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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. Transformation protocols using particle bombardment-mediated method (at the ARO), or *Agrobacterium*-mediated method (at ARC-Roodeplaat) to deliver genes into the plant tissue were developed.

At the ARO, *O. dubium* cell clusters were bombarded with four target gene constructs (the viral coat protein (CP) and replicase (Nib) genes, each under the control of the UBQ3 and ΔSV promoters). Since stable GUS expression was evident in the cultures bombarded with all the four constructs, the cultures were transferred to a selective medium supplemented with 80 mg/l kanamycin. Following a selection period in the dark, the cell clusters were transferred to the light for a regeneration of putative transgenic plantlets. The regenerated plantlets recovered from the cultures showed high level of hyperhydricity. They required several months of recovery on a solid medium to develop into normal-looking plants, and this caused an unforeseen delay in the project. GUS expression was evident in most of the plants recovered from the cultures.

The South African team calibrated the system for *Agrobacterium*-mediated transformation of leaf tissue as an alternative to nicroprojectile-driven transformation of callus. Plants recovered following selection on kanamycin were GUS-positive and the presence of the transgenes was confirmed by PCR analysis. The optimized *Agrobacterium* method was used to transform leaf disks of the South African *Ornithogalum* clone with the same constructs used by the Israeli team to transform cell clusters of *O. dubium* using particle bombardment. Most of the recovered plantlets showed GUS expression.

Two very important issues which were stated in the original objectives of the research plan remain to be dealt with. The putative transgenic plants developed both in South Africa and in Israel will have to pass two important tests: The first, being the molecular verification for the presence of the transgenes. In the second test, the transgenic plants will have to be challenged with virus infection using either mechanical means or by virus-carrying aphids, in order to determine whether the selected *Ornithogalum* plants carrying the various transgenes are, infect, resistant to virus infection

Section I

A. Research Objectives.

The objectives for the third year of the project were:

1. Continued selection on kanamycin of putative transgenic *Ornithogalum* plants regenerated from the cell clusters bombarded with microprojectiles carrying the various target-genes constructs. .
2. Optimization of *Agrobacterium* mediated transformation of breeding line A2 leaf material (at ARC-Roodeplaat).
3. Continued cloning of construct developed at ARC-Roodeplaat including constructs of the South African isolate of OrMV
4. *Agrobacterium*-mediated transformation of selected *Ornithogalum* A2 clone (in South Africa) with vectors carrying the target genes using callus and/or leaf disks and selection of transgenic plants.
5. Biolistic bombardment-mediated transformation to obtain stable transformants of the South African *Ornithogalum* with the constructs from Israel.
6. Molecular verification of the presence of the target-genes in the regenerated plantlets.
7. Challenging the putative transgenic plants with virus infection and estimation of the level of virus resistance conferred by the target genes.

B. Research accomplishments

B.1. Activities at the ARO.

Kanamycin as a selectable marker

Our attempt to use the herbicide Basta as a plant selectable marker in the initial stages of the research did not yield any transgenic *Ornithogalum* plants. Obvious difficulty in obtaining putative transgenic plants resulted from the drastic response of the explants. The presence of 1 mg/l Basta yielded too many "escapes" while 2 mg/l caused the tissue to bleach and excrete oxidation products into the medium with no obvious shoot regeneration. We had similar difficulty in our attempt to transform *Lilium longiflorum*, which we overcame after changing the selectable marker to kanamycin. We have decided to use the

kanamycin as the alternative marker also for *Ornithogalum*. It was clear that when *Ornithogalum* cells were exposed to kanamycin there was a strong reduction in cell proliferation and a total block in regeneration but the medium remained clear with no obvious excretion of oxidation products or cell death even in concentration as high as 150 mg/l. The blocking effect of kanamycin on the differentiation and development was obvious even when it was applied at a later stage when some differentiation had already initiated. In view of the very efficient regeneration after a prolonged selection in the presence of 80 mg/l kanamycin, we decided to check the effect of kanamycin on the later stages of regeneration and plantlet development, when the regenerated plantlets were subcultured to an agar-solidified medium. The results are presented in figure A-1. A control tissue that was maintained on a non-selective medium during cell proliferation and plant regeneration (Fig.A-1, upper left) was exposed for two weeks to a liquid medium supplemented with 20 mg/l kanamycin and then placed on either a solid MS medium with no kanamycin (upper right) or on a solid medium with 20mg/l kanamycin (lower right). Alternatively, the regenerated plantlets where transferred directly to a solid medium with 20 mg/l kanamycin (lower left). It became obvious that a transfer of the tissue to a relatively low concentration of kanamycin at this rather late stage of development totally inhibits further growth resulting in the death of the tissue. A two-week exposure of the tissue to kanamycin was sufficient to cause bleaching and death. Kanamycin was proven to be the appropriate selective marker for *Ornithogalum*. It also indicated that the plants regenerated following the prolonged selection on 80 mg/l kanamycin are probably transgenic.

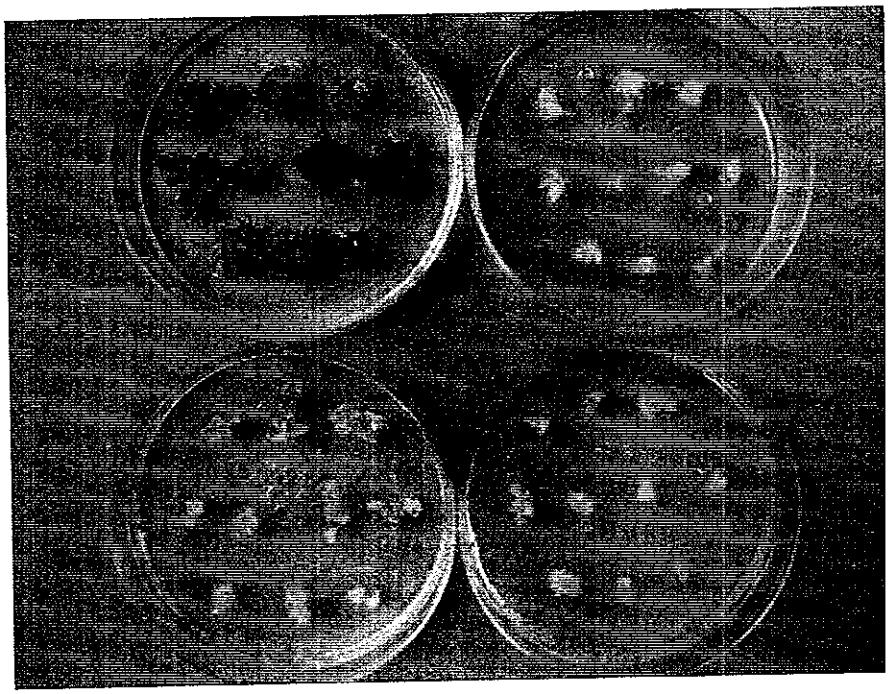

Figure A-1: The effect of 20 mg/l kanamycin on continued development of regenerating *Ornithogalum dubium* cultures (see the text for details). Plantlet development stopped and the cultures died when exposed to kanamycin.

Selection and regeneration of transgenic plants

In an attempt to obtain transgenic plants resistant to virus infection, cell clumps were grown in darkness in a liquid medium 206 (MS medium supplemented with 0.1 mg/l NAA, 2mg/l BA and 3% sucrose) on a gyratory shaker (100 rpm), for more than a year with successive subcultures every two weeks. The cells were bombarded with 4 constructs that included the two target genes: OMV coat protein (CP) and OMV-replicase (Nib), under the control of UBQ3 and DSV promoters. The cloning strategies used for the construction of the plasmids were made as well their respective restriction maps were reported in details in the 2nd annual report US-AID CDR Project C18-006, sept.2002. Following the bombardment, the cell were maintained in a selective liquid 206 medium in the presence of 80 mg/l kanamycin in the dark, with successive subcultures to a fresh selective medium every two weeks. Samples of cell clusters were taken from the culture flasks during the subcultures and these were checked for GUS expression. After the initial selection for two months, 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 darkness on medium 206 for continued selection on kanamycin. The regeneration in the first cell sample was slow and inefficient. It took more than a month

before the development of green centers of regeneration was apparent. In addition, the number of regenerants was very small despite the obvious presence of meristematic centers as was evident from the GUS expression in the cell samples taken from the culture flasks. The second part of the cells continued their growth under selective pressure with 80 mg/l kanamycin for additional 4 moths before being transferred to a regeneration medium in the light. Regeneration in this second batch of cells was rapid and by far more efficient. Within a week following the transfer to the light, the appearance of hundreds of green regeneration centers was apparent. These centers developed into shoots that showed the expression of the GUS reporter gene (Plate I). However, the developed plantlets were highly vitreous. It took several months on an Agar-solidified medium for these plants to recover from the hyperhydricity and develop into normal-looking transgenic plants. This caused an unforeseen delay in the project. The entire pathway for the development particle bombardment mediated transformation of *Ornithogalum* dubium is presented in plate I.

Putative transgenic plants with all four target gene constructs were recovered. Most of these plants show very strong GUS expression. Tissue samples are currently being collected from those that reached appropriate size in order to perform molecular verification to the presence of the transgene. It will take, however several more months for these plants to develop sufficient size before they could be challenged with virus infection and assessed for their virus resistance.

B.2. Activities at ARC-Roodeplaat

Cloning of ARC-Roodeplaat constructs

During the previous year the efficiency of transient expression of different plasmid vectors in *Ornithogalum thyrsoides* callus were compared These plasmids contained the *bar*GUS reporter genes under control of either the CaMV35S promoter (pDM327), enhanced CaMV35S promoter (pDM327-e35S), eCaMV35S promoter followed by the tobacco etch virus (TEV) translational leader sequence (pDM327-TEV), or *ubq*10 promoter (pDM327-ubq10). A negative control without a promoter (pDM327-neg) was also used. The best results were obtained with the e35S-TEV leader promoter, followed by the eCaMV35S, *ubq*10 and CaMV35S promoters. The coat protein gene of the South African isolate of *Ornithogalum* mosaic virus (SA-OrMV) was cloned under the control of the enhanced CaMV35S and e35S-TEV control elements of the pRTL2 plasmid vector.

In previous experiments *Ornithogalum* plant tissue performed much better with less oxidation on kanamycin selection. It was decided, therefore, to clone the SA-OrMV construct in pCAMBIA 2301 under the control of the CaMV35S or e35S-TEV promoters.

Cloning of the OrMV constructs

In the previous year, the South African team reported on an attempt to clone the coat protein (CP) 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 sub-cloned from pA7 (pBI221 containing the SA-OrMV coat protein gene) into pRTL2. These efforts resulted in the construction of two recombinant plasmids named pRTLTEVOMVCP and pRTLe35SOMVCP (see 2nd annual report US-AID CDR Project C18-006, sept.2002, for details of the procedure and restriction enzyme maps of the two recombinant plasmids). The next step was to sub-clone the expression cassettes into pCAMBIA 2300 that includes multiple cloning sites, GUS reporter gene and nptII gene conferring kanamycin resistance as a selectable marker.

Cloning of OrMVCP downstream of the e35S-promoter (with or without the TEV leader) in pCAMBIA2300.

Attempts were made at ARC to sub-clone the SA-OrMVCP gene from pRTLTEVOMVCP (pRTL2 containing the SA-OrMV coat protein gene under the control of the e35S promoter and TEV leader control elements) and from pRTLe35SOMVCP (pRTL2 containing the SA-OrMVCP gene under control of the e35S promoter) into pCAMBIA2300 as *Hind*III fragments. *Hind*III excises the SA-OrMVCP gene together with the upstream and downstream control elements as an expression cassette. The pCAMBIA2300, pRTLTEVOMVCP and pRTLe35SOMVCP plasmids were prepared and restricted with *Hind*III. The appropriate fragments were extracted from the gel and the required fragments legated to the vector. After transformation of *E. coli* cells, no colonies were obtained. Re-ligated vector controls also yielded no colonies. The same results were obtained in subsequent repetitions.

We tried to determine whether the *Hind*III digested vector could re-ligate with greater efficiency if it hadn't been extracted from the gel. *Hind*III digestion reactions of vector plasmid were heat inactivated and used for ligation reactions. It was found that the yield of colonies transformed with ligated *Hind*III-digested vector plasmid was greatly increased

compared to the unligated background control. It was therefore decided to use the *Hind*III digested vector for ligation of the inserts without cleaning up the vector first by extraction from the gel, to try and increase the number of the recombinant colonies obtained (which was previously zero). When these ligation reactions were transformed into *E. coli*, only blue colonies were obtained. PCR screening using the OMVCP primers of eight of each type of insert indicated that four colonies putatively contained the e35S-OMVCP-pCAM2300 constructs. No putative positive TEV-OMVCP-pCAM2300 colonies were obtained, even after PCR screening of an additional twenty-one colonies.

When these four putative e35S-OMVCP-pCAM2300 colonies were screened by miniprep plasmid isolation and *Hind*III restriction digestion, none contained the expected insert. Blunt ligation was attempted in case there was a problem with the *Hind*III sticky overhangs that prevented the fragments from ligating. The two inserts and pCAMBIA2300 vector, previously prepared with *Hind*III and gel-extracted, were filled in by a Klenow reaction, cleaned-up, ligated and transformed into *E. coli*. This strategy however also proved to be unsuccessful, since no colonies were obtained.

Biolistic bombardment of Ornithogalum A2 callus for transient transformation with different promoters.

The following constructs were received form Israel during August 2002. These plasmids contained the barGUS reporter genes under control of either the UBQ3 (pDM327-UBQ3) promoter, the strawberry vein-bending virus promoter (pDM327-ΔSBV) and the rice Act1 (pDM327- Act1) promoter. Six different promoters were compared for their efficiency of transient expression in *Ornithogalum* A2 callus by microprojectile bombardment. These promoters include the three Israeli and three ARC-Roodeplaat constructs, CaMV35S (pDM327), enhanced CaMV35S (pDM327-e35S) and the eCaMV35S followed by the tobacco etch virus (TEV) translational leader sequence (pDM327-TEV). No one promoter performed discernibly better than the others. All the promoters were functional and gave more or less the same results.

Biolistic transformation for stable transformation with the Israeli constructs.

Due to the unsuccessful cloning of the ARC-Roodeplaat constructs into the pCAMBIA 2300 vector, it was decided to continue the experimental work with the constructs designed by the Israeli team. These constructs, IS-OrMV-CP (*Ornithogalum* mosaic virus coat protein gene) and SA-OrMV-NIB (*Ornithogalum* mosaic virus replicase gene), under the control of either the ΔSV or the UBQ3 promoter were sent to South Africa during August 2002. The constructs were transformed into *E. coli* and DNA was isolate with a Qiagen® mega plasmid kit. *Ornithogalum* callus was bombarded with the different constructs from Israel using the Biorad PDS Helium device® for stable transformation. Two days after bombardment the tissue was transferred to regeneration medium (0.1mg/l NAA and 2mg/l BA) supplemented with 80mg/l kanamycin (for selection). These plates were cultured in a growth room with a 16 hr photoperiod at 23°C. Every 2 weeks the plants were transferred to fresh medium containing kanamycin. Although there was some shoot formation on the selection medium soon after bombardment, the callus and shoots eventually become discolored and died off.

Optimization and transient transformation of breeding line A2 leaf material with *Agrobacterium* mediated transformation

During 2002 an *Agrobacterium tumefaciens*-mediated transformation protocol for *Ornithogalum* A2 was developed and optimized. A comparison was made between different *A. tumefaciens* strains (LBA 4404, C58C1, and AGL0 or AGL1) and plasmids (pCSV1.6, pCAMBIA3301, pCAMBIA2301, and pGA482) in their ability to transform *Ornithogalum*.

Leaves of *in vitro* plants were cut 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 suspensions and co-cultivated for 3-5 days. Some leaves were stained for GUS expression and the rest of the leaves were transferred to pre-selection medium with 250 mg/l cefotaxime. Eleven days after transformation some of the leaves were stained and the rest were transferred to selection medium containing 80mg/l kanamycin and 250 mg/l cefotaxime. The best results were obtained with *Ornithogalum* A2 that was co-cultivated with *Agrobacterium strain* AGL0 containing the recombinant plasmid pSCV1.6, and it appeared that after 11 days the GUS expression was greater than after 5 days. The *Agrobacterium* strain AGL0 seemed to give rise to higher transient expression levels than

strains C58C1 or LBA4404. Very little blue staining was observed for explants co-cultivated with the bacterial strain LBA4404.

The transformed explants were selected on 80mg/l kanamycin during regeneration. The regenerated plants were transferred to MS medium with 80mg/l kanamycin. The shoots that regenerated from the leaf disks that survived the kanamycin selection were stained for GUS activity. Only two shoots transformed with the *Agrobacterium* strain C58C1 plasmid pSCV1.6 grew on the selection medium with 80mg/l kanamycin. The leaves and the roots of the 2 plants, A_2G1 and A_2G2 were stained for GUS activity and were both were found to be positive. These two plants were multiplied from leaf explants on MS medium with 2mg/l BA, 0.1mg/l NAA supplemented with 80mg/l kanamycin. The shoots were transferred to MS medium with 80mg/l kanamycin.

DNA was isolated from A_2G1 and A_2G2 plants and also a control A2 plant using a SIGMA Genelute plant genomic DNA kit®. Verification of the presence of the *nptII* (kanamycin) and the GUS gene was done by PCR. The presence of the GUS gene was confirmed using the following GUS primers: GUS intron L and GUS intron R. The presence of the *nptII* gene was confirmed using the following *nptII* primers: *nptII* L and the *nptII* R. The GUS gene fragment is approximately 1 200 bp long and the *nptII* gene fragment is approximately 680 bp long. Fifty ng of DNA was used per reaction. The $MgCl_2$ concentration was 4mM. Both primers sets anneal at 64 °C. The following cycle profile was used:

Temperature	Time (seconds)	Number of cycles
94.5 °C	90	1
94.5 °C	15	35
64 °C	20	
72 °C	30	
72 °C	120	1
4 °C	600	1

Figures B-2 and B-3 show the bands obtained from the PCR reactions on 1% agarose gels.

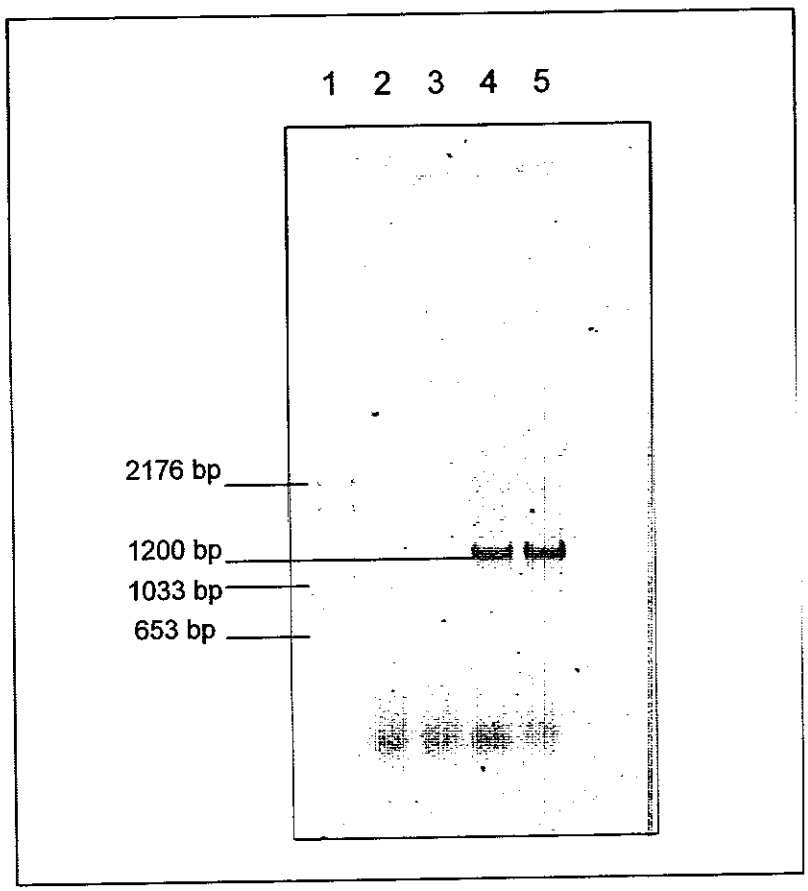

Figure B-2: The presence of the GUS gene was confirmed using the GUS intronL and GUS intronR primers. Lane 1; molecular marker VI, lane 2; water control, lane 3; negative control, lane 4; A2G1 (transformation event 1), lane 5; A2G2 (transformation event 2).

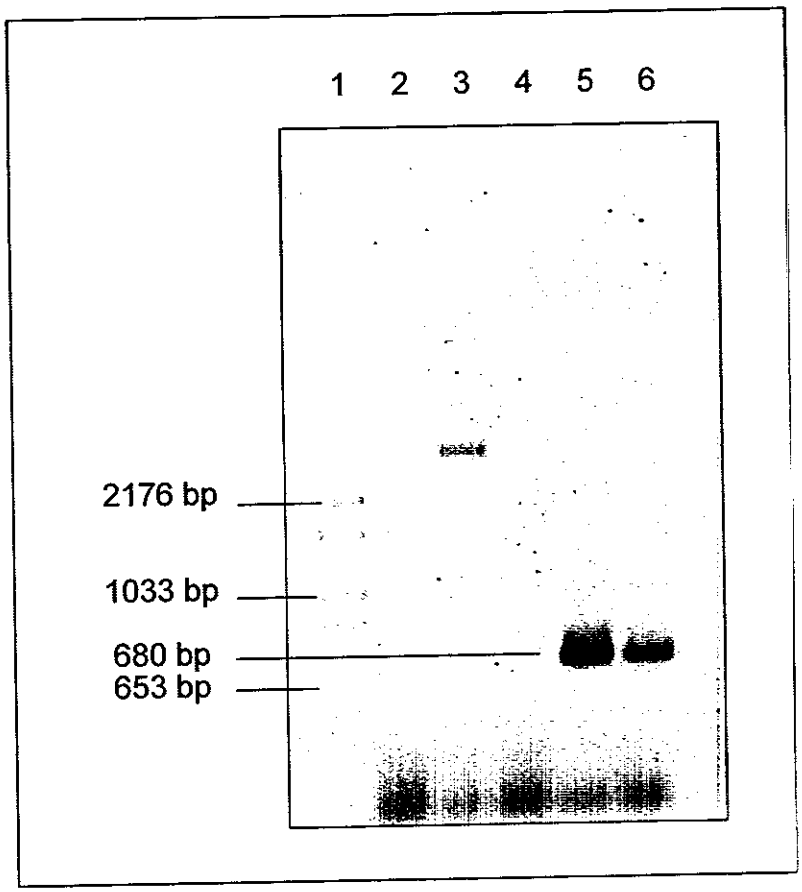

Figure B-3: The presence of the NPT II gene was confirmed using the NPT IIf and the NPT IIr primers. Lane 1; molecular marker VI, lane 2; water control, lane 3; positive control, lane 4; negative control, lane 5; A2G1 (transformation event 1), lane 6; A2G2 (transformation event 2).

These results showed that *Agrobacterium tumefaciens* could be used for transforming *Ornithogalum* breeding line A2.

Leaf disk transformation of *Agrobacterium* with the Israeli promoters

Using tri-parental mating, *Agrobacterium tumefaciens* strain AGL1 was transformed with the constructs cloned in Israel, i.e. IS-OrMV-CP and SA-OrMV-NIb under control of either the ΔSV promoter (isolated from the strawberry vein-bending virus) or the UBQ3 promoter. The optimized *Agrobacterium tumefaciens* protocol was used to transformed *Ornithogalum* A2 leaf disks.

The shoots that developed on MS medium with 2mg/l BA, 0.1mg/l NAA supplemented with 80mg/l kanamycin were subcultured to MS medium with 80mg/l kanamycin. Table 1 shows the number of shoots that regenerated on kanamycin selection.

Table 1. Number of shoots that regenerated on kanamycin selection (80mg/l).

Name of construct	ΔSV Is-OrMV-CP	ΔSV SA-OrMV-NIb	UBQ3 SA-OrMV-CP	UBQ3 Is-OrMV-NIb
Number shoots	221	178	163	177

The shoots (Table1) that survived on 80mg/l kanamycin were tested for GUS activity. These shoots were transferred to fresh selection medium. After a month some of the shoots began to discolor and the shoots that remained green were stained again for GUS activity. The results are summarized in table 2.

Table 2: Shoots that survived on selection medium and were stained for GUS activity, transformed with either the *Ornithogalum* replicase Gene, OrMV-NIb or the *Ornithogalum* Coat protein gene, IS-OrMV-CP.

Plant #	Promoter	NIb gene	CP gene	Expression of GUS activity	Plants survived on selection	After 2 month on selection medium 80mg/l kanamycin
1	ΔSV			Faint	Yes	Discolored
2	ΔSV			Faint	No	-
3	UBQ3	X		-	Yes	Discolored
4	UBQ3		X	Strong	Yes	Green
5	UBQ3		X	-	No	-
6	ΔSV	X		Faint	Yes	Discolored
7	ΔSV	X		Edge of the leaf	No	-
8	ΔSV	X		Faint	Yes	Discolored
9	ΔSV	X		Moderate	Yes	Green
10	UBQ3		X	Faint	Yes	Discolored
11	UBQ3		X	Faint	No	-
12	UBQ3	X		Moderate	Yes	Discolored
13	UBQ3	X		Moderate	Yes	Discolored
14	UBQ3	X		-	Yes	Discolored
15	UBQ3	X		Faint	No	-
16	UBQ3	X		Faint	Yes	Discolored
17	UBQ3		X	Faint	Yes	Discolored
18	ΔSV	X		-	Yes	Discolored
19	ΔSV	X		Faint	No	-
20	ΔSV	X		Faint	No	-
21	ΔSV	X		-	No	-
22	ΔSV	X		Faint	Yes	Discolored
23	ΔSV	X		Faint	No	-
24	UBQ3		X	Strong	Yes	Green

Replicase Gene: SA-OrMV-Nib

Coat protein gene: IS-OrMV-CP

Only plants number 4 transformed with the UBQ3 IS-OrMV-CP gene, number 9 transformed with the ΔSV SA-OrMV-NIb gene and number 24 transformed with the UBQ3 IS-OrMV-CP gene survived the prolonged on selection medium with 80mg/l kanamycin. These shoots will be multiplied and PCR will confirm the insertions of the genes.

C. Scientific Impact of Collaboration

In order to find the most efficient promoters for the use in *Ornithogalum* transformation, six different promoters were compared at ARC-Roodeplaat for their efficiency to bring about transient GUS expression in *Ornithogalum* callus by microprojectile bombardment. Three of them were constructed by the Israeli team and the other three were constructed in South Africa. The South African compared the functioning of six promoters in callus of Ornithogalum A2. Since no one promoter performed discernibly better than the others and all of them were functional and gave more or less the same results, it was decided to use UBQ3 and ΔSV promoters. UBQ3 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 Israeli team cloned both the Israeli isolate of OrMV coat protein and the NIb (replicase) gene that was isolated by the South African team, under the control of UBQ3 and ΔSV. These four constructs were used by both collaborating groups in the attempt to transform *Ornithogalum* clones using Agrobacterium-mediated transformation (in South Africa), or the particle acceleration method (in Israel). All the construct use include kanamycin as a selectable marker following the mutual decision made to discontinue the use of the herbicide Basta as a selectable marker for transgenic *Ornithogalum*, due to the narrow margin between concentrations that allow "escape" of non-transgenic plants and the one that caused necrosis and cell death.

D. Description of Project Impact

Since no promoter was found more effective by the transient GUS expression test, ARC-Roodeplaat, based on the previous year's results, went ahead and cloned the SA-OMV CP gene under control of the enhanced CaMV 35S promoter and the e35S-TEV control elements. The cloning of these constructs to pCAMBIA2300 was unsuccessful and so the work continued with the Israeli constructs.

The Biorad PDS Helium Device® was not as successful as hoped in transforming the South African *Ornithogalum* A2 callus. The *Agrobacterium* method seemed to be more efficient for the South African selection than the biolistic transformation method. In contrast, the Israeli self-constructed particle inflow gun (PIG) used for biolistic transformation, was found very efficient in transforming the Israeli selection of Ornithogalum *dubium*. In fact, the system is so efficient that the number of putative transgenic plants is too large to handle. Following molecular verification and the challenge with virus infection, plants proven to contain the genes for virus resistance could either be propagated as such, or be used in a breeding program to transfer virus resistance to future breeding lines.

E. Strengthening of Developing Country Institutions

The optimized *Agrobacterium*-mediated transformation of *Ornithogalum* A2 tissue, lead to the stable transformation of *Ornithogalum* A2 for virus resistance.

Ornithogalum mosaic virus also infects *Lachnelia*, indigenous South African flower bulb, and is one of its major diseases. *Lachnelia* is one of the flower bulb species that is part of ARC-Roodplaat breeding program with a market potential for the small scale growers. Techniques learned and the knowledge gained from the project using either the microprojectile technique or *Agrobacterium*-mediated transformation could be used for future transformation protocols for other indigenous South African flower bulbs especially for Lachenalias. In addition, the system could also be used to transform other *Ornithogalum* breeding lines with other genes of merits, like ones that confers resistance to bacterial diseases such as Erwinia, which is a major cause of yield losses in *Ornithogalum*, in *Lachenalia*, as well as in many other bulbous crops.

F. Future Work

Due to unforeseen delays resulting from the unsuccessful sub-cloning of the South African OrMV CP gene into the appropriate plasmid (in South Africa) and the need to recover from hyperhydricity (vitreousity) in the Israeli cultures, two main objectives of the project have yet to be met. The first objective is the molecular verification using PCR for the presence of the OrMV CP and Nib (replicase) genes, *as well as nptII* and GUS transgenes in the putative transgenic plants in both South Africa and Israel. The second step is the confirmation whether the transgenic plants are, in fact, resistant to virus infection. Transgenic plants will

undergo small-scale *in-vitro* propagation and the plants will be challenged, in the glasshouse, with virus infection using either mechanical means or by virus-carrying aphids. The presence or absence of the virus in the challenged plants will be checked by ELISA.

New attempts will be made in ARC-roodeplaat to subclone the SA-OrMVCP gene from pRTLTEVOMVCP and from pRTLe35SOMVCP into pCAMBIA2300 as *Hind*III fragments. If pCAMBIA2300 continues to give negative results an alternative vector will be selected.

Section II

A. Managerial issues

Staff changes: Dr. Michele Cloete left ARC-Roodeplaat. Her function as project manager was taken over by Mr. Gurling Bothma. Dr Lynelle van Emmenes started work at ARC-Roodeplaat at the beginning of August 2003.

B. Budget

Procurement of equipment

The following laboratory equipment was purchased at ARC during the year:

Air conditioner for growth room: USD 1632.00

Gel documentation system: USD 10486.00

This amount includes budget for equipment not used from year one as well as years two and three combined.

Funds requested from USAID

ARC-Roodeplaat requests the re-imbursement of the amount spent on the project that is documented in the financial report.

C. Special concerns

None

D. Collaboration, Travel, Training and Publications

Travel: None

E. Requests for American Embassy Tel Aviv or AID actions

1. Refer to budget section

2. Due to the delays in obtaining the results in the project and the need for future work we request that we will be granted an extension that will be allow us the use of the unused funds allocated for the project for another year.

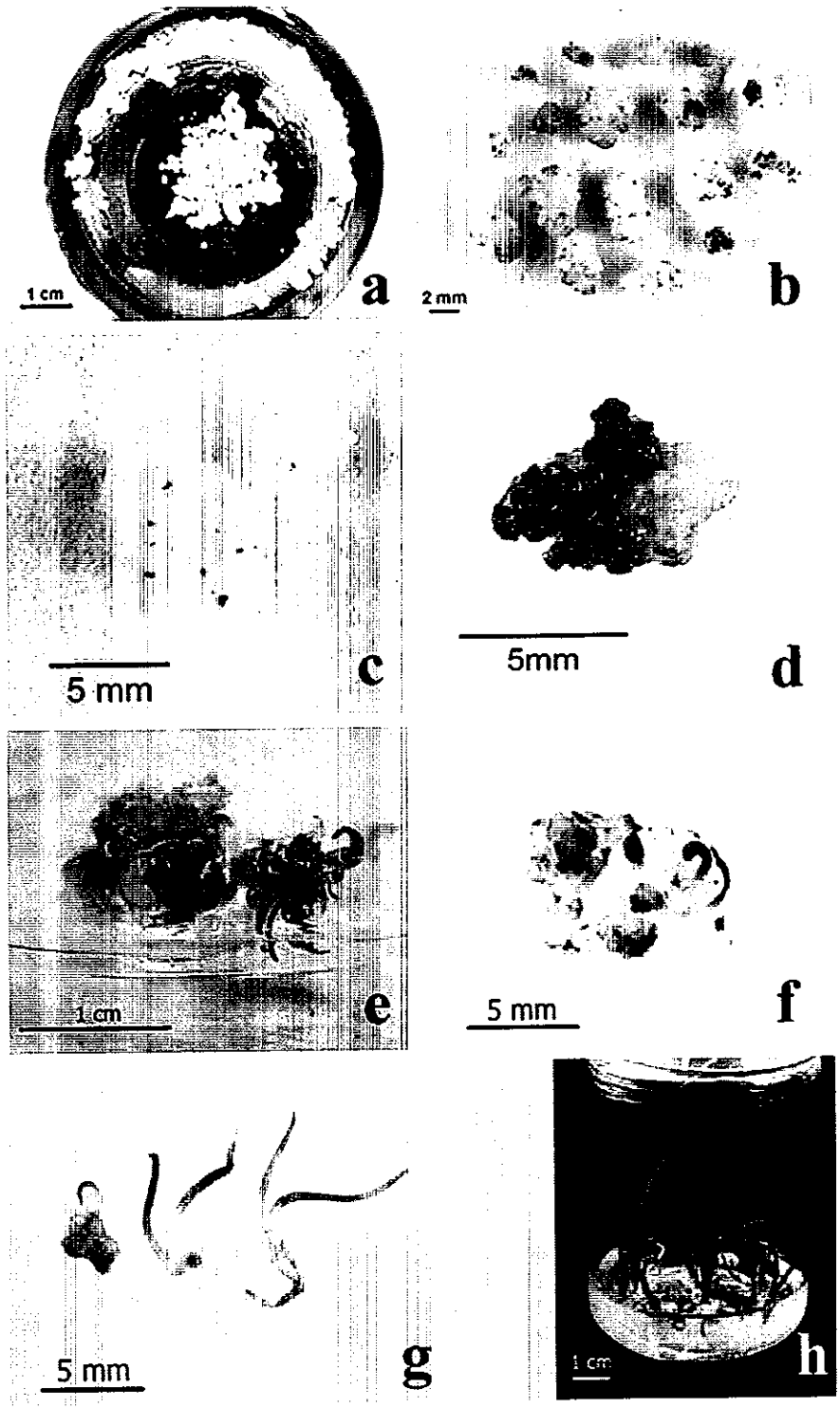

Plate I: Genetic transformation of *O. dubium*: **a**: Cell clusters of *Ornithogalum* grown in liquid medium 206 in darkness prior to the bombardment. **b**: Transient GUS expression in competent cells bombarded with pUBQ3genGUS. More than 1300 GUS expressing foci were counted per g FW. **c**: Stable expression of the reporter gene in cells grown in a selective medium containing 80mg/l kanamycin for 54 days. **d**: The development of organized transgenic meristematic centers in the liquid medium after prolonged selection. **e**: Plantlet regeneration in light-grown cultures in liquid MS medium containing 80 mg/l kanamycin. **f**: GUS expression in regenerating cell clumps from the culture presented in plate I-e. **g**: GUS expression in transgenic plantlets regenerated in the liquid cultures. The plantlet on the right in a non-transgenic control. **h**: Transgenic Ornithogalum plants in a solid MS with 50mg/l kanamycin.