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ANNUAL REPORT

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SUBMITTED TO THE U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT

TRANSGENIC SEEDLESSNESS IN GRAPES BY USING GENETIC ABLATION OF SEEDS

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Grantee Institution: Volcani Center

Collaborator: Dr. Phyllis Burger

Institution: ARC-Fruit, Vine and Wine Research Institute

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2000/2001-RESEARCH REPORT : US-AID

FRUIT TYPE : Grapes **PROJECT NUMBER** : WW 14/13

ORGANISATION : ARC Infruitec-Nietvoorbij **RESEARCHER** : P. Burger

PROJECT TITLE : Transgenic seedlessness in grapes by using genetic ablation of seeds.

OBJECTIVE OF THE PROJECT :
Currently, at the institute, seedless grapes are being developed through crosses involving seedless parents. However, the application of cross-breeding is limited as progeny characteristics are unpredictable, because both parents contribute genetic material. Therefore, a single characteristic of a certain variety cannot be altered in this way. The use of specific biotechnological techniques enable researchers to change a single characteristic. The aim of this project is the development of seedless varieties by employing genetic transformation techniques to incorporate a "seed ablation" gene that changes the seeded characteristic to one of seedlessness.

OBJECTIVES FOR PRESENT YEAR :
Continuous multiplication of somatic embryos of Redglobe, Bonheur and Majestic to supply enough material for genetic transformation with the "seed ablation" gene. Selection of transformed cells and regeneration there-of. Initiate somatic embryos of Dauphine.

FINDINGS TO DATE :
The first payment was received from the US-AID in February 2001. Redglobe embryos were received from Dr Perl, the Israeli partner in the project. These embryos as well as Bonheur and Majestic embryos were multiplied during the report period to supply sufficient material for genetic transformation experiments. The gene was received from Dr Perl in January 2001 and the first transformation experiments were done recently. Currently embryos are in the selection process for transformants. Somatic embryos were not initiated from *in vitro* leaves and petioles of Dauphine as it was put in culture too recently.

ADVANTAGES FOR INDUSTRY :
Seedlessness is a desired characteristic and to date seedless varieties were developed at the institute by cross-breeding with seedless parents. In this project genetic transformation techniques are employed in an attempt to develop seedless varieties from existing seeded varieties by altering this specific characteristic only. In this way a contribution is made towards the development of a greater variation of seedless grapes.

RECOMMENDATIONS FOR COMING YEAR :
Somatic embryos of other important South-African varieties (Dauphine and La Rochelle) should be developed. Transformation of varieties of which sufficient embryos are available and development of plantlets from transformed embryos. The transformation process should be optimised.

INDUSTRY FUNDING FOR CURRENT YEAR :
This is a contract project in collaboration with Dr Avi Perl from the Volcani institute in Israel and runs over a period of three years. Funding is received from the American government (US-AID).

2000/2001 – RESEARCH REPORT

PROJECT NUMBER	RESEARCHER	PRINCIPAL PARTNER
WW 14/13	P. Burger	Dr A Perl

PROJCT TITLE

Transgenic seedlessness in grapes by using genetic ablation of seeds

ACKNOWLEDGEMENTS

Acknowledgement is given to the American government for funding of the project.

PROBLEM ADDRESS/OBJECTIVES OR HIPOTHESIS

Currently, at the institute, seedless grapes are developed by conventional breeding and embryo rescue techniques involving seedless parents. As crosses are made progeny characteristics are unpredictable and differ from that of both parents. This implicates that a single characteristic of a certain variety cannot be changed in this way. For example, the seeded characteristic cannot be altered to seedlessness, without changing other characteristics as well. However, by implementing certain specific biotechnological techniques it is possible to incorporate this specific characteristic into a known variety. The objective of this project is the development of genetic transformation techniques for important seeded varieties and the development of seedless variants from these by using a seed ablation gene to alter the seeded characteristic to one of seedlessness.

RESEARCH NEED ADDRESSED

Foreign funding

OBJECTIVES OF REPORT PERIOD

1. Develop somatic embryogenesis systems of important seeded South African varieties.
2. Multiply somatic embryos of Bonheur and Redglobe.
3. Do transformations with the "seed ablation" gene on embryogenic material of above mentioned varieties.

MATERIAL AND METHODS

1. **Cultivation of embriogenic callus cultures for transformation**

Somatic embryos of Bonheur and Redglobe were multiplied every six to eight weeks on a multiplcation medium (SEM). During the report period embryos were also developed from the variety Majestic in project WW14/11. Currently these embryos are multiplied for genetic transformations in projects WW14/11 and

WW14/13. Results obtained recently with Majestic indicated that higher rates of somatic embryo initiation per explant were obtained when *in vitro* petioles were used as compared to leaflets. Currently leaflets and petioles of Dauphine are being cultivated on various media to initiate somatic embryos. The composition of these media are given in Table 1.

Table 1. Media used to initiate somatic embryos from *in vitro* leaflets and petioles of the variety Dauphine.

1	SEI (Nitsch medium with 1 µM BA + 5 µM 2,4-D) + 10uM ABA
2	SEI + 3 g/l Gelrite (instead of agar) + 10 uM ABA
3	SEI + 3 g/l Gelrite + 20 uM 2,4 D + 9 uM BA
4	SEI + 3 g/l Gelrite + 10 µM 2,4 D + 4,5 uM BA
5	Half MS + 0,8 g casein acid hydrolysate + 60g/l sucrose + 0,1 g/l inositol + 5 mg/l IASP + 2 mg/l NOA + 0,2mg/ BA + 2g/l activated charcoal
6	N + Bouquet & Davis amino acids and 1 g/l caseien acid hydrolysate + 1 µM BA + 5 µM 2,4-D
7	SEI + 266 mg/l $Ca(NO_3)_2$ instead of $CaCl_2$ + 3 g/l Gelrite + 20 µM NOA + 4 µM TDZ (no PHE)
8	N macro + MS micro + N vit. + 60 g sucrose + 3 g/l Gelrite + 2,5 uM 2,4 D + 2,5 uM NOA + 5 uM CPPU
9	SEI (Nitsch medium with 1 µM BA + 5 µM 2,4-D) Control

SEI = Nitsch medium with 1 µM BA + 5 µM 2,4-D
N = Nitsch medium
MS = Murashige and Skoog medium

Young *in vitro* leaflets and petioles (five of each per petridish) were cultivated on each of these different media and sixteen dishes were used per medium. Leaflets are cultured in dark at 25 ± 2 °C.

2. Genetic transformation

Somatic embryos of Redglobe, Bonheur and Majestic were used in transformation. As few embryogenic material of Bonheur and Majestic are available these two varieties were used in small quantities. The *Agrobacterium* culture was received from Dr Perl in January 2001 and is maintained at the Institute for Wine Biotechnology (University of Stellenbosch), where it is also prepared for use in transformations. The procedure is as follows (it was adapted from a procedure of Dr Perl's).

1). Put a volume of an *A. tumefaciens* culture, with the "seed ablation" gene, in 20 ml liquid SEM.

2). Transfer young somatic embryos to this, shake lightly and let stand for 10 minutes.

3). Decant liquid and plate embryos on solid SEM medium in petridishes. Leave for three days in growth conditions of 16 h light at 25 ± 2 °C.

4). Transfer embryos to 50 ml liquid Nitsch medium with 20g/l sucrose, 100 mg/l DTT and 300 mg/l claforan (cefotaxime) in an erlenmeyer flask. Put on a shaker (80 – 100 rpm) for 1 hour. Replace the medium every hour for three times.

5). Transfer embryos to 50 ml liquid Nitsch medium with 50 mg/l kanamycin and 400 mg/l claforan. Cultivate in dark at 25 °C and 100 rpm. Replace the medium every second day, gradually increasing the kanamycin concentration to 100 mg/l.

6). After about one month embryos are selected and are transferred to a solid germination medium (Nitsch, 1969 medium with 1 mg/l BA).

This procedure was followed only once and is currently in the selection phase (step 5). Approximately 35 petridishes were used in the experiment.

RESULTS AND DISCUSSION

1. Cultivation of embryogenic callus cultures for transformation

Somatic embryos of Redglobe and Bonheur were multiplied to supply in the need for genetic transformation. The leaflets and petioles of Dauphine were put in culture too recently to obtain results as it was found previously that it approximately takes between six to eight months before embryos develop from explants (leaflets on the control medium). Results will be reported in full in the next report.

2. Transformation

Embryos that were transformed are currently in the selection process for transformants and will be transferred to a germination medium to induce shoot growth after it has been selected. Plantlets developing from germinating embryos will be tested for successful genetic transformation.

The transformation process used, has the advantage of better control of *Agrobacterium* over-growth as with a solid medium method that was used previously in out laboratory. However, a higher percentage embryos turned brown as compared to using the solid medium (Project WW14/11). It may be ascribed to various causes including that gene transfer to plant tissue might have been less successful. It could also be that the change from a liquid to a solid medium, with a different constituency had a big influence on the embryos (previously the same solid medium with the addition of antibiotics was used throughout the transformation process). It should be investigated to adapt the SEM medium on which embryos are multiplied prior to transformation for use in a liquid phase instead of liquid Nitsch medium that is being used currently.

RECOMMENDATIONS

!. Somatic embryos of other important seeded South African varieties (Dauphine en La Rochelle) should be developed.

2. The possibility of keeping the *Agrobacterium* culture at Nietvoorbij and preparation thereof for transformation should be investigated.
3. Genetic transformations should be done with varieties when sufficient material are available (Redglobe, Bonheur and Majestic).
4. Investigate the exchange of liquid Nitsch medium with liquid SEM medium in the selection phase.

OBJECTIVES FOR 2001/2002

1. Initiate and multiply somatic embryos from Dauphine and La Rochelle.
2. Multiply somatic embryos of Bonheur, Redglobe and Majestic.
3. Execute transformations on embriogenic material of above-mentioned three varieties.
4. Germinate and regenerate transformed embryos.
5. Develop plantlets from transformed embryos.
6. Do DNA testing on possible transformants.

PROJECT RISKS

Risk factors that may have an effect on the project are the following :

1. The antibiotic cefotaxime (claforan) may have detrimental effects on normal development and germination of somatic embryos.
2. The possibility that transformed embryos fail to germinate and do not develop into plants.
3. The possibility exists that transformed plants fail to be seedless or may be partially seedless.
4. It is possible that transformed plants may exhibit viticultural qualities that differ from that of the original varieties.

BUDGET: 2001/2002 **PROJECT : WW 14/13**

TIME PERIOD	Running	Direct salaries	Overhead costs	Total
2001/2002	124 809	238 107	71 142	434 058
2002/2003				
2003/2004				

EXTERNAL FUNDING

The following budget is as approved by US-AID and is given in American dollar.

Item	Year 1	Year 2	Year 3	Total
Salaries ###	10 000	14 000	14 000	38 000
Equipment	7 000	8 000	-	15 000
Material	8 000	9 000	7 500	24 500
Travel	3 000	2 000	3 000	8 000
Computer etc.	1 000	1 000	1 000	3 000
Field tests	-	3 000	3 000	6 000
Total Direct costs	29 000	37 000	28 500	94 500
Indirect costs	4 350	5 500	4 394	14 244
Total	**33 350**	**42 500**	**32 894**	**108 744**

It is not allowed that salaries be paid to permanent personnel and these funds are therefore applied for a contract worker.

(b) Other organisations approached for funding for 2001/2002:

None

(c) Funding received from other organisations for 2000/2001:

None

TRANSGENIC SEEDLESSNESS IN GRAPES USING GENETIC ABLATION OF SEEDS

SCIENTIFIC REPORT FOR THE FIRST YEAR

ISRAELI PROGRESS

Introduction and Description of the Problem

From the point of view of the consumer, seeds are an unattractive aspect of these fruits. Many high yielding, fine tasting grape cultivars have already been produced by conventional breeding, yet, some of these cultivars are often seedy, and their market value is low. It would be advantageous to eliminate the seeds but maintain the other favorable characteristics of the cultivar unaltered. It has been demonstrated that selected genes can be introduced into grapes by molecular transformation, and plants were successfully regenerated. This proposal aims to develop new grapes cultivars which produce seedless fruits. This purpose will be achieved by introducing heterologous lethal genes from other origins, which are specifically expressed in seeds leading to the destruction of seeds as they develop inside the fruit.

Research Objectives

The overall aim of this proposal is to examine the feasibility of utilizing the genetic ablation methodology in order to transform seeded table grapes into seedless, via one gene insertion. The introduction of such new modified cultivars into the market will be relatively easy because the overall properties of the fruit, except its seed bearing, will not be altered. Moreover, no special concern with respect to growth conditions, plant protection and agrotechnical treatment will be required.

The specific research objectives of this proposal are the following:

1. The construction of plant expression vectors that upon transformation into plants will express lethal genes (e.g. *RNase T1*), under the control of the seed specific promoter, in the developing seed.
2. Production of transgenic plants that will express the specific lethal genes, only in the developing seeds.
3. Analysis of the transgenic plants with respect to their berry and seed development during fruit maturation.

The Research Accomplishments During the First Year:

The general strategy

To produce transgenic grapes that express a lethal gene under the control of a seed specific promoter. The toxic gene product will interfere the normal development of the seed, leading consequently to seed abortion and seedlessness.

Engineering of expression vectors for *Agrobacterium* - mediated transformation of grape

For engineering these expression vectors we followed the general strategy of Armitage et al. (1988). Briefly, the coding DNA sequence of a given suicide gene was cloned into a pUC-derived vector. The gene was placed under the control of the seed coat specific promoter. The engineered cassette was then be removed from the modified pUC derived vector and cloned into a pGA492 binary vector (an 1986). The latter vector will then be mobilized into an appropriate *Agrobacterium* strain.

All the genes of interest will be placed under the control of this seed coat promoter. The vector contained the *RNase* gene as a lethal gene. This type of vectors will serve for the application of other ablation technique in commercial grape cultivars. **Construct was transferred to the South African group for transformation experiments**.

Plant material

Transformation experiments were carried out In Israel using the important grape cultivar "Red Globe ". Embryogenic cultures of this seeded cultivar were produced in our laboratory. **Embryogenic culture were transfer to the South African group for transformation experiments**.

Transformation of tomato

Since the evaluation of grape berries will take place behond the scope of this project, we tested the feasibility of our hypothesis as well as our recently prepared construct - in tomatoes. Tomatoes were transformed using the Seed-Coat - RNase construct. Transgenic plants were transferred to the greenhouse and set fruits. Mature fruits were cut and in order to study seed ablation. Out of 12 independent transgenic plants, 9 were totally seedless while 3 had few seeds within the fruits. These promising data supported our hypothesis that a functional *RNase* gene under the control of a seed specific promoter may induce transgenic seedlessness.

Transformation of Grape

We have started the transformation of Red-Globe. **Working protocols for transformation were transferred to the South African group for experiments**.