial science. SCRI was established at Invergowrie in 1981 by the amalgamation of the Schottish Horticultural Research (a SHRI founded at Invergowrie in 1951) by the amalgamation of Plant breeding station (SPBS founded in 1921 at East Craigs Edimburgh). This institute have well equipped laboratory, glasshouse and field facilities in which to conduct excellent research. Research focuses on processes that regulate the growth of plants and their responses to pests, pathogens and the environment. It includes genetics to breed crops with improved quality and nutritional value as fast as possible. I visited this institute from 19th November to 7th December 2006. The aim of this STMS was to improve my knowledge concerning compatible and incompatible plant-pathogen interaction at molecular level. In particular, the methods of investigation inducible disease resistance mechanisms in *Rubus* species. I worked one project proposed by Dr Stuart MacFarlane, researcher in SCRI is expert on plant virus disease. He has developed important study concerning the transmission mechanism and identification of tomato, potato and raspberry virus.

Objectives

One of the objectives of the work program of STMS was the study of expression of Raspberry bushy dwarf virus (RBDV) protein in the plant.

Viruses are both initiators and targets of a defence mechanism that is similar to post-transcriptional gene silencing (PTGS), and in transgenic plants involves sequence-specific degradations of RNA. Viruses of plants encode pathogenicity determinants that suppress this defence mechanism. It has been proposed that PTGS is a manifestation of a natural virus resistance mechanism in plants, and they are activated when the transgene, or its RNA, is perceived as a virus.

RBDV virus has two positive-strand genomic RNAs that encode at least three viral proteins (replicase protein, movement protein and coat protein). In addition, other open reading frames that could encode putative proteins are present in the virus genome.

The aim of this research was to know the potential of PTGS suppression activity of replicase, movement protein (MP), RNA-dependent RNA polymerase (RdRP), and RBDV - coat protein (CP) in transformed plants exhibiting PTGS of the green fluorescent protein (GFP) transgene. In this STMS period we have started to clone all of these virus genes into binary vectors for expression in plants using *Agrobacterium tumefaciens*.

The GFP expression was monitored in GFP-transgenic *Nicotiana benthamiana* plants, that had been pre-treated to silence expression of the transgene. Three weeks after initiation of GFP silencing these plants were infected with RBDV. Suppression of silencing by RBDV would be identified by the recovery of expression of the GFP transgene in these plants.

Another objective has been to develop a PCR assay for the specific diagnosis in blackcurrant reversion virus (BRAV). Specific primers were analyzed with different PCR methods. Amplified fragments were cloned in pGemT vectors for future sequence analyses.

Description of the work

Cloning of Raspberry bushy dwarf virus (RBDV) helicase protein, movement protein (MP), RNA-dependent RNA polymerase (RdRP), and coat protein (CP) in *Agrobacterium tumefaciens* using Gateway technology.

Raspberry bushy dwarf virus (RBDV, genus *Idaeovirus*) has a bipartite genome that consists of a 5.4kb RNA-1 and a 2.2kb RNA-2. RNA-1 encodes for the proteins that are involved in virus replication. The RBDV genes used in this experiment were organized in three different plasmids: po826 including RNA-1 RBDV, po819 containing RNA-2 RBDV and po864 including BCRV -CP.

Each of the virus genes were cloned using Gateway technology (Invitrogen).

This technology is based on the bacteriophage lambda site-specific recombination system, which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathway. In the Gateway technology, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system. Lambda-based recombination involves two major components:

- the DNA recombination sequences (ATT sites) and
- the proteins that mediate the recombination reaction (i.e. Clonase™ enzyme mix).

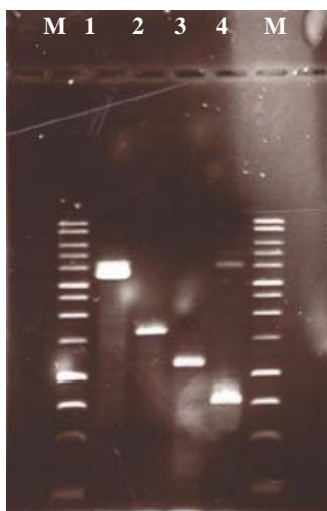
The gateway Technology uses the lambda recombination system to facilitate transfer of heterologous DNA sequences (flanked by modified att sites) between vectors. Two recombinations reactions constitute the basic of the gateway technology:

- BP Reaction: Facilitates recombination of an attB substrate (attB-PCR product or a linearized attB expression clone) within attB substrate (attB-PCR product or a linearized attB expression clone) with an attP substrate (donor vector) to create an attL-containing entry clone (see diagram below). This reaction is catalyzed by BP Clonase™ enzyme mix.
- LR Reaction: facilitates recombination of an attL substrate (entry clone) with an attR substrate (destination vector) to create an attB-containing expression clone (see diagram below). This reaction is catalyzed by LR Clonase™ enzyme mix.

In the first step the RBDV viruses were isolated by PCR performed in 50 µl of volume containing Buffer MF 5X (Finenzyme), 10 µl; dNTP 0.25 mM 1 µl, 0,4 pmol of each primers, 2.5 units of Enzyme Phusion (Finenzyme). The primers used in the first step were homologous at sequence gene and at *att* sequence of the Gateway techenology:

| Primers | Amplicons |
|-----------------------------------|-----------------------------|
| 1024 RBDV 5' helicase +att | RBDV RNA1 po 826 -3465 bp |
| 1025 RBDV 3'elicase -att | |
| 1026 RBDV 5' RDRP +att | RBDV RNA1 po 826 -1560 bp |
| 1029 RBDV 3'RDRP -att | |
| 1028 RBDV 5' MP+att | RBDV RNA2 po 819 -1050 bp |
| 1023 RBDV 3'MP -att | |
| 1032 RBDV 5'BCRV-CP +att | genT/BCRV CP. Po 864 bp 850 |
| 1033 RBDV 3 BCRV-CP -att | |

Figure1-Isolation of RBDV genes; 1° PCR.M=LADDER;1=HELICASE;2=RDp;3=MP;4=BCRV-CP

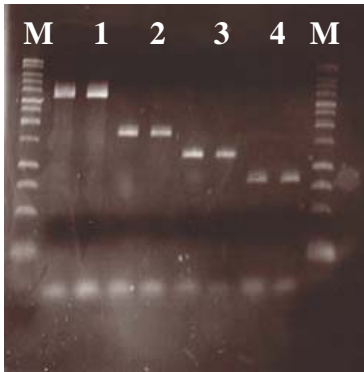


For helicase, after the initial denaturation of 30'' at 98°C thirty amplification cycles (98°C, 5''; 60°C, 20''; 72°C, 2'') were performed. The RDRP, MP and BCRV-CP gene were amplified after the initial denaturation at 98°C, 30'' followed by thirty cycles at 98°C, 5''; 65°C, 20''; 72°C, 1'.

Samples amplification was assessed by an agarose mini-gel electrophoresis (1%) and EtBr stained (Figure 1).

1/100 and 1/20 dilutions were used as template for a second PCR necessary to complete the site of replication according with Gateway technology. This PCR was performed using universal primers 636-637.

Figure 2 – 2° PCR for compile replications site. M= LADDER; 1= HELICASE (1/20-1/100); 2=RDRP(1/20-1/100); 3=MP(1/20-1/100) 4=BCV(1/20-1/100)



The reaction was performed in 50 µl of volume containing 10 µl of Buffer MF 5X (Finenzyme), 1 µl of 0.25 mM of dNTP, 0,4 pmol of primers, 2.5units of Enzyme Phusion (Finenzyme). The cycle used for PCR consisted in 5 initial cycles at 98°C, 5''; 50°C, 5''; 72°C, 2' for helicase gene or 1' for RDRP, MP and BCRV-CP gene, following by twenty-five cycles at 98°C, 5''; 72°C, 2' for helicase gene or 1' for RDRP, MP and BCRV-CP genes. The PCR fragments were analysed by an agarose mini-gel electrophoresis (1%) and EtBr stained (Figure 2)

After controlling in agarose gel the fragments were purified according to the Promega kit. Every RBDV gene was cloned in the plasmid p0553 DONR207 according to gateway technology. This plasmid present the resistance to gentamicin and the fragment lost by the recombination produces toxic protein for *E. coli*.

The BP clonase reaction was performed in 10 µl according to the following table:

| Gene (PCR products 300 ng) | Buffer BP 5X (µl) | po553DONOR207 (254 ng/ µl) (µl) | BP Clonage enzyme (µl) |
|----------------------------|-------------------|---------------------------------|------------------------|
| helicase 4µl | 2 | 1.5 | 2.5 |
| RDRP 4,5 µl | 2 | 1.5 | 2 |
| MP 5 µl | 2 | 1.5 | 1.5 |
| BCRV-CP 5 µl | 2 | 1.5 | 1.5 |
| 2b 5 µl | 2 | 1.5 | 1.5 |

The

reaction has proceeded overnight at 25 °C.

The next day the mix was digested with 1 µl of protease K for 10 min. The plasmid was transferred in *E. coli* competent cells. For every gene, tubes containing 75 µl of the competent cells were added with 3 µl of BP clonase reaction. The mix was incubated on ice for 30' than at 42°C for 50'' and then on ice for 50''. Samples were mixed with 250 µl of SOC media and incubated for 1 h at 37°C at 125 rpm. The cells were spread in the Petri disch containing LB medium with gen50 and incubated at 37°C overnight. All colonies grown were then transferred in LB liquid medium, and incubated at 37°C overnight.

The plasmidial DNA was extracted from the bacteria (Promega mini prep), and it was quantified by spectrophotometer. The DONR207 recombinant was controlled digesting with BstG1. The reaction was carried out using 200-250 ng of DNA for 1 hour in 15 µl of volume containing 10X buffer 2 (Biolabs), BSA 10X, 20U BstGI enzyme. This enzyme cuts the plasmidial DNA in 4 sites as indicated in the following scheme.

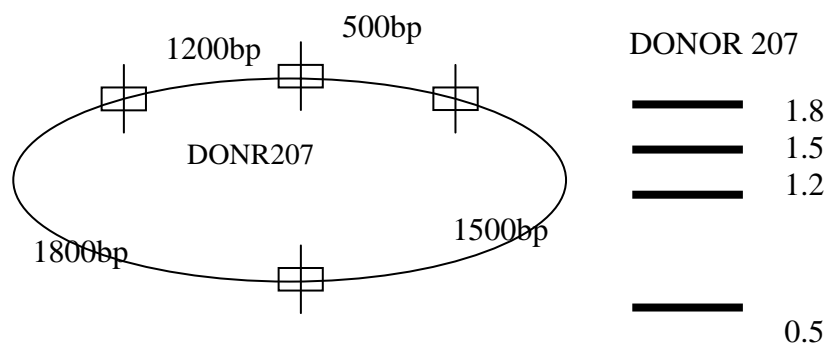
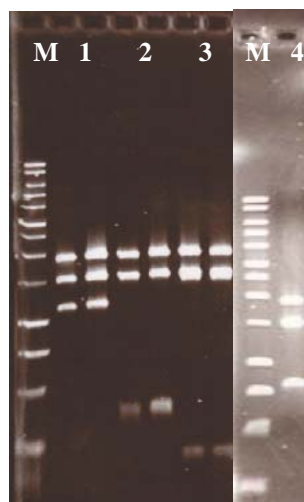


Figure 3. pDONOR207 recombinant whit , MP, 2b, RdRP and CP genes



After recombination two BstG1 sites were lost and the fragments 1.2 and 0.5 were not more present. In this work we have analyzed 16 different *E. coli* recombinant colonies, grown on selective LB media. The new patterns obtained were showed in the Figure 3.

LR recombinations were performed cloning 2b, MP, RdRP and BCRV-CP genes in pmDC32 plasmid. According to the following reaction.

| Gene (PCR products 300 ng) | Buffer BP 5X (μ l) | pmDC32 (137 ng/ μ l) (μ l) | LB Clonage enzyme (μ l) |
|--|-------------------------|-------------------------------------|------------------------------|
| 2b 4.5(μl) | 2 | 2 | 1.5 |
| RDRP 4.5 (μl) | 2 | 2 | 1.5 |
| MP 4.5(μl) | 2 | 2 | 1.5 |
| BCRV-CP 4.5 (μl) | 2 | 2 | 1.5 |

the reaction has proceeded overnight at 25 °C

The next day the mix was digested with 1 μ l of protease K for 10'. The plasmid was transferred in *E. coli* using the competent cells. For every gene, tubes containing 50 μ l of the competent cells were added with 2 μ l of LR reaction gene. The mix was incubated on ice for 30' following by 42°C for 50'', and then on ice for 50''. Samples were mixed with the 250 μ l of SOC medium, and incubated for 1 h at 37°C at 125 rpm. The cells were spread in the Petri disch containing LB medium with Kan50 and incubated at 37°C over night. All the recombinant genes obtained will be transferred in *A. tumefaciens*. The transfer in this binary vector is important for gene agro-infiltration methods in the plants.

Reversion of GFP silencing in *N. benthamiana* GFP plants by RBDV virus

The virus gene expression in the plants was monitored in GFP-transgenic *Nicotiana benthamiana* plants, pre-treated with silence expression of the GFP gene. The *A. tumefaciens* used for silencing gene contained inverted repeat GFP sequence that was able to induce silencing of

GFP. After three weeks of agro-infiltration, these plants were infected with RBDV. Suppression of silencing was identified by an extended period of expression of the GFP gene in these plants.

Five 16C clones GFP-transgenic *Nicotiana benthamiana* were inoculated with RBDV infected *Chenopodium*, after three weeks of previous agro-inoculation with *dsRNA* which showed different silent GFP effect at UV light according to the following table.

| plant | Silent GFP effect | Inoculated |
|-------|-------------------|------------------------------|
| 1 | GFP+ not showed | No inoculated |
| 2 | GFP- | Inoculated with RBDV |
| 3 | GFP- | Inoculated with RBDV |
| 4 | GFP+/- | Inoculated with RBDV |
| 5 | GFP- | Inoculated with RBDV |
| 6 | GFP- | Inoculated with leaf healthy |

Both leaves of *Chenopodium* which were infected with RBDV and leaves healthy, were ground in different mortars. The 16C clones GFP-transgenic *Nicotiana benthamiana* were treated with corundum with abrasive action. We have selected 4 leaves for every plant. Two of these have been marked by one hole and the other two with two holes. The second ones were inoculated. These leaves were infected with RBDV (2-3-4-5 plants) and the number 6 with *Chenopodium* healthy. The plants were monitored in this period for evaluation.

BRAV detection

The new primers were designed for the detection 101 bp amplicon of black currant reversion virus (BRAV) in the raspberry plants. The primers detection was tested in the RNA raspberry plants using two different methods:

1. RNA was retrotranscribed in 15µl of reaction using RNA 5 µl, IF primer (20 picoM) 1µl and of H₂O 9 µl. This mix was incubated at 70°C for 10', centrifuged and mixed with 5X SuperscriptII buffer 5 µl, IF 10 mM primers, 1.25 µl, RNasin 0.625 µl, RT enzyme 1µl, H₂O 2.125 µl for a total volume of 50 µl. After incubation for 1 h at 42°C, 1 µl of cDNA was added at buffer 5X 5 µl, 10mM dNTP 5 µl, taq enzyme 2.5 units, specific primers IF 20 picoM 1 µl, 4R 20 picoM 1 µl and H₂O 36.5 µl.
2. The RT-PCR beads methods (Amersham) is a very easy technology where the RT-PCR tube contains lyophilized dNTP, buffer, taq enzyme, RT enzyme, and Mg⁺⁺. At this tube we have added RNA 5 µl, specific primers IF 20 picoM 1 µl, specific primers 4R 20 picoM, 1 µl primers and H₂O 43 µl

At this step, PCR reactions were performed using the same cycle: one initial cycle at 94°C for 5', followed by 30 cycles at 94°C 30''; 56°C, 30''; 72°C 1'. The last cycle was at 72°C for 10'. 1 µl of the previous PCR specific primers 2F, 20 picoM, 1 µl, primers 3R 20 picoM, 1 µl and H₂O 22 µl were added at the PCR beads technology (Amersham) containing lyophilized dNTP, buffer, taq enzyme and Mg⁺⁺. Traditional PCR was performed mixing cDNA 1 µl, buffer 5X, 5 µl, dNTP 10mM 5 µl, taq enzyme 0.5 µl, specific primers 2F, 20 picoM, 1 µl, specific primers 3R 20 picoM, 1 µl and H₂O 36.5 µl. The PCR cycle was the same used for the first

Detection BRAV in raspberry. Clone in pGem-T Easy Vector of the PCR 101 bp fragment. DNA digest with EcoRI enzyme of 8 colonies grown in LB-AMP100.



PCR. The PCR beads generated a big fragment (101 bp) at the 2° PCR; nucleotidic sequence was determined. With this aim the gel band was cut and purified using Promega Kit according the protocols. pGem-T Easy Vector Systems was used for cloning the raspberry BRAV fragment. 3 µl of purified fragment was used with 5µl of 2X Ligation Buffer, 1µl of pGem-T Easy Vector (50ng/µl), 1 µl of T4 DNA ligase. The reaction mixture was incubated at 12°C overnight. The pGemT rapid recombinant vector was inserted in *E.coli* using JM109High Efficiency Competent cells. 2µl of ligation reaction was added at 50 µl of JM109High Efficiency Competent cell was, incubated 20' on ice, and heat-shock at 42°C for 50'' was performed and immediately returned on ice for 2'. After that, 950 µl of the SOC medium was added, the cells were incubated at 37°C for 1,5 h at 150 rpm, they were then cultured overnight at

37°C in LB medium containing AMP100. The grown colonies were transferred and cultured in LB liquid medium with selective antibiotic AMP100. Mini prep Promega was used for DNA plasmidial extraction, and 8 different recombinants were analyzed with EcoRI enzyme digestion.

The patterns showed the presence of 101 bp in all colonies analyzed.

References

Brigneti G, Voinnet O, Li WX, Ji LH, Ding SW, Baulcombe DC. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* Nov 16;**17**(22):6739–6746

Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses. **Proc. Natl. Acad. Sci. USA** 96, 14147-14152.

Ratcliff F.G., MacFarlane S.A., and Baulcombe D.C. (1999) Gene Silencing without DNA: RNA-Mediated Cross-Protection between Viruses. *Plant Cell*, Vol. 11, 1207-1216,

Constantin, G.D., Krath, B.N., MacFarlane, S.A., Nicolaisen, M., Johansen, I.E. and Lund, O.S. (2004). Virus induced gene silencing as a tool for functional genomics in a legume species. *Plant Journal*, 40, 622-631.

Attended seminars

21/11/06

‘Mycorrhizal networks of power and influence their roles in biogeochemical cycles and ecosystem functions’.

Prof. Jonathan Leake – University of Sheffield.

30/11/06

‘Multiple pathways of innate resistance of virus’

Prof. Peater Palukaitis, Ju-Yeon Yoon, Minoru Takeshita and Tomas Canto

Scottish Crop Research Institute

05/12/06

‘Soil biogeochemistry at an environmental extreme’

David Hopkins.

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