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BARD

FINAL REPORT
PROJECT NO. IS-2251-93

**Transgenic Endochitinase Producing
Grapevines for the Improvement of
Resistance to Powdery Mildew (*Uncinula
necator*)**

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**הספריה המרכזית
למדעי החקלאות
בית דגן**

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Signature
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Refereed (published, in press, accepted)		2	2	4
Submitted, in review, in preparation		1		1
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Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
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Licensed				

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	2	2	1	5
Sabbaticals	1			1
Postdoctorates				

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Supportive cooperation having the US partner working mainly on wine grapes and the Israeli partner on table grapes. Synergistic cooperation based on sharing of transformation technologies and anti-fungal genes. _____

**Transgenic endochitinase producing grapevines for improvement
of resistance to powdery mildew (*Uncinula necator*)**

BARD Final Report

ABSTRACT

The original objectives are listed underlined below:

1. Design vectors for constitutive expression of endochitinase from *Trichoderma harzianum* strain P1. Design vectors with signal peptides to target gene expression.
2. Extend transformation/regeneration technology to other cultivars of importance in the U.S. and Israel.
3. Transform cultivars with the endochitinase constructs developed as part of objective 1.
 - A. Characterize foliar powdery mildew resistance in transgenic plants.

BACKGROUND OF THE TOPIC

Conventional breeding of grapevines is a slow and imprecise process. The long generation cycle, large space requirements and poor understanding of grapevine genetics prevent rapid progress. There remains great need to improve existing important cultivars without the loss of identity that follows from hybridization. Powdery mildew (*Uncinula necator*) is the most important fungal pathogen of grapevines, causing economic losses around the world. Genetic control of powdery mildew would reduce the requirement for chemical or cultural control of the disease. Yet, since the trait is under polygenic control, it is difficult to manipulate through hybridization and breeding. Also, because grapevines are heterozygous and vegetatively propagated, cultivar identity is lost in the breeding process. Therefore, there is great need for techniques to produce transgenic versions of established cultivars with heterologous genes conferring disease resistance. Such a gene is now available for control of powdery mildew of grapevines. The protein coded by the Endochitinase gene, derived from *Trichoderma harzianum*, is very effective in suppressing *U. necator* growth. The goal of this proposal is to develop transgenic grapevines with this antifungal gene, and to test the effect of this gene on resistance to powdery mildew.

CONCLUSIONS, ACHIVEMENTS AND IMPLICATIONS

Gene transfer technology for grape was developed using commercial cultivars for both wine and table grapes. It paved the way for a new tool in grapevine genetic studies enabling the alteration of specific important traits while maintaining the essential features of existing elite cultivars. Regeneration and transformation technologies were developed and are currently at an advanced stage for USA wine and Israeli seedless cultivars, representing the cutting edge of grape genetic engineering studies worldwide. Transgenic plants produced are tested for powdery mildew resistance in greenhouse and field experiments at both locations. It is our ultimate goal to develop transgenic grapes which will be more efficient and economical for growers to produce, while also providing consumers with familiar products grown with reduced chemical inputs.

ACHIEVEMENTS

World production of grapes is in excess of 65 million metric tons, exceeding the production of all other temperate fruits, and surpassed only by *Citrus* and banana among all other fruit crops around the world (FAO Production Yearbook, 1990).

Grapes are grown on over 9 million hectares. While the major portion is consumed as wine and spirits, grapes are also used in quantity as fresh fruit and dried as raisins. The importance of powdery mildew resistance in grapevines cannot be underestimated.

The disease causes damage worldwide. Where more organic measures such as sulfur are used for control, weekly applications are often necessary, and the level of control is not sufficient. Frequently applied pesticides also require a large expenditure in time, labor, and fuel costs. Resistance would reduce production costs and increase crop quality.

The following scientific achievements resulted due to this BARD program.

1. Development and improvement of embryogenesis and tissue culture manipulation in grape, while extending these procedures to several agricultural important cultivars both at Israel and US.
2. Development and improvements of novel transformation procedures while developing transformation techniques for grape and other recalcitrant species.
3. Production of transgenic grape plant, characterization of transformed grape plants while studying the expression patterns of a marker gene under the control of different promoter as the 35S CaMV in different part of the plants including flowers and fruits.
4. Expression of anti-fungal genes in grape: establishment of transgenic plants and evaluation of gene expression.

POTENTIAL FOR APPLICATION

We assume that transgenic grapes resulting from this BARD program might be available to both the USA and Israeli producers in about 5-10 years. It mainly depends on two factors: 1. The success in field experiments. 2. The public acceptance of GM food. Note that the US group has already started field experiments with transgenic grapes that resulted from this program. Data regarding this field experiment can be reached at <http://www.isb.vt.edu/cfdocd/fieldtest3/cfm> permits # 98-093-13N and 98-090-04N.

ESTIMATED IMPACT

Production costs of grape are in Israel between 8000-10000 US\$ per hectare. About 30% of these costs are due to the need for fungal protection performed mainly by spraying. In case the transformed grape plants would exhibit a full protection against fungal diseases; a significant reduction in production costs (about 30%) might be expected. If only a limited protection will be obtained using the transgenic grapes produced in this program, one might expect a 10-15% reduction in production costs.

DETAILS OF COOPERATION

The proposed project was planned in order to provide valuable information on the possibilities for the development of transgenic grape plants, which control fungal pathogens with heterologous genes. Our eventual goal was to improve powdery mildew resistance of important varieties with the insertion of the endochitinase gene. At the end of this proposed project developed transgenic plants available for testing. These should provide data on field resistance and stability of expression of the foreign gene. Endochitinase may be sufficient to control powdery mildew either alone or in combination with other pathogen control procedures. Whether endochitinase alone is sufficient to control powdery mildew will have to be determined in later testing. It is our ultimate goal to develop transgenic grapes which will be more efficient and economical for growers to produce, while also providing consumers with familiar products grown with reduced chemical inputs.

Since 1993, the PI are collaborating on this subject and share the grape cultivars to be transformed. In USA wine commercial cultivars were chosen as the primary target for gene insertion and in Israel seedless table grape were selected as the target for transformation. Both PI's are keeping an open email line for real time discussions. Dr. Reisch visited Israel during this BARD program and recently spent a 6 months sabbatical in the lab of Dr. Perl performing a collaborative work within the frame of the BARD program. Dr. Perl visited Geneva twice. In the frame of these visits the following activities took place: 1. intensive discussions and further ideas were exchanged. 2. Some of the constructs developed in Israel were transferred to the US for future transformation studies.

3. Both the US and the Israeli PI's presented lectures describing their progress in grape transformation.

BODY OF REPORT

Background

The production of new grape cultivars by conventional breeding is a complex and time-consuming process. The fact that breeding methods require a cross to be made between parent plants necessitates the interaction and sorting of two genomes. Progeny of grapevine combine both the positive and negative traits from each parent. Undesirable characters are eventually identified during growth and testing and undesirable seedlings are discarded. In grape, the final assessment of progeny performance is made in relation to the quality and quantity of the fruit produced. Moreover, grapes are highly heterozygous and the characters which constitute a good cultivar are polygenic in their inheritance. Unfortunately, under common horticultural practice, most grape seedlings undergo a juvenile period of about two to three years, before fruit is produced consistently. Clearly, reliable assessment of the results of a particular grape breeding strategy may not be possible until many years after the cross has been made. Given this significant investment of time, the most economical breeding strategy would be to transfer individual traits as single genes into an already available and desirable genetic background. In this case only the desired trait would be transferred, with minimum disturbance to the genome. This proposal combined novel methodologies and recent discoveries in order to alleviate a major problem in grape production

In brief: The recent availability of effective genetic transformation procedures for grapevines, the existence of genes able to reduce pathogen stress, and improved in vitro culture methods for grapes, were combined to serve the objective of this proposal.

Powdery mildew is the most economically damaging fungal pathogen of grape worldwide (Pearson and Goheen, 1990). The importance of powdery mildew resistance in grapevines cannot be underestimated. The disease causes damage worldwide. Where more organic measures such as sulfur are used for control, weekly applications are often necessary, and the level of control is not sufficient. Frequently applied pesticides also require a large expenditure in time, labor, and

fuel costs. Resistance would reduce production costs and increase crop quality. Endochitinase genes offer the potential for effective genetic control of powdery mildew. There are several requirements for such genes and their products to be of maximum utility, i.e. (a) they should code for proteins so that the gene can directly produce an active product, (b) the gene product should be highly active against the target organism, and (c) they should not be toxic to higher plants or vertebrates.

Enzymes that degrade fungal cell walls, especially chitinolytic enzymes, are attractive alternatives to pesticides for several reasons. (1) They attack chitin, which is a constituent of the cell walls of higher fungi but which is not found in higher plants or vertebrates. Therefore, these enzymes should not be toxic to crops or higher animals. (2) Proteins with similar activity are probably part of the natural defense mechanisms of plants against infection (Legrand, et al., 1987, Roberts and Selitrennikoff, 1988, Roby and Esquerre-Tugaye, 1987, Schlumbaum, et al., 1986). This suggests that future transgenic crops containing genes that encode for cell wall degrading enzymes will be healthy and have little or no detrimental effect on the environment. (3) These enzymes are proteins and should be directly coded for by specific genes. Therefore, either the gene(s) or the protein(s) can be used in pest control strategies. (4) Enzymes from *T. harzianum* are very strongly antimycotic, especially in combination, and so low levels of these proteins should provide effective control. *Trichoderma* and *Gliocladium* species produce a complex mixture of proteins with chitinolytic and glucanolytic activities. A series of such cell wall degrading enzymes have been isolated (Harman et al., 1993; Lorito et al., 1993). Studies have shown that: (1) These enzymes are more effective as biocontrol agents than similar enzymes from other sources (Lorito et al., 1993a); (2) they act synergistically *in vitro* to inhibit spore germination and fungal growth of a wide range of chitinous plant pathogenic fungi (Lorito et al., 1993); and (3) synergy also exists between these enzymes and other fungitoxicants (Di Pietro et al., 1993) and biocontrol bacteria (Lorito et al., 1993b). The full length cDNA sequence, consisting of 1613 bp, contains an open reading frame of 424 amino acids.

Methodologies and Materials and results

Establishment of Embryogenic Cell Cultures:

I. Culture initiation

For grapevines, transgenic plants can be produced only when embryogenic cell cultures have been used as the starting material. Thus, we have invested a large effort to establish embryogenic cultures of the several commercial both table and wine grape cultivars. To initiate somatic embryogenesis, anthers and ovaries were extracted from unopened flower buds and then cultured in Petri plates on a semi-solid growth medium. During the past two years, three different growth media were tested. Medium "A" is that used by R. Goodman (unpublished) for 'Chancellor' and consists of Murashige and Skoog basal salts, supplemented with 5 μ M 2,4-D and 1 μ M BA. Medium "K" was developed by Robacker (1993, HortSci 25:53-55) and consists of Nitsch and Nitsch basal salts, supplemented with 9 μ M 2,4-D and 4.4 μ M BA. Medium "S" was developed by Schaeffers (PhD thesis, Cornell University, 1995) and consists of Nitsch and Nitsch basal salts, supplemented with 2.5 μ M 2,4-D, 2.5 μ M NOA, and 5 μ M 4-CPPU.

More than 20% of the Chancellor and Chardonnay ovaries on S medium produced embryogenic callus. A small number of other cultivars and treatments also yielded embryos, but many did not. Although we would like to develop reproducible methods to establish embryogenic cultures, only one explant need produce embryogenic callus to establish a multiplying culture for transformation experiments. A similar experiment was set up in 1996 (Table 2) for the purpose of obtaining a second year's data, as well as to target the establishment of new cultures of Merlot, Pinot noir, and Concord, and reduce potential problems with loss of regenerative capacity and somaclonal variation which occur over time. Because of the expected low frequency of embryogenesis, large numbers of explants were used. We have already observed somatic embryos from Concord ovaries, however, the experiment will require several more months to complete.

Currently, in our lab we have embryogenic cultures of Merlot, Concord, Chardonnay, Pinot noir, Chancellor, and Niagara (in USA). IN Israel the same procedures were utilize to establish embryogenic lines of Superior Seedless, Prime,

Novomuscat Seedless, Gamay, Chardonnay, Red-Globe and Velika (in Israel). Additionally, liquid suspension cultures of both Merlot and Chardonnay were established using the medium described by Mauro et al. (1995, Plant Science 112:97-106). The suspensions provide large numbers of uniform proembryos for transformation experiments.

Table 1. Percent of 1995 Explants that Formed Embryogenic Callus

Variety	Medium		
	A	K	S
<i>Anthers</i>			
Chancellor	0	0	0
Chardonnay	2.6	1.2	0.1
Concord*	0	0	0
Merlot	0	0	0
Niagara	0	1.5	0
Pinot Noir	0	0.2	0
<i>Ovaries</i>			
Chancellor	0	0	21.7
Chardonnay	0.9	3.1	24.1
Concord*	0	0	0
Merlot	0	0	0
Niagara	0	0	0
Pinot Noir	0	0	0

*High rates of contamination

Media:

A (Goodman)	MS	5 μ M 2,4-D, 1 μ M BA
K (Robacker)	NN	9 μ M 2,4-D, 4.4 μ M BA
S (Schaeffers) NN	2.5 μ M 2,4-D, 5 μ M 4-CPPU	2.5 μ M NOA

Table 2. Number of anthers and ovaries extracted in 1996.

	Medium			
Cultivar	A	K	S	Total
<i>Anthers</i>				
Chancellor	616	547	674	1837
Chardonnay	566	471	431	1468
Concord	1876	1700	1959	5535
Merlot	1439	1479	1323	4241
Niagara	638	529	607	1774
Pinot Noir	1609	1567	1641	<u>4817</u>
				19,672
<i>Ovaries</i>				
Chancellor	126	105	107	338
Chardonnay	120	126	121	367
Concord	325	305	307	937
Merlot	341	322	282	945
Niagara	95	98	106	299
Pinot Noir	288	265	297	<u>850</u>
				3,736

II. Long term maintenance of embryogenic cell lines in bioreactors

Suspension cultures in 125 ml Erlenmeyer flasks with 20 ml liquid medium were developed from embryogenic tissue cultures established more than three years ago from anthers of four commercial cultivars of *Vitis vinifera* L. ["Superior seedless" (SR), cv. 49, cv. "Red Globe"(RG) and cv. 902] (Perl et al., 1995). The cultures were grown on two orbital shakers (90 and 130 rpm) at $25\pm 1^{\circ}\text{C}$ in a diffused 16 h light. The proliferation liquid medium consisted of MS salts and vitamins and was also supplemented with 3-4% sucrose, 1 ppm 2,4-D, 2 ppm NOA, 5 ppm IAA, 0.2 ppm BA, with or without 1 ppm ABA. For morphogenic induction, the GM+NOA medium (Lebrun & Branchard, 1987) was used. It contained MS salts and vitamins and was also supplemented with 18 g/l maltose, 4.6 g/l glycerol and 1 ppm NOA. Regeneration and subsequent plant germination was obtained on MS medium supplemented with 0.1 ppm NAA, 0.25% Gelrite and 3% sucrose, with or without 0.1 ppm TDZ. The conical bubble type bioreactors (Lipsky & Chernyak, 1983) with working volume from 0.2 l to 0.7 l and an aeration rate 1-1.5 vvm were used.

All the 17 obtained cell suspension lines retain a high embryogenic potential, but they differ from each other by the number of subcultures required before regeneration and germination took place. Some lines demanded for regeneration an addition stage of preliminary cultivation on the induction medium. Long-term cultivation on proliferation medium resulted in blockage of embryogenesis at early development stages after transfer onto the regeneration medium. Utilization of the GM+NOA medium for the sub cultivation of these suspension culture lines resulted in shift of the block to later stages of development or its cancellation. Table 3 summarizes the different chart-flow established in order to regenerate plantlets from the different lines. The possibility of growing the suspension cultures of cvs. 902 and RG in the conical bubble type bioreactors in batch regime, and with further four (902) or six (RG) cycles of draw-fill culture regime was demonstrated in course of half a year cultivation. High

level and low fluctuation of embryogenic activity of these suspension cultures grown in the bioreactors confirm susceptibility of this system for genetic transformation. Cell suspensions originating from cv. RG

Table 3 - Protocol for regeneration of the different lines of suspension cultures from four commercial cultivars of *Vitis vinifera* [cv. "Superior seedless" (SR), cv. 49, cv. "Red Globe" (RG) and cv. 902].

Line designation	Type of suspension	Subcultures required on media		% of regeneration culture up to ...
		A	B	
RG - 1	Fine cells	6-10	0	50
RG - 2	Globular	3-10	0	50
RG - 3	Globular	1-7	0	70
(bioreactor)				
RG - 5	Embryoids	0	4-6	90
902 - 1	Fine cells & small aggregates	7-8	0	60
902 - 1.1	Fine cells & small aggregates	1-5	0	65
(bioreactor)				
902 - 2	Fine cells	0	3-4	70
902 - 3	Embryoids	0	4-7	85
SRI - 1	Fine cells	2-3	0	50
SRII - 1	Fine cells	5-10	0	55
SRII - 4	Embryoids	0	3-6	85
SRIII - 1	Fine cells	2-3	0	40
SRIII - 2	Fine cells	0	1-2	0
49 - 1	Fine cells	3-5	0	75
49 - 3	Fine cells	0	1-2	0

A - proliferation liquid medium;

B - morphogenic induction liquid medium (Lebrun & Branchard, 1987);

C - regeneration solid medium.

III. Vector construction with anti-fungal genes

A. Vectors containing the endochitinase gene

The transformation protocol previously developed for 'Superior Seedless' (Perl et al., 1996, Nature Biotechnology 14:624-628) was applied this year in order to transform the same cultivar with the endochitinase gene. Two of the genes constructed in the previous year were utilized in this study. The

III. Vector construction with anti-fungal genes

A. Vectors containing the endochitinase gene

The transformation protocol previously developed for 'Superior Seedless' (Perl et al., 1996, Nature Biotechnology 14:624-628) was applied this year in order to transform the same cultivar with the endochitinase gene. Two of the genes constructed in the previous year were utilized in this study. The first one (fig 1A) was the original construct obtained from our BARD partner and the second one was modified in Israel (Fig. 1B). The latter has an omega enhancer element and the potato proteinase inhibitor II (PI II) appoplast targeting peptide. These two elements should enhance expression and efficient appoplast targeting of the endochitinase respectively.

35S CaMV

Fig 1. Schematic representation of the original (A) and modified (B) endochitinase constructs.

B. Vectors containing the endochitinase gene and other antifungal genes

Additional vectors were constructed. Most of them contain several antifungal genes of the same binary vector in order to achieve the expected synergism able to confer antifungal tolerance.

The following vectors were produced:

1. 35S-CaMV- Endochitinase; 35S-CaMV- Glucanase; 35S-CaMV – Ribosome Inactivating Protein (RIP)
2. 35S-CaMV – Antimicrobial protein CAE; 35S-CaMV- Glucanase; 35S-CaMV – Ribosome Inactivating Protein (RIP)
- C. 35S-CaMV – El301

D. PR-Promoter - stilbene synthase

35S-CaMV - Antimicrobial protein CAE

IV. Transformation of embryogenic cells

A. Transient expression of endochitinase

There were four separate experiments using Superior embryogenic cultures that had been grown either in solid media, in liquid suspensions, or in liquid bioreactors.

Transient endochitinase assays were performed using the muchT assay. Expression was assayed 1-5 days after *Agrobacterium* transformation under different co-cultivation conditions. It was interesting to note that the expression of the endochitinase gene as measured by the muchT assay (procedure obtained from B. Reisch) can actually serve as a novel reporter gene in a transformed tissue. Thus we studied the involvement of different co-cultivation conditions on the expression of the introduced endochitinase. Transformed embryogenic tissues were homogenized and assayed for endochitinase activity. The influence of the following conditions were studied:

1. The influence of the pH of the media during co-cultivation (Fig. 3).
2. The effect of acetosyringone on the time and level of endochitinase expression (Fig. 4).

In a parallel study, several stable transformed plants were selected and evaluate for their endochitinase activity from leaves harvested from *in vitro* shoots. The results are summarized in Fig. 6. In order to obtain comparable results between different plants, all endochitinase activities were normalized to the average activity in leaves of the control (Plant designation # 1 in Fig. 5) which was given the value of 1.

B. Transformations utilizing different *Agrobacterium tumefaciens* strains.

The inoculum for co-cultivation was prepared from an overnight culture of *A. tumefaciens* in LB liquid medium. After 15 min vacuum infiltration of the grape cells with *A. tumefaciens* or followed by 1 h on a gyratory shaker (130 rpm), the cells were co-cultivated (48 h) on the regeneration medium supplemented with 20 μ M acetosyringone and then transferred on to the same regeneration medium supplemented with 50 ppm kanamycin. In case of using the GUS reporter gene, 24 h later, GUS expression was determined by staining of the cells with 5-bromo-3-chloro-3-indolyl- β -D-glucuronic acid (X-gluc).

C. Additional transformation of cultivars with anti-fungal genes

Embryogenic lines of different *Vitis vinifera* were used for *Agrobacterium* mediated transformation. Selection systems utilized the inhibitors hygromycin and paromomycin, as appropriate to the vectors constructed. The following table summarizes the different transformation experiments done and the state of each transformation study.

<u>Grape cultivar</u>	<u>Construct used</u>	<u>Selection system</u>	<u>Current status</u>
Superior	CAE	Paromomycin	Regenerated plantlets
Superior	CAE+Glu+RIP	Paromomycin	Regenerated plantlets
41B	Glu+RIP	Hygromycin	Regenerated plantlets
41B	Stilbene synthase	Paromomycin	Regenerated plant
Chardonnay	EL 301	Paromomycin	Regenerated plantlets
Chardonnay	stilbene synthase	Paromomycin	Regenerating calli
	+Glu+RIP	Hygromycin

II. Biolistic bombardment of the endochitinase gene into grapevines.

The transformation protocol previously developed for 'Chancellor' (Kikkert et al., 1996, Plant Cell Rep. 15:311-316) was applied to Merlot, Chardonnay, and Concord. There were eight separate bombardment experiments using Merlot embryogenic cultures that had been grown either in Petri plates or in liquid suspension. A total of 88 plates were bombarded with p35SEn42 which contains a genomic endochitinase gene under control of a single CaMV 35S promoter. Similarly, 58 plates were bombarded with pBin19ESR which contains the endochitinase gene from a cDNA library under control of a double CaMV 35S promoter and leader sequence from alfalfa mosaic virus. Both plasmids also contain the NPTII gene for kanamycin resistance as a selectable marker. Appropriate negative controls as well as a GUS containing plasmid were also included.

Transient GUS expression was assayed 2 days after bombardment, and there was an average of approximately 3,000 blue cells per bombarded plate. Other cells were cultured on selective media with different concentrations of kanamycin (25-100 mg/L). The Merlot embryos have a tendency to germinate rapidly on the post-bombardment medium and many germinated embryos were obtained from both the endochitinase-bombarded and the negative control plates. The embryos were spread out on medium with 50 to 100 mg/L kanamycin and then transferred from the dark to a lighted shelf. Only a small portion of the embryos turned green and continued growing. These were selected and multiplied in baby food jars as shoot cultures. To date a total of 26 lines have been regenerated. These are being analyzed by PCR to confirm the presence of the genes. Already, several lines have tested positive for the NPTII gene. PCR tests are also being conducted using primers for the endochitinase gene, and Southern blotting will be done as soon as enough plant material can be generated. Furthermore, there are hundreds of additional embryos being selected and regenerated from more recent bombardments.

Two bombardments of Chardonnay have been conducted. There were 6 plates bombarded with p35SEn42 and 10 plates of pBin19ESR. Selection on

kanamycin containing medium has been clean with no escapes on non-bombarded plates. Two separate clumps of germinating embryos have been obtained from pBin19ESR bombarded plates. Additionally, there are other growing calli on the plates that are potentially transformed.

V. Expression of the GUS gene in flowers and fruit of 'Chancellor'

Previously transformed plants of 'Chancellor' (Kikkert et al. 1996, Plant Cell Rep. 15:311-316) were further analyzed for GUS expression. One plant line which expressed GUS strongly in vegetative tissues was induced to flower by a cold treatment. All floral tissues (anthers, ovaries, ovules, etc.) expressed GUS strongly. Nearly mature berries were also tested for GUS expression. Chancellor berries have endogenous GUS activity in the endosperm tissue which could be partially overcome by including methanol in the assay solution. Additionally, true GUS expression in the berries could only be detected when the berries were soaked overnight in a pH 9 phosphate buffer. The acidity of non-soaked berries interferes with the GUS reaction (Avi Perl, unpublished). It is important to know that GUS expression under control of the 35S promoter occurs both in vegetative and floral tissues, as both can be the site of pathogen infection. This gives us an indication that the pathogen-resistance genes will be expressed constitutively also.

VI. Expression of the endochitinase gene in Merlot and Chardonnay

A total of 101 'Merlot' and 93 'Chardonnay' plants were recovered after selection on kanamycin containing medium after biolistic transformation. Ninety-four percent were positive for the *nptII* gene and 79% were positive for endochitinase gene (*ThEn42*). A small number of 'Chardonnay' plants were tested by PCR (Table 1). Nearly all of the plants at both locations were screened for chitinase expression. Forty to fifty percent had 10 to 100 fold higher chitinase expression than did non transformed controls

Plants were further analyzed by Southern blotting. DNA samples digested by *EcoRI* and probed with either *ThEn42* or *nptII* fragments were expected to yield a 12 kb or larger fragment if they contained the entire plasmid. However, since the point of insertion of the plasmid is random when using

biolistic transformation, smaller fragments could contain intact genes. The minimum fragment size that would contain both genes is 3.6 kb. DNA samples digested with *NcoI* and probed with *ThEn42* yield a 1.4 kb band if the gene insertion was intact. Nearly all of the plants tested contained fragments of the *ThEn42* gene. The plants that did not express chitinase either did not contain *ThEn42* at all or had only small fragments of the gene. Only one plant analyzed to date contained the intact *ThEn42* gene, but did not express chitinase. All of the plants tested contained the *nptII* gene.

A *Botrytis* resistance assay was used to determine the biological activity of the gene in transgenic grapevine leaves. *Botrytis* is normally considered only a fruit disease in grapevines, but the leaf assay can be used to detect endochitinase activity at an early stage in plant growth. In preliminary tests, the lesion size after *Botrytis* infection was 65% less in a high chitinase expressing line as compared to a non-bombarded line.

The endochitinase gene *ThEn42* from *Trichoderma harzianum* strain P-1 was successfully introduced into 'Merlot' and 'Chardonnay' grapevines by biolistic transformation of embryogenic cultures. The gene is expressed at high levels in some of the transgenic lines and in preliminary tests appears to have activity against *Botrytis cinerea*. The plants have been successfully transferred to the greenhouse and field (USA location). Plants will be screened for resistance to *Uncinula necator*. Other antimicrobial genes are being tested in our laboratory, alone and in combination with the *Trichoderma* endochitinase gene. Grapevines that contain multiple genes for resistance are likely to have a higher degree of disease tolerance and there will be less chance for the pathogens to overcome resistance.

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Publications and patents resulting during and from this project

Subject:

Improvement of embryogenesis and tissue culture manipulation in grape, while extending these procedures to several agricultural important cultivars both at US and Israel:

Perl, A., Saad, S., Sahar, N. and Holland, D. (1995). Establishment of long term embryogenic cultures of seedless *Vitis vinifera* cultivars - a synergistic effect of auxins and the role of abscisic acid.
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