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TRANSFORMATION OF ORNITHOGALUM BREEDING LINES FOR VIRUS RESISTANCE

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Executive summary

The project aims to develop virus-resistant *Ornithogalum* plants of two varieties, one from South Africa (*O.thyrsoides* x *O.dubium*) and one from Israel (*O.dubium*) using pathogen-derived resistance. A transformation protocol was developed and tested for efficiency by optimizing a regenerable tissue culture protocol suitable for transformation, constructing plasmid vectors to optimize transformation efficiency and attempting transient and stable transformation. At ARC-Roodeplaat, four plasmids were constructed which contained a *bar*GUS fusion as selectable marker- and reporter genes, under control of either the CaMV35S, enhanced CaMV35S (eCaMV35S), eCaMV35S followed by a TEV leader sequence or ubq10. In addition, The South African team cloned the coat protein (CP) gene South African isolate of the Ornithogalum Mosaic virus driven by enhanced-35S and e35S-TEV into pRTL vector. At the ARO, a variety of different promoters: enhanced CaMV35S, SVBV, UBQ3, Act 1 were introduced into the pDM327 transformation vector. In Addition, two target genes the coat protein (CP) of the Israeli isolate of OrMV and the virus replicase gene (NIb) under the control of either UBQ3 or ΔSV promoter were cloned into pCAMBIA vector and used for bombardment of the target tissue. The ARC team also calibrated the system for *Agrobacterium*-mediated transformation of leaf tissue as an alternative to nicroprojectile-driven transformation of callus. Several *Agrobacterium* strains carrying a variety of plasmids were tested for their ability to cause transient and stable transformation. Findings- Several *Agrobacterium tumefaciens* strains have been found to be effective in causing transformation. The strain AGL0 containing the recombinant plasmid pSCV1.6 was found the most efficient one. At the ARO, *O. dubium* cells were bombarded with four target-gene constructs (CP and replicase-NIb, each under the control of the UBQ3 and ΔSV promoters. Stable GUS expression is evident with all the constructs. The cultures have been grown in a selective medium supplemented with Kanamycin. They are currently growing in the light on a hormone-free medium to initiate plantlet regeneration.

In the following year – Promoters that remain to be tested will be evaluated for their efficiency in *Ornithogalum*. Simultaneous transformation of varieties will be done to determine if they act the same. Collaborative cloning of virus constructs behind the best promoter. Continued transformation with target genes using *Agrobacterium*-mediated or the microprojectile bombardment technique and a continued selection of putative transgenic plants. Plant regenerated under selection will be tested for the presence of the transgene.

Section I

A. Research Objectives.

The objectives for the second year of the project were:

1. Complete the cloning of various promoters into a suitable vector, pDM327 to determine the best one for transformation of *Ornithogalum*.
2. Testing Kanamycin and Geneticin as an alternative selectable marker
3. Continue to optimize bombardment parameters for efficient delivery of transgenes to *Ornithogalum* cultures.
4. Clone various OrMV cDNA constructs ("target genes") under control of the most efficient promoter for *Ornithogalum*.
5. Optimization of *Agrobacterium* mediated transformation
6. Transformation of Ornithogalum tissue with target genes and selection for transgenic plants.

B. Research accomplishments

B.1. Activities at the ARO.

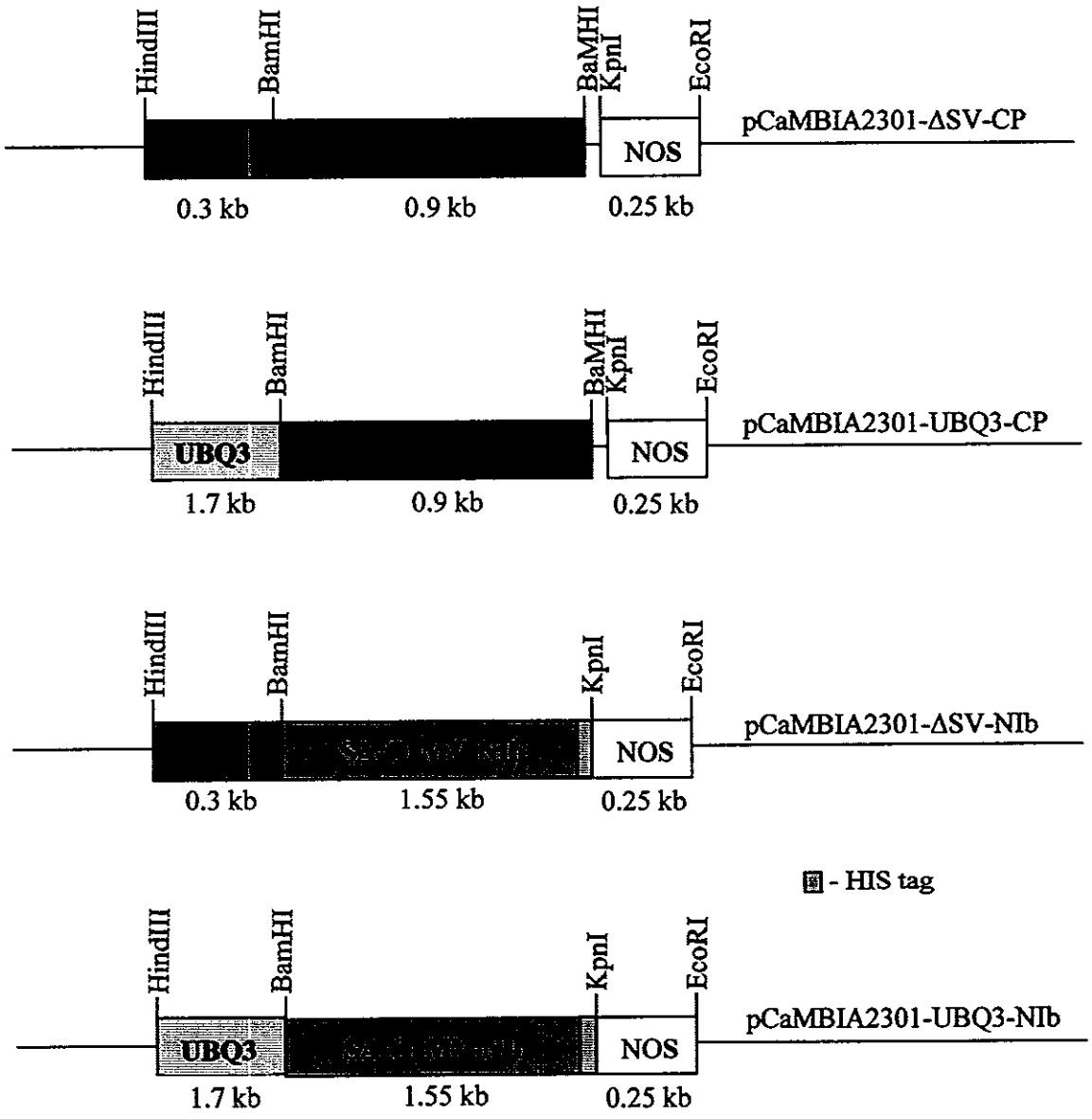
Construction of various plasmids.

Two different plasmids groups were constructed at the ARO. The first group intended for assessing relative efficiency of several promoters to control gene expression in *Ornithogalum* tissue. The four promoters used were: UBQ3 (which was previously tested successfully in *Lilium longiflorum* transformation), ΔSV (ΔSBV) (isolated from Strawberry Vein Banding Virus), Act1, and 35S. These promoters were introduced into pDM327 cloning vector, which includes GUS and barGUS, the same vector used by ARC for the construction with other promoters (see 1st Annual Report, US-AID CDR Project C18-006, sept.2001). The cloning strategies were as follows: For the introduction of UBQ3, PCR products of the promoter with HindIII – BamHI flanking sites was inserted to pDM327 digested with HindIII – BamHI. An addition of 1.7 kb to the length of the plasmid was verified by both by restriction enzyme digestion and DNA sequencing. Similarly, PCR products of ΔSBV promoter (0.38 kb) were introduced into pDM327 pre-digested with the same combination of enzymes. The promoter Act1 was

isolated from pDM302. A 1400 bp fragment digested with EcoRI and HindIII was introduced by three-piece ligation into pDM327 pre digested with EcoRI and HindIII. Finally, the 35S promoter was isolated from a pCAMBIA vector (a fragment of ca. 1kb using HindIII - BglII) and inserted into pDM327 pre digested with Hind III-BamHI.

Constructs with target genes: The second group of plasmids constructed at the ARO included two target gene intended to be used for transformation of Ornithogalum, each under the control of one of the two promoters UBQ3 and ΔSV (ΔSBV). The use of the herbicide Basta as a selectable marker was discontinued in both ARC and ARO because of a very narrow margin between a concentration that is harmful to *Ornithogalum* and one that allows a regeneration of non-transgenic plants. The system was calibrated for the use of Kanamycin as a selectable marker. We decided to use a pCAMBIA2301 vector that has nptII gene, conferring Kanamycin resistance, and a GUS reporter gene (both under control of 35S promoter), with which we were successful in transforming lilies. The two target genes used were: the coat protein gene (CP) of the Israeli isolate of Ornithogalum Mosaic Virus (OMV), and the virus replicase gene (NIb). The CP gene was isolated and sequenced by the Israeli research team and the NIb (replicase) gene was cloned and sequenced by the South African team. In the construction of the chimeric gene Standard DNA procedures of DNA restriction, subcloning and propagation in *E. coli* strains were employed. The vector containing all constructs was pCaMBIA2301. Firstly, a NOS terminator was PCR cloned into pCaMBIA2301 KpnI-EcoRI sites to create pCaMBIA-2301-NOS. The strawberry vein-banding virus deleted promoter (ΔSV) and the *Arabidopsis* polyubiquitin promoter (UBQ3) were then PCR cloned into the HindIII-BamHI sites of pCaMBIA2301-NOS to create pCaMBIA2301-ΔSV-NOS and pCaMBIA2301-UBQ3-NOS. The Israeli OrMV coat protein gene (CP) and the South African OrMV Nib gene were then PCR cloned into the BaMHI site or BamHI-KpnI sites respectively of either pCaMBIA2301-ΔSV-NOS or pCaMBIA2301-UBQ3-NOS to create pCaMBIA2301-ΔSV-CP or NIb and pCaMBIA2301-UBQ3-CP or NIb. Sense orientation of the CP gene was verified by PCR and restriction analysis. All other constructs were verified by restriction analysis. The restriction maps of the four constructs is outlined in figure A-1. All 8 constructs were handed over to the research team at ARC-Roodeplaat. The four constructs with the two target genes gave very good indication of transient GUS expression after bombardment of cell clusters

grown in liquid cultures, and are currently being used to transform the *Ornithogalum* tissue.



* A Kozak sequence (**CCA/GCCATGG**) was inserted in the 5' of CP and Nib - CCACCATGG

Fig. A-1. Map and restriction sites of the promoter-target-gene inserts cloned into the pCAMBIA2301 vector

Particle bombardment with target genes.

Following the establishment of Ornithogalum cells with high competency for transformation we have tested the cloned vectors that contained the target gene for their ability to cause transient GUS expression in the cultured cells. The cells have been maintained in a dark-grown liquid culture for more than a year in medium #101 (MS salt and vitamins with additional 0,4 mg/l thiamine-HCl + 1 mg/l NAA+ 0.1 mg/l BA+ 3% sucrose) with successive subcultures every two weeks. The cultures were checked periodically to ensure that they retain their potential for regeneration into plants upon a transfer to the light. The cell clusters were bombarded with gold particles carrying the target gene constructs using the particle-acceleration inflow gun. In each experiment, two plates containing cell clusters were bombarded three times each with one construct. GUS transient expression was checked 48 hours following the bombardment. For the assessment of the efficiency of each bombardment, cell samples of the same culture were bombarded with pUC-UBQ3genGUS containing the *uid*A gene encoding for the enzyme ß-glucuronidase (GUS) under the control of the ubiquitin3 promoter. This plasmid was used for the initial calibration of the bombardment apparatus as well as for evaluating the competency of the *Ornithogalum* cells. Selected data of transient GUS expression is presented in Table A-1. The relative efficiency of each bombardment was evaluated compared to the number of foci in cells of the same culture bombarded with pUC UBQ3 gen GUS as a control. The competence of the cells maintained in culture for more than a year ("old" culture) is assessed compared to the transient expression results obtained with the results with the same plasmid a year ago when the culture was relatively "young" (row 1). It was obvious that although we obtained relatively high transient GUS expression with the various target genes represented in table A-1 by pCAMBIA ΔSV-NIb-NOS-35S-GUS (row 3) and pCAMBIA UBQ3-NIb-NOS-35S-GUS (row 6), it was far less than the results obtained with the young culture (results in row 1 vs. those in row 2). We have tested the possibility that the level of sucrose in the culture medium may have affected the number of observed foci. We found essentially the same results when the level of sucrose used was 9% (medium 101S, row 5) or 3% (medium 101, row 7). In the previous year we found that the cells grown on medium #206 (MS salts and vitamins with 2 mg/l BA + 0.1 mg/l NAA) were by far more competent for transient GUS expression than the ones grown in medium #101. However, medium #206 failed to support a long-term maintenance of actively growing cells and the cultures slowly deteriorated. Therefore, the use of medium #101 was preferred. We have tested the effect of a short transfer of the culture to medium #206 prior to the bombardment on the level of GUS expression. When the cells were sub-cultured onto a

fresh #206 medium two weeks prior to the bombardment (row 8), the level of competence of the cells increased dramatically. The results were even better when cells were bombarded only one week after being exposed to fresh #206 medium (row 9). By the short subculture on medium #206 the cells were able to regain the level of competence observed in a young culture.

Table **A-1**. Selected data from transient GUS expression experiments with *Ornithogalum dubium* cells following particle bombardment with various plasmids (See the text for details).

Row #	Plasmid vector	Date of experiment (Number)	Medium	# foci per experiment	# foci per g FW	Relative efficiency (% of exp. I)	Relative efficiency within the same experiment
1	pUC UBQ3 gen GUS	17.04.2001 (I)	101S*	6500	1320	100	
2	pUC UBQ3 gen GUS	12.04.2002 (II)	101S	1280	660	50	100
3	pCAMBIA ΔSV-Nib-nos-35S-GUS	12.04.2002 (II)	101S	1040	510	38	77
4	pCAMBIA2301 -35S-GUS	12.04.2002 (II)	101S	500	360	27	55
5	pUC UBQ3 gen GUS	01.05.2002 (III)	101S	2500	500	38	100
6	pCAMBIA UBQ3-Nib-nos-35S-GUS	01.05.2002 (III)	101S	1600	370	28	75
7	pUC UBQ3 gen GUS	01.05.2002 (III)	101	2490	550	41	109
8	pUC UBQ3 gen GUS	26.05.2002 (IV)	206S**	4000	1180	89	
9	pUC UBQ3 gen GUS	23.06.2002 (V)	206S**	6350	1160	88	

*Medium #101: MS salt and vitamins with additional 0,4 mg/l thiamine-HCl + 1 mg/l NAA+ 0.1 mg/l BA+ 3% sucrose, #101S is the same medium but supplemented with 9% sucrose.

**Medium #206S : MS salts and vitamins with 2 mg/l BA + 0.1 mg/l NAA +9% sucrose.

Stable GUS expression and selection of transgenic tissue. Following the bombardment with the four target gene constructs and the verification of transient GUS expression ((plate I, a; b), the bombarded cells were placed in the dark in a liquid #101 medium supplemented with 80 mg/l Kanamycin for selection. The cells were sub cultured onto a fresh medium every two weeks. A sample of cell clusters was removed and stained for GUS reaction. A stable GUS reaction was evident in all the tissue samples taken from the culture flasks as presented in Plate I. The selection on Kanamycin was continued for two months in the dark, at which time the volume of the bombarded tissue was nearly doubled. Transformed cells need to reach a certain critical mass before they are able to continue their meristematic development and regenerate plants upon the transfer to hormone-free medium in the light. It was obvious that *Ornithogalum* cells grow slower than cells of Lilium when exposed to Kanamycin we could not assess when it is the right stage to initiate the regeneration phase. Therefore, after two months the cells were divided into two parts. One half was transferred to the light to hormone-free MS medium (supplemented with 80 mg/l Kanamycin) for initiation of plantlet regeneration. The second half was placed back in dark on medium #101 for continued selection on Kanamycin.

Regeneration and selection in liquid cultures.

The callus lines previously grown on medium #101 were transferred to liquid MS medium without any growth regulators and continued their growth in the light (16 hrs photoperiod). The non-bombarded (control) cell lines previously maintained on a non-selective medium, turned green shortly after the exposure to the light and regenerate into plantlets starting 4 weeks after the transfer. Cultures that were subcultured onto medium #206 one week prior to the transfer to light grew and regenerated plantlets faster than the ones subcultured to the same medium two weeks prior to the transfer to light as illustrated in Plate II (a: and b), or the ones that were transferred directly from medium #101 in the dark to MS medium in the light (data not shown). The transfer of the control cell line to a selective regeneration medium (MS + 80 mg/l Kanamycin) totally inhibits cell growth and plantlet regeneration (Plate II-c). Cell lines that are constantly maintained on a selective medium hardly show any growth, there is little sign of browning but no obvious necrosis and no oxidation products are excreted into the medium (Plate II-e). The cells bombarded with the target genes showed relatively active growth in the presence of Kanamycin (Plate II-d). Their volume increased two fold in 4 weeks. Control cell lines growing on a non-selective medium exhibited a six-fold increase in volume during the same period.

The bombarded cells are currently maintained on a selective medium. There are the first signs of green centers in the culture indicating the initial stages of putative transgenic plants.

B.2. Activities at ARC-Roodeplaat

Biolistic bombardment for stable transformation at the USDA and at ARC-Roodeplaat

USA

During the previous year different plasmid vectors were constructed at ARC-Roodeplaat that contained the *bar*GUS reporter genes under control of either the CaMV35S (pDM327), enhanced CaMV35S (pDM327-e35S), eCaMV35S followed by the tobacco etch virus (TEV) translation leader sequence (pDM327-TEV), or *ubq*10 (pDM327-ubq10), as well as a negative control not containing any promoter region (pDM327-neg).

During Ms Adri Veale's study at the USDA in July 2001 the five different promoters were compared for their efficiency of transient expression in *Ornithogalum thyrsoides* callus, which were transformed by microprojectile bombardment. In this experiment the best results were obtained with the e35S-TEV leader promoter, followed by the eCaMV35S, *ubq*10 and CaMV35S promoters. The *ubq*10 promoter gave slightly better results than the CaMV35S promoter (results not shown). These results were comparable to previous experiments, and showed all ARC-Roodeplaat promoters were functional.

During her stay at the USDA Ms Veale bombarded different *Ornithogalum* callus with the different promoter constructs pDM237, pDM327-e35S, pDM327-TEV and pDM327-ubq10 for stable transformation. Two days after bombardment the tissues were transferred to regeneration medium (0.1mg/l NAA and 2mg/l BA) supplemented with 1.5 mg/l Basta (for selection) and 300 mg/l Cefotaxime. These plates were transported from the USA to South Africa to further select for stable transformation. The antibiotic Cefotaxime was included into the medium to prevent internal contaminants from overgrowing the material as it was found previously that flight does stress the plant material.

The callus was transferred to fresh medium every 2 weeks. Although some shoots regenerated soon after bombardment on the selection medium, the selection agent (PPT) started to have an effect and the shoots become discolored and died off. These experiments were repeated using the Roodeplaat biolistic device on return from the USA.

South Africa

*O. thyrsoides xO.dubium (*line A2) callus was bombarded with pDM327-e35S and pDM327-TEV for stable transformation. The bombarded callus was then transferred to selection medium containing 2 mg/l PPT selection medium, but to date no stable transformation was achieved. ARC-Roodeplaat also experience difficulty to select in the presence of 1.5mg/l PPT, since this concentration caused the tissue to bleach.

A decision was then made to rather investigate whether Kanamycin or Geneticin (a structural analogue of Kanamycin) could be used as selectable agents.

Testing of Kanamycin and Geneticin as alternative selectable agents

The Neomycin phospho-transferase gene enables plant tissue to grow in the presence of antibiotics, Kanamycin or Geneticin. The plant tissues perform much better and less oxidation products get secreted. The sensitivity of the plant tissues to the antibiotics were determined on both leaf and callus tissue of A2 *(O.dubium x O.thrysoides)* cultured on *Ornithogalum* regeneration medium. The effect of 10mg/l, 20mg/l, 40mg/l and 50mg/l Kanamycin or Geneticin were tested on the callus, and concentrations of 25mg/l, 50mg/l and 80mg/l were tested on the leaves, a control lacking Geneticin and Kanamycin was also included.

After 8 weeks little to moderate shoots regenerate formed on the callus at 50mg/l Kanamycin and the inhibition concentration can be even higher with Kanamycin, maybe 80mg/l – 100 mg/l. With Geneticin the callus was completely inhibited at a concentration of 40mg/l and no regeneration occurred. In the leaves, 80 mg/l Kanamycin or 40 mg/l Geneticin was sufficient to inhibit growth.

In vitro* multiplication and transient transformation of *O. dubium

Ornithogalum dubium plantlets were obtained in the USA and multiplied at ARC-Roodeplaat. Callus was initiated from leaf disks to compare the transient transformation efficacy of A2 (*O.thyrsoides x O.dubium)* and *O.dubium* using the ARC-Roodeplaat Bio-Rad biolistic device.

A2 and *O.dubium* callus were bombarded with pDM327, pDM327-e35S and pDM327-TEV. In comparison to A2, very little transient expression was obtained on *O.dubium.* The highest number of spots obtained on *O.dubium* callus was obtained with pDM327-e35S (60 spots per

bombardment), with pDM327 and pDM327-TEV each giving an average of 30 spots per bombardment. In contrast, the A2 callus the average number of spots obtained per bombardment ranged between 150 – 250, with pDM327-e35S giving the highest level of transient expression.

Cloning of the OMV constructs

ARC-Roodeplaat has to date not been able to compare the transient expression efficacy of the Israeli promoters. Hence, ARC-Roodeplaat decided to clone the coat protein gene of the South African isolate of *Ornithogalum* mosaic virus (SA-OrMV) under the control of the enhanced CaMV35S and e35S-TEV control elements of the pRTL2 plasmid vector. The SA-OrMVCP gene was subcloned from pA7 (pBI221 containing the SA-OrMV coat protein gene) into pRTL2 as an NcoI/BamHI fragment, and the fidelity of the resultant plasmid, named pRTLTEVOMVCP, was verified by restriction enzyme analysis as well as DNA sequencing. In order to obtain the construct with the SA-OrMVCP gene under control of the e35S promoter, the CP gene was amplified by PCR using primers containing EcoRI and BamHI restriction enzyme sites respectively. The PCR product was then subcloned into EcoRI/BamHI digested pRTL2 and the resultant recombinant plasmid was named pRTL2e35SOMVCP. The fidelity of the pRTL2e35SOMVCP was verified by restriction enzyme analysis and DNA sequencing. Figure B-1 shows the restriction enzyme maps of the two recombinant plasmids. The next step is to subclone the expression cassettes into pCAMBIA 2301.

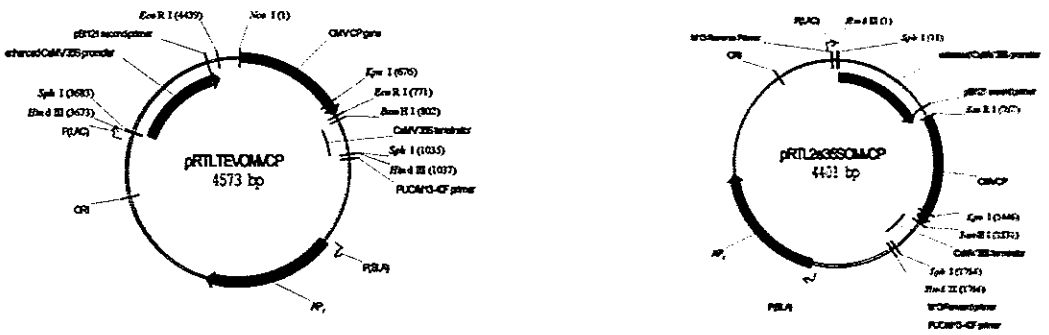


Figure B-1. Restriction enzyme maps of pRTLTEVOMVCP and pRTL2e35SOMVCP

In vitro multiplication and transient transformation of *O. dubium*

Dr Santie de Villiers developed a basic *Agrobacterium tumefaciens* mediated transformation protocol for *Ornithogalum* A2 during her visit to Plant Research International, in Utrecht the Netherlands in May-November 2000. She made attempts to transform *Ornithogalum* with *Agrobacterium tumefaciens*. At ARC-Roodeplaat a comparison was made between different *A. tumefaciens* strains (LBA 4404, C58C1, and AGL0 or AGL1) and plasmids (pCSV1.6, pCAMBIA 3301, pCAMBIA 2301, and pGA482).

Leaves of *in vitro* plants were dissected into pieces and incisions were made on the surface to create wounding sites for transformation. The leaf explants were then dipped into the *Agrobacterium* cell suspension and co-cultivated for 3 – 5 days. The leaves were stained for GUS expression and the rest of the leaves were transferred to pre-selection medium with 250 mg/l cefotaxime. After 11 days of transformation some of the leaves were stained and the rest were transferred to selection medium containing 80mg/l Kanamycin and 250 mg/l cefotaxime.

Table B-1 summarizes the *Agrobacterium tumefaciens* strains and the recombinant plasmids that were tested. Table B-2 summarizes the results that were obtained with the transient transformation, which is also shown in Figure B-2.

Table **B-1**. Bacterial strains and plasmids used in *Agrobacterium*-mediated transformation of *Ornithogalum*.

Agrobacterium tumefaciens strain	**Plasmid**	Transient transformation	Test for Stable transformation
AGL0	pSCV1.6	YES	YES
AGL0	pCAMBIA3301	YES	NO
C58C1	pSCV1.6	YES	YES
C58C1	pCAMBIA3301	YES	NO
LBA4404	pSCV1.6	YES	YES
LBA 4404	pGA 482	YES	YES
LBA 4404	pCAMBIA 2301	YES	NO
AGL1	pCAMBIA2301	YES	NO

Table **B-2**. Transient expression in *Ornithogalum* A2 leaf tissue transformed by means of *Agrobacterium tumefaciens* mediated transformation

Agrobacterium strain	Plasmid	Stained (5days) Reaction	Stained (11 days) Reaction
AGL0	pSCV1.6	+++	++++
AGL0	pCambia3301	+	+
C58C1	pSCV1.6	++	++
C58C1	pCambia3301	-	+
LBA4404	pSCV1.6	-	-
LBA 4404	pGA482	+	-
LBA 4404	pCambia2301	+	-
AGL1	pCambia2301	-	-

+ Faint reaction, ++++ Positive dense reaction

The *Agrobacterium* mediated transformation seemed to be more efficient than the biolistic transformation. The blue spots occurred at the positions of wounding, where regeneration is expected to occur. The best results were obtained with the *Ornithogalum* A2 that was co-cultivated with *Agrobacterium strain* AGL0 containing the recombinant plasmid pSCV1.6, and it appeared that after 11 days the reaction was even denser than in 5 days (Figure B-2A). The *Agrobacterium* strain AGL0 seemed to give rise to a higher transient expression levels than strains C58C1 (Figure B-2B) or LBA4404 (Figure B-2C). Very little blue staining was observed for explants co-cultivated with the bacterial strain LBA4404 (Figure B-2C).

The transformed explants were selected on 80mg/l Kanamycin for regeneration. The regenerated plants were transferred to MS medium with 80mg/l Kanamycin. The stable transformation work is still ongoing.

C. Scientific Impact of Collaboration

ARC-Roodeplaat has not been able to compare the transient expression ability of all the promoters identified for use in the project, since the Israeli promoter constructs were only sent to Roodeplaat at the end of the reporting year. ARC-Roodeplaat could therefore not make an informed decision on which promoter to use for the expression of the viral gene. However, ARC-Roodeplaat went ahead and cloned the SA-OMV CP gene under control of the enhanced

CaMV 35S promoter and the e35S-TEV control elements. The Israeli team cloned both the Israeli isolate of OrMV coat protein and the NIb (replicase) gene under the control of UBQ3 and ΔSV promoters. The first promoter was used after proving to be very efficient in driving GUS reaction. The ΔSV was used because it was found as efficient as 35S in other systems as well as being free of patent restrictions. The constructs were handed over to the ARC team to be used in their attempt to transform the South African cultivars. The teams made mutual decision to discontinue the use of the herbicide Basta as a selectable marker for transgenic Ornithogalum, due to the narrow margin between concentrations that allow an "escape" of non-transgenic plants and the one that caused necrosis and cell death. This calls for a change in the cloned construct to be used and may cause a delay in achieving the goal. On the other hand it may save a lot of work we might have spent on an inefficient and superfluous technique.

D. Description of Project Impact

The constructs prepared by ARC-Roodeplaat are being tested for their efficiency for both transient transformation as well as stable transformation studies. The conditions used for optimal bombardment that Ms Veale learnt during her visit in the USA, will be used to study both transient as well as stable transformation efficiencies of the different promoter constructs. The calibration of the methods for Agrobacterium-mediated transformation of Ornithogalum tissue, if proven to be efficient should add an alternative technique to be used with other genes when they become available.

E. Strengthening of Developing Country Institutions

The skills that Ms Veale learnt in the USA have been instrumental in ensuring that the biolistic transformation of the *Ornithogalum* tissue can be carried out on a routine basis. Ms Veale has also successfully optimized the *Agrobacterium*-mediated transformation of *Ornithogalum* A2 tissue, and it is hoped that this will lead to the stable transformation of Ornithogalum for virus resistance.

F. Future Work

Not all the objectives were met due to that not all the promoters were available for the evaluation of the most efficient promoter for expression in *Ornithogalum* tissue. The constructs were received at the end of the reporting period and ARC-Roodeplaat will now continue to compare the transient expression efficiency of all the promoters on A2 and *O. dubium* callus. The two *Ornithogalum* varieties will be transformed simultaneously with all

the different promoters to determine whether they act the same. The selection on Kanamycin or Geneticin will continue with the aim of stable transgenic plants with the target genes using the alternative transformation systems.

The Israeli collaborators have also supplied ARC-Roodeplaat with constructs containing the Israeli OrMV CP and Nib genes under control of either the ΔSV promoter or the *ubq*3 promoters in the pCAMBIA 2301 backbone. These constructs will be used in both biolistic and *Agrobacterium*-mediated transformation of A2 for virus resistance.

The selection of stable transformants following the bombardment with target gene constructs will continue as well as the attempt to regenerate transgenic plants. Putative transgenic plantlets will be tested for GUS activity as for the presence of the target gene in a hope to obtain virus resistant Ornithogalum plants.
South Africa needs to clone the SA-OrMV-CP expression cassettes into pCAMBIA 2301. Dr Avner Cohen will visit ARC-Roodeplaat to evaluate the progress and to plan future experiments.

Section II

A. Managerial issues

Staff changes: Dr. Tzahi Arazi, a molecular biologist, joined the Israeli team. He is responsible for the cloning of the target gene constructs.

B. Budget

Procurement of equipment:

Funds requested from USAID:

1. ARC-Roodeplaat requests that the outstanding amount of $3000, from year one as well as the outstanding amount of $30000 for year two be paid as soon as possible.
2. An amount of $3000 was budgeted on year 3 of the project for the purchase of a Stratalinker. However, ARC-Roodeplaat requests permission to rather purchase a shaker apparatus to enable ARC-Roodeplaat to also test shaker cultures.

C. Special concerns

None

D. Collaboration, Travel, Training and Publications

Travel: None

E. Requests for American Embassy Tel Aviv or AID actions

Refer to budget section

References

Kamo, K., McElroy, D., and Chamberlain, D. (In Press). Transforming embryonic cell lines of *Gladiolus* with either a *bar-uidA* Fusion Gene or Co-Bombardment

McElroy, D. Chamberlain, D.A., Moon, E. and Wilson, K.J. (1995). Development of *gusA* reporter gene constructs for cereal transformation: Availability of plant transformation vectors from the CAMBIA Molecular Genetic Resource Service. *Molecular Breeding* **1**, 27-37.

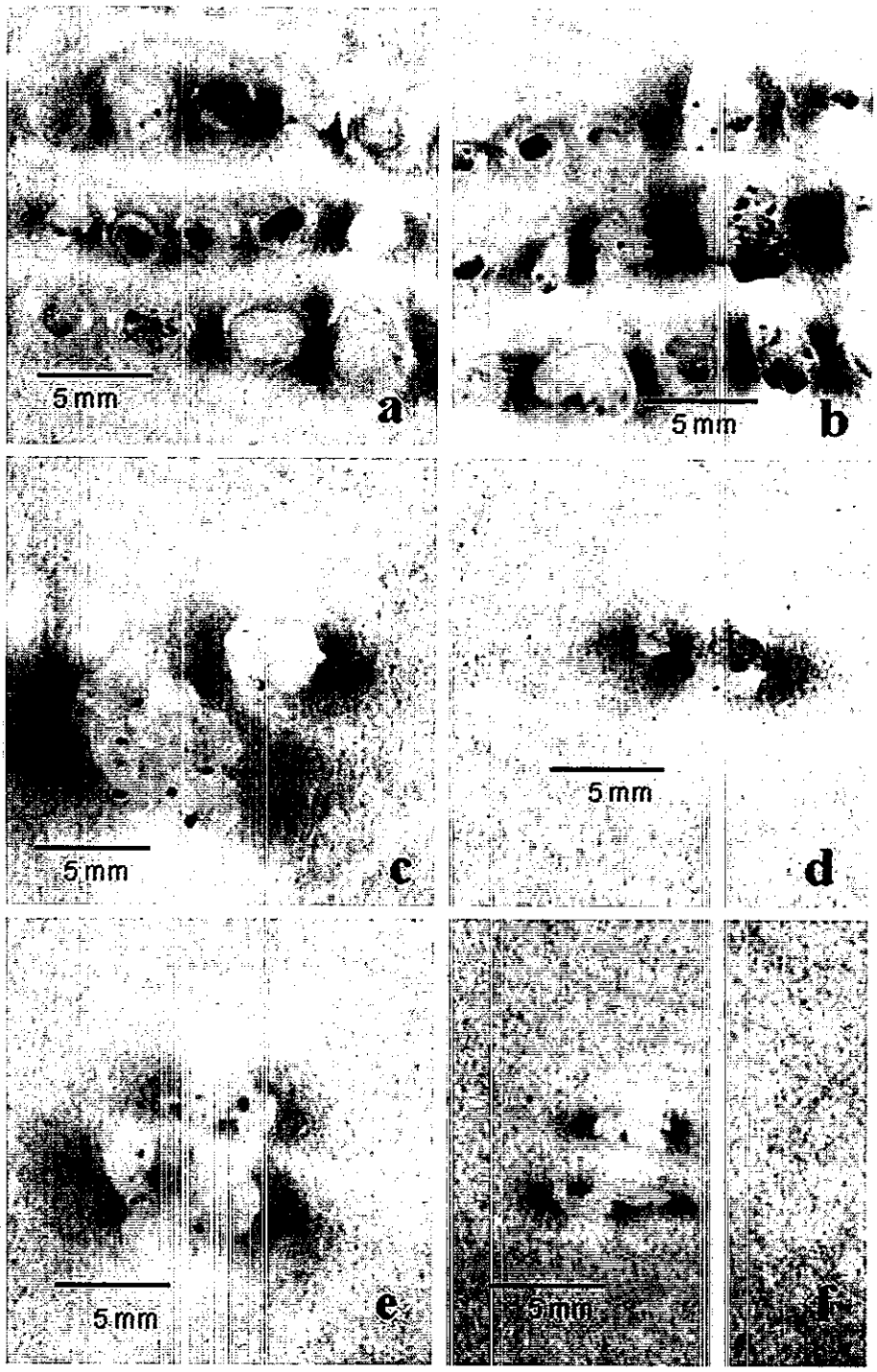


Plate I. GUS expression in Ornithogalum dubium cell clusters following microprojectile bombardment with various target-gene constructs. **a.** and **b.** Transient expression following bombardment with pCAMBIA ΔSV-NIb-nos-35S-GUS and pCAMBIA UBQ3-Nib-nos-35S-GUS, respectively. **c-f:** stable expression following bombardment with the plasmids containing ΔSV-CP (**c**); UBQ3-CP (**d**); ΔSV-NIb (**e**); UBQ3-NIb (**f**), and selection in a liquid medium containing 80 mg/l Kanamycin for 54, 77, 58, and 65 days, respectively.

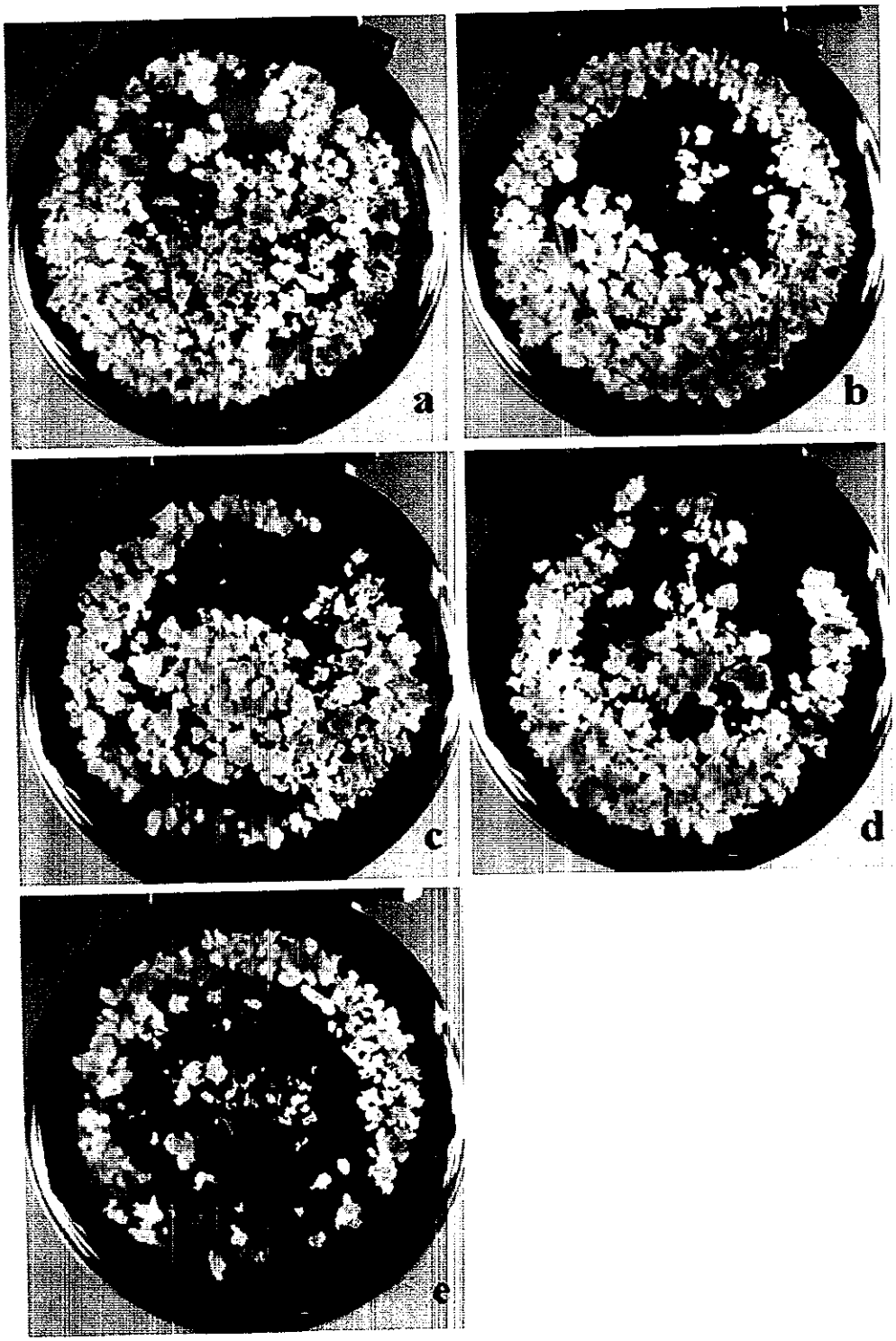


Plate II. Patterns of plantlet regeneration and the effect of Kanamycin in *Ornithogalum* cells grown in liquid MS regeneration medium in the light. All the cultures were previously maintained in medium #101. **a**: Onset of plantlet regeneration 4 weeks after the transfer to the light in control culture that was transferred to medium #206 for one week prior to the transfer to the light. **b**: Similar culture that was transferred to medium #206 two weeks prior to the transfer to regeneration medium in the light showing considerable delay in regeneration. **c**: Culture of the same origin as in **a** and **b**, that was transferred to a selective MS medium (with 80 mg/l Kanamycin) in the light showing a total block in plant regeneration. **d**: Cells bombarded with ΔSV-CP construct and maintained on a selective medium, 3 weeks after the transfer to selective regeneration medium in the light. There is no greening or regeneration at this stage but the cells continue to grow and proliferate. **e**: Non-bombarded (control) cells that have been maintained under the same conditions as in **d**, showing virtually no growth in the presence of Kanamycin.

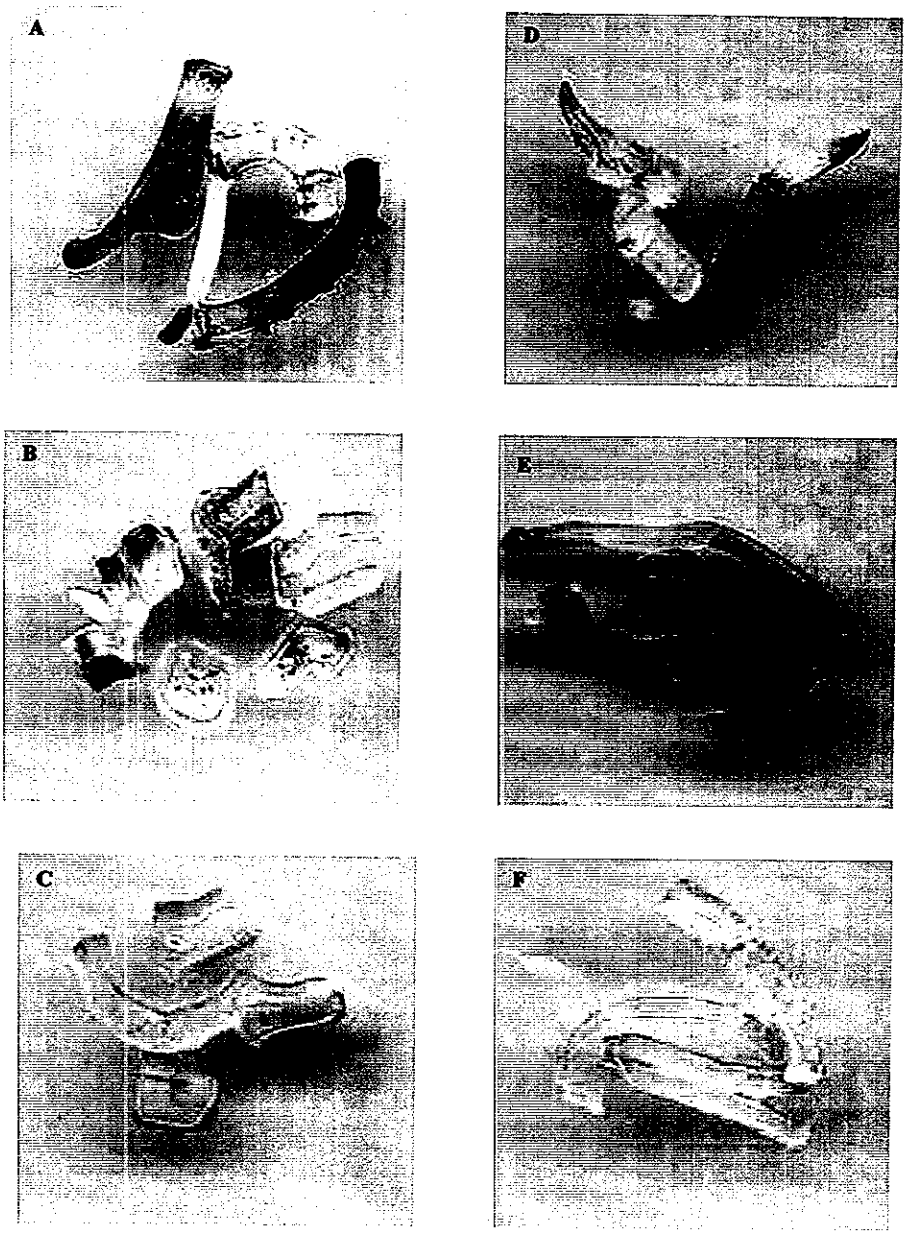


Figure B-2. Transient expression of putative transgenic *Ornithogalum* (A2 –*O.thyrsoides* x *O. dubium)* by means *Agrobacterium tumefaciens* mediated transformation. A2 callus was bombarded as follows: A. *Agrobacterium* AGL0 containing pSCV1.6; B. *Agrobacterium* C58C1 containing pSCV1.6; C. *Agrobacterium* LBA4404 containing pSCV1.6; D. *Agrobacterium* C58C1 containing pCambia3301; E. *Agrobacterium* AGL0 containing pCambia3301 and F. *Agrobacterium* LBA 4404 containing pGA 482.