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למדעי החקלאות
בית-דגן

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Title

**TISSUE CULTURE AND GENE TRANSFER FOR GENETIC IMPROVEMENT
OF GRAPES**

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Chapter 2. Manuscript published in Proc. of the 5th International Conference on Grapevine Breeding, Vitis special issue, 1990:

Regeneration from grapevine petioles: Extension to new genotypes.

B.I. Reisch, M.H. Martens and Z.M. Cheng

Introduction:

Improved ability to produce plants from tissue cultures of *Vitis* would expedite efforts in genetic engineering, in vitro mutant isolation, rapid multiplication of elite stocks, and the production of haploids and then homozygous diploids.

In grapes, varying degrees of organogenesis and somatic embryogenesis have been obtained. Shoot organogenesis and embryogenesis have been obtained from vegetative tissues, such as shoot apices (Barlass and Skene 1978, 1980a, 1980b), leaf callus (Favre 1977; Hirabayashi 1985; Stamp and Meredith 1988), and internode callus (Krul and Worley 1977; Rajasekaran and Mullins 1981). Yet there is still a need for an easy to use system which applies to a large number of genotypes while inducing improved levels of regeneration.

The objective of our research was to extend the regeneration system developed for *Vitis X labruscana* 'Catawba' (Cheng and Reisch 1989) to other genotypes. We also examined auxin and cytokinin effects on regeneration from petiole tissues.

Materials and Methods.

The grape cultivars 'Catawba', 'Vanessa' and Ravat 51 ('Vignoles') were established in vitro from vines grown at the New York State Agricultural Experiment Station, Geneva, NY. All explants for regeneration experiments were derived from in vitro shoot cultures.

Axillary buds from the basal part of growing primary shoots were isolated and washed for 30 minutes in tap water with Micro detergent (International Products Corp., Trenton, NJ, USA). After woody parts and outer layers of bud scales were removed, buds were submerged in 70% ethanol for 2 to 3 min and then were transferred to 1.85% sodium hypochlorite for 15 min, followed by 3 to 5 washes in sterile distilled water. Apex tissues, 2 to 4 mm long, were isolated from buds and inoculated for shoot multiplication on MS basal medium (Murashige and Skoog 1962) supplemented with 4.0 μ M BA. Conditions for further growth of shoot tip cultures were described earlier (Reisch 1986). The micropropagated shoots were later cultured in MS medium or C2D (Chée and Pool 1987) with 2.0 or 4.0 μ M BA prior to excision of explants. All media were solidified with agar (Difco bacto) at 7.0 g/l.

The basic medium for regeneration experiments was NN69 (Nitsch and Nitsch 1969). This medium was supplemented with various plant growth regulators or other components as described below. Vitamin concentrations were those of Murashige and Skoog (1962). Myo-inositol (100 mg/l), sucrose (20 g/l), 6-benzylaminopurine (BA), indole butyric acid (IBA), thidiazuron (TDZ), naphthalenacetic acid (NAA) and amino acid conjugates of indole acetic acid (IAA) were added to the media prior to autoclaving.

Petioles from shoot cultures were isolated with great care to exclude nearby axillary buds. Each treatment included 6-12 Petri dishes (25 x 100 mm) with 10 explants each.

The cultures for regeneration were grown in the dark (in cardboard boxes) in a culture room at 24-26°C. After 6 weeks, cultures were transferred to BA containing media under a 16:8 light:dark photoperiod to induce further shoot growth. Experiments were statistically analyzed with ANOVA procedures.

Results and discussion.

Auxin effects on grapevine regeneration from petiole cultures.

Earlier results with 'Catawba' suggested that regeneration could be greatly improved with the addition of autoclaved IBA to the culture medium (Cheng and Reisch 1989). In this first experiment, the effects of IBA as well as other auxin sources were examined. Petioles were excised from in vitro shoots of both 'Vanessa' and 'Catawba'. Cultures were incubated in the dark for 6 weeks on five media which varied in auxin content: 1. 10 μ M BA, 2. 10 μ M BA + 4.0 μ M IAA-dl-aspartate, 3. 10 μ M BA + 2.0 μ M IAA-glycine, 4. 10 μ M BA + 0.5 μ M IBA, and 5. 10 μ M BA + 0.5 μ M NAA. Preliminary experiments with these compounds were used to determine the most promising concentrations which were then tested in this experiment.

Analysis of variance indicated that auxin effects were highly significant. However, the large increase seen in 'Catawba' regeneration on IBA containing medium was not reconfirmed (Table 1). In fact, addition of all auxin sources tested except NAA resulted in improved regeneration from 'Catawba' petioles. The IAA-dl-aspartate conjugate and IBA were the most effective auxins for 'Vanessa' regeneration. The use of NAA at the level tested prevented regeneration from both cultivars.

A second experiment concentrated on defining the optimum levels of IAA conjugates in the medium. Conjugates of IAA are reported to stabilize the levels of IAA in tissue culture media and provide for a slower, steadier release of auxin over a longer period of time (Hangarter et al. 1980). They are often used at very high levels since they form an equilibrium resulting in a free IAA concentration in the medium which is much lower. Preliminary results in our lab have shown that these conjugates are extremely active in promoting callus growth in grape tissue cultures. Using petioles from in vitro shoots of Ravat 51, three conjugates of IAA were tested in combination with 10 μ M BA at the following levels: 10 μ M BA (control), 10 μ M BA + 4.0 μ M IAA-dl-alanine, 10 μ M BA + 2.0 μ M IAA-glycine, 10 μ M BA + 20.0 μ M IAA-glycine, 10 μ M BA + 4.0 μ M IAA-dl-aspartate, and 10 μ M BA + 80.0 μ M IAA-dl-aspartate. These auxin levels were chosen based on preliminary results.

The percentage of explants forming shoots was significantly better on media with either 2.0 μ M IAA-gly or 4.0 μ M IAA-dl-aspartate than on auxin free medium (Table 2). In the latter case, the effective concentration is 2.0 μ M since only the "l" form of the conjugate can be naturally hydrolyzed in vitro.

Cytokinin effects on grapevine regeneration from petiole cultures:

Preliminary experiments with thidiazuron in the range of 0.0-2.0 μ M showed that it was an effective cytokinin like compound when used with grape petiole cultures. We then sought to determine its optimum effective level and to test it at higher levels. Earlier results suggested that it would be effective alone or in combination with BA. This present experiment tested TDZ in the absence of other growth regulators. Petioles were derived from in vitro cultured Vanessa shoots and incubated in the dark at the indicated concentrations of TDZ for 6 weeks followed by transfer to the light (16 hr photoperiod).

Without TDZ, no callus growth or regeneration was observed (Table 3). All levels of TDZ tested (2.0 - 8.0 μ M) produced shoots from the cut basal ends of the petioles. Due to variability in response at each level, there was no significant difference in regeneration response between all levels

of TDZ tested. It was clear, however, that TDZ induced regeneration and was effective even at 8.0 μM which is considered to be very high for such an active compound.

In the present experiments, two new genotypes, 'Vanessa' and Ravat 51, have been shown to be regenerable via the petiole regeneration system. The frequency of regeneration is lower than that reported for 'Catawba', yet it was comparable to the frequency for 'Catawba' in the one experiment (Table 1) in which 'Catawba' was run side by side with 'Vanessa'. For reasons unknown, the level of regeneration may vary considerably from experiment to experiment. This variability causes difficulties in determining the optimum levels of the auxin and cytokinin treatments examined in the present work. This uncontrolled variation may be due to explant physiological status on the shoot proliferation medium, node position of the petiole, and other *in vitro* factors which may affect the hormone or nutritional status of petioles prior to explanting to regeneration medium.

Summary. Auxins at low concentrations were found to enhance shoot organogenesis when used in a high cytokinin medium. This enhancement was genotype specific. In the presence of 10.0 μM BA, 'Vanessa' regenerated best with the inclusion of either 4.0 μM IAA-dl-aspartate or 0.5 μM IBA, while 'Catawba' regenerated well with either the same two auxins or with 2.0 μM IAA-glycine. The inclusion of 0.5 μM NAA in the medium inhibited regeneration. With Ravat 51, 14-15% of explants regenerated in the presence of either 2.0 μM IAA-gly or 4.0 μM IAA-dl-aspartate, whereas there was no regeneration in the absence of auxin. All levels of TDZ tested (2.0 - 8.0 μM) produced shoots from the cut basal ends of 'Vanessa' petioles, indicating that TDZ could be substituted for BA to induce shoot formation from petiole explants. Our results clearly show that high cytokinin media can be easily extended to petiole explants of other genotypes to facilitate shoot organogenesis and that low levels of auxins, especially IAA conjugates, can enhance the level of regeneration.

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VARIETY	AUXIN SOURCE	% REGENERATION
Vanessa	none	3.3
Vanessa	4.0 μ M IAA-dl- asp	9.0
Vanessa	2.0 μ M IAA-gly	2.5
Vanessa	0.5 μ M IBA	8.3
Vanessa	0.5 μ M NAA	0.0
Catawba	none	5.0
Catawba	4.0 μ M IAA-dl-asp	7.0
Catawba	2.0 μ M IAA-gly	10.8
Catawba	0.5 μ M IBA	7.5
Catawba	0.5 μ M NAA	0.0

Table 1. Regeneration from petioles of Vanessa and Catawba after 8 weeks on media with 10.0 μ M BA plus the indicated auxin (60-120 petioles/treatment).

BA (μ M)	AUXIN (μ M)	% REGENERATION
10.0	0.0	0.0c
10.0	4.0 IAA-dl-ala	5.9bc
10.0	2.0 IAA-gly	15.0a
10.0	20.0 IAA-gly	2.9c
10.0	4.0 IAA-dl-asp	14.4ab
10.0	80.0 IAA-dl-asp	5.0c

Table 2. Regeneration from Ravat 51 petioles after 6 weeks (40-90 petioles/treatment). Means followed by the same letter are not significantly different at $p < 0.05$ by Duncan's Multiple Range Test.

TDZ (μ M)	% REGENERATION
0.0	0.0a
2.0	4.6ab
4.0	10.0b
6.0	6.1ab
8.0	7.3ab

Table 3. Regeneration from 'Vanessa' petioles after 8 weeks (65-100 petioles/treatment). Means followed by the same letter are not significantly different at $p < 0.05$ by Duncan's Multiple Range Test.

Chapter 3. Regeneration of *vinifera* genotypes from petiole culture; Embryogenesis from nucellar cultures; Miscellaneous tissue culture experiments

Organogenesis from petiole tissues

Petioles from *in vitro* grown plants cvs. Grenache, Muscat Roy and Sivan were explanted onto a medium based on Nitsch and Nitsch (1969). Cultures were grown in the dark, for 4-6 weeks at 24°-26C. In a few cases callus formation was observed, without subsequent shoot formation. Shoot formation occurred at the basipetal end of the explants, at the swollen cut edge. Recovered shoots were cultured on a medium containing zeatin riboside 1.0 or 2.5 pp, (zeatin riboside proved more effective than benzyladenine). Rooting of the shoots was accomplished in a medium free of cytokinins. Muscat Roy gave the highest percentage of regenerated plants as seen from the table below:

Table 1.

Cultivar	No. of Explants	No. of explants forming callus	No. plants regenerated
Grenache	275	9	5
Muscat Roy	277	5	63
Sivan	90	2	-

In 1989, a further experiment was conducted with petioles taken from *in vitro* plants of Muscat Roy and Sivan. The petioles were taken from the first fully expanded leaf to the fifth fully expanded leaf. Two media, NN 69 and MS, were tested. They were supplemented with 0.225 μ M BA and 1.0 μ M TDZ (Thidiazuron). Results are summarized in Table 2.

In further experiments, petioles were taken from cvs. Superior, Flame Seedless, Muscat Roy and Grenache. Use of petioles from vineyard grown plants did not prove successful, in spite of several decontamination procedures employed. Success with petioles or leaves from *in vitro* grown material was also less consistent than in the first experiments. Several growth substances were tried in the medium; benzyladenine, thidiazuron, benzoyladenine as well as a combination of two substances.

Muscat Roy gave the highest number of forming shoots and subsequently plants. In one experiment, 6 out of 21 explants yielded shoots and plants with 1.0 ppm BA and 0.2 ppm TDZ in the medium. In the same experiment Grenache yielded 4 plants out of 21 explants. Petioles from the second or third leaf from the apex gave usually the best results. In a further experiment with Muscat Roy petioles, it was difficult to conclude whether node position affected regeneration frequency since frequencies were low from all node positions (Table 3).

Table 2.

Cultivar	Medium	Petiole node positioning	No. explants	No. explants forming callus	No. plants regenerated
Muscat Roy	NN 69	1 ^x	81	3	3
Muscat Roy	NN 69	1 ^x	81	3	3
Muscat Roy	NN 69	2	85	2	9
Muscat Roy	NN 69	3	76	3	4
Muscat Roy	NN 69	4	96	3	8
Muscat Roy	NN 69	5	84	2	4
Muscat Roy	MS	1	50	1	2
Muscat Roy	MS	2	50	8	3
Muscat Roy	MS	3	40	4	5
Muscat Roy	MS	4	40	5	3
Muscat Roy	MS	5	35	1	2
Sivan	NN 69	1	33	1	1
Sivan	NN 69	2	40	2	1
Sivan	NN 69	3	39	2	-
Sivan	NN 69	4	36	-	-
Sivan	NN 69	5	26	2	-

^x Indicates position closest to apex.

Table 3.

Medium	No. of explants	No. leaf from apex	No. plantlets obtained
Nitsch	81	1	3
+BA+TDZ	85	2	2
	76	3	3
	96	4	3
	84	5	2
MS+BA+TDZ	50	1	2
	50	2	3
	40	3	5
	40	4	3
	35	5	2

With Sivan, only 2 plants were obtained out of a total of 174 explants, in Superior 2 plants out of 26 explants on the MS medium with 0.7 ppm BA and 1 plant out of 25 explants on MS with 0.5 ppm BA. Similar results have been obtained with explants from 'Grenache' petioles, 3 plants were also obtained (out of 150 explants) on a medium of MS/2 with BA and NAA. Submerging the leaves in the medium did not give higher regeneration though a high percentage of leaves did form roots (nearly 40% with Sivan, 32% with Muscat Roy, 29% with Grenache).

Regeneration of additional Vitis vinifera cultivars

The regeneration system with 'Catawba' is suitable as a model system in New York where this cultivar is widely grown, but it is important to extend this system to other genotypes,

particularly the classic *Vitis vinifera* varieties of world-wide importance. Unfortunately, experience has shown that optimal medium and hormone concentrations are highly variety-dependant, requiring much testing to develop the best system for each variety. We chose to test our best regeneration system on several other cultivars of both local and world-wide interest.

Table 4. Regeneration of 3 Genotypes

Basic Medium: Nitsch and Nitsch 69, 10 μ M BA, 0.5 μ M IBA
Tissue: Merlot, Cabernet, and Concord - Leaf blades and petioles

Cultivar	Explant	Condition after 6 weeks
Merlot	Leaves	No regeneration, still green
Merlot	Petioles	2/10 petioles regenerating, sl. callus, healthy
Cabernet	Leaves	No regeneration, slight white callus on cut surface
Cabernet	Petioles	1/11 petioles regenerating, much callus, healthy
Concord	Leaves	No regeneration, sl. callus
Concord	Petioles	No actual regeneration but several small round embryoids on callus surface. Two of seven regenerated small shoots after 6 additional weeks.

No change was noted in regeneration rate in Merlot or Cabernet after the 6 week point, although the shoots formed grew into healthy plantlets. By 12 weeks, the embryoid-like structures on the Concord petiole callus did indeed form small shoots, with 2/7 remaining petioles regenerating.

From these results, we can see that while this medium that works well for 'Catawba' may not be ideal for the 3 genotypes tested here, even under less than optimal conditions, some regeneration may occur. This is encouraging, and will eventually hasten the incorporation of other more widely accepted cultivars into future biolistic experiments.

Polyamine Effects

Various reports have indicated that polyamines affect plant growth and development and as well as embryogenesis in several species (e.g. Feirer et al., 1984; Meijer and Simmonds, 1988). We tested spermidine and putrescine for their effect on regeneration rates from two varieties of grape at various concentrations.

Table 5 - The Effect of Putrescine and Spermidine on Grape RegenerationBasic Medium: Nitsch and Nitsch 69, 10 μ M BA, 0.1 μ M IBA

Tissue: Petioles from 'Vanessa' and 'Catawba', 100 explants per treatment

Polyamine Compound	Concentration	% shoot formation after 8 weeks	
		'Vanessa'	'Catawba'
0	0	0	29
Putr	0.5 μ M	2	25
	1.0 μ M	8	7
	2.0 μ M	0	15
	0.5 mM	0	4
	1.0 mM	2	8
	2.0 mM	1	6
Sperm	0.5 μ M	0	22
	1.0 μ M	2	31
	2.0 μ M	0	19
	0.5 mM	1	4
	1.0 mM	3	8
	2.0 mM	0	4

From these results, it can be shown that low levels of either polyamine compound (0.1-2.0 μ M) differ non-significantly in their effect from the control, although it appears that the 1.0 μ M putrescine treatment might have had a slightly beneficial effect on Vanessa. However, at the higher levels (0.5-2.0 mM), inhibition occurs and few shoots are regenerated. The difference in cultivar, with Catawba regenerating better than Vanessa at all treatments, is not surprising, since Catawba has shown itself to be highly regenerable under a number of diverse conditions. It is enlightening to see that the highest level of regeneration occurs on the Catawba control. Therefore, it appears that polyamines would be of no use in enhancing organogenesis in grape.

Effects of Gelling Agents and Temperature

A report by Clog et al.(1990) indicated that regeneration may occur at an increased rate if the temperature of the culture room is held at a constant 28 °C throughout the regeneration period. Though our culture facility approximates this, there is variation over the course of day and night as the lights tend to produce some heat and as the door is occasionally opened and shut. In this experiment, we tested whether regeneration is superior in a precisely temperature-controlled incubator. We also tested 2 different medium gelling agents, the standard Difco Bacto agar and a new product, Gelrite, which produces a more transparent gel at a lower cost.

Table 6. Effects of Gelling Agents and Temperature on Regeneration

Cultivar	Medium	Location	% Regeneration After 10 weeks
Catawba	0.6% Agar	Culture Room	16
Catawba	0.4% Agar	Culture Room	8
Catawba	0.8% Gelrite	Culture Room	14
Catawba	0.4% Agar & 0.3% Gelrite	Culture Room	16
Catawba	0.6% Agar	Incubator	17
Catawba	0.4% Agar	Incubator	16
Catawba	0.8% Gelrite	Incubator	10
Catawba	0.4% Agar & 0.3% Gelrite	Incubator	9
Vanessa	0.6% Agar	Culture Room	5
Vanessa	0.4% Agar	Culture Room	8
Vanessa	0.8% Gelrite	Culture Room	7
Vanessa	0.4% Agar & 0.3% Gelrite	Culture Room	6
Vanessa	0.6% Agar	Incubator	0
Vanessa	0.4% Agar	Incubator	3
Vanessa	0.8% Gelrite	Incubator	3
Vanessa	0.4% Agar & 0.3% Gelrite	Incubator	4

When compared by analysis of variance on a per plate basis, there were no significant differences between the treatments within each variety. The obvious difference between cultivars was expected. Cultures in the incubator were much more likely to develop fungal contamination than those plates on the culture room shelves. When the cultures were held, not subcultured, for 14 weeks, many more of the cultures on Gelrite-containing medium were browned and appeared to have stopped growing entirely. Therefore, the decision of which gelling agent to use does not seem critical, nor does the absolute temperature during regeneration, as long as an approximate level is maintained.

Leaf regeneration from nucellus derived embryos

Leaf segments from Grenache noir shoots originating from nucellus derived embryos were excised and planted on media with 0.5 ppm BA and 1.0 ppm NAA. Regeneration of shoots was obtained from 3-5% of the explants, with some callus and subsequent plant formation. Shoots or small plantlets were transplanted from the BA + NAA to medium without phytohormones. Most explants not regenerating shoots formed roots. An attempt to obtain shoot callus from culture derived roots has not been successful. Several combinations of BA, IAA and GA₃ have been tried.

Regeneration from roots

Roots were taken from *in vitro* stem cuttings of Grenache noir. Two hundred roots were placed in MS/2 medium with 1.0 ppm BA + 0.2 ppm TDZ. After 4 months two of the roots formed shoots which developed into plants. In another experiment, a similar low rate of regeneration was obtained.

Regeneration efforts via nucellus culture

Ovules of the following cultivars were cultured: Grenache noir, Cabernet Sauvignon, Muscat Roy and Superior. The technique of Mullins and Srinivasan (1976) was followed. A combination of NOA and BA was used, followed by transfer to media with lower concentrations such as 5 ppm NOA + 2.5 ppm BA and later to 1.0 ppm NOA + 0.5 ppm BA. The use of 2,4-D proved to be much inferior to either NOA or NAA. In all cases when callus was formed, it proved to be non embryogenic. With Grenache, and on a somewhat smaller scale with Muscat Roy and Cabernet Sauvignon, many embryos formed from the nucellus, this gave rise by proliferation to further embryos on media containing 0.5 BA + 1.0 ppm NOA.

Embryo proliferation continued successfully with every passage for two years with Grenache noir, with decline setting in during the third consecutive year. High auxin concentrations caused in Muscat Roy formation of too many roots and fewer shoots. No embryos were formed with Superior.

Somaclonal plants from Grenache noir

Plants regenerated directly from nucellar cultures of Grenache noir were transplanted to the field after hardening. During the second year all 79 somaclonal plants started to flower and set fruit. Plants were set out at half the distance adapted for the normal grafted clone (on Richter 110 rootstocks). The distances for the latter were 3.5 x 1.0 m, while for the somaclonal own rooted plants distances were 3.5 x 0.5 m. All somaclonal plants as well as 3 plants of the normal grafted clone were scored for 3 consecutive years from cluster weight, berry weight, sugar and total acidity as well as color (on an ascending 1-7 scale). In most cases, yield per vine and number of clusters were also determined.

Fruit color determinations have confirmed the significantly better coloring of most somaclonal plants compared to the original vegetatively propagated clone.

Table 7.

	1988	1989	1990
Somaclonal plants			
Green	0	0	0
Green-red	1	0	1
Red-green	3	0	3
Red	0	1	1
Red-black	1	2	3
Black-red	8	6	14
Black	23	25	48
Clonal plants			
Green-red	3	0	0
Red-green	0	0	3
Red	0	1	0
Red-black	0	2	0

The better external color in the majority of somaclonal variants was accompanied by a significantly higher sugar content as can be seen from Table 5.

The acidity level was similar in both cases with a much higher sugar/acidity ratio in the somaclonal plants. While the yield of clonal Grenache was higher, as well as the number of

clusters per vine, one has to take into account that the distances from the clonal plants were twice larger (3 x 1m) as for the somaclonal plants (3 x 0.5 m). Still, many somaclonal plants exceeded the clonal plants in many characters, apart from exceeding very significantly concerning color and sugar/acid ratio. Seventy-four out of 79 somaclonal plants exceeded the clonal plants in sugar content, 25 out of 79 in acid content. Berry weight was higher in 45 somaclonal plants, average cluster weight in 54 plants. The number of somaclonal plants exceeding the average of the clonal plants in yield was smaller 15 exceeding the average. Eleven somaclonal plants had a larger number of clusters than the clonal grafted Grenache noir.

Table 8. Yield, number of clusters, average berry weight (g), sugar content, total acidity, sugar/acidity and color of berry skin^x. Average of 3 seasons (1988, 1989, 1990).

	Yield ^{xxxx} kg/vine	Number clusters	Mean wt/ cluster	Berry wt/g	Sugar	Acidity	Ratio Sugar/acidity	Color
Clonal Grenache noir ^{xx} (on Richter 110)	4.28	26.3	161.0	1.68	17.33	0.59	29.4	3.2
Somaclonal Grenache plants ^{xxx} (Nucellus derived)	3.28	19.0	181.3	1.77	19.52	0.57	34.2	6.4

x On a 1-7 scale from green (1) to black (7)

xx Average of 3 plants

xxx Average of 79 plants

xxxx Somaclonal vines planted at 1/2 distance in the row compared to clonal plants.

On the basis of the detailed examinations on yield, fruit quality and color, five somaclonal plants were selected and propagated on rootstocks after the 1989 yield. These selections yielded between 3.2-4.7 kg per vine, their sugar content was between 18.5 and 19.5. Brix acidity 0.55-0.68 and the color of fruit skin was black in all cases. One of the plants selected, number 42, yielded highly comparable yields every year while another selection, number 71 excelled in high average yield (4.7 kg) and very low pH of the fruit during maturity. First samples of fruit for vinification were taken in 1990.

Callus Production Experiments

1. Hormone/Medium Effects

Conditions that optimize callus production usually are quite different from those that enhance regeneration. Different hormones control callus growth, and other growth factors, including nutrients, may be involved. Usually the auxin/cytokinin balance is critical in determining amount and character of callus produced. In this experiment, we tested one auxin source (NAA) that we have used rarely in past work at 2 levels, 2 cytokinin sources (kinetin and BA), and 2 media (B5 and MS), each supplying different levels of nutrients.

Table 9. Callus Production on 2 Media

Tissue: Catawba, Riesling, and Vanessa - Leaf, Petiole, and Internode

Medium A - B5 with 1 mg/l kinetin and 1 mg/l NAA

Medium B - MS with 4 μ M BA and 5 μ M NAA

Medium	Cultivar	Explant	Weight after 9 weeks (g)	Type of Callus
A	Catawba	Internode	1.01 ± 1.05	Soft, wet, brown
A	Catawba	Leaf	*	Contamination
A	Catawba	Petiole	1.19 ± 0.76	Soft, wet, brown
A	Riesling	Internode	0.48 ± 0.40	Soft, dry, white
A	Riesling	Leaf	0.40 ± 0.20	Firm, wet, brown
A	Riesling	Petiole	0.28 ± 0.36	Soft, dry, white
A	Vanessa	Internode	0.92 ± 1.07	Firm, wet, white
A	Vanessa	Leaf	0.61 ± 0.31	Firm. med-wet, brown
A	Vanessa	Petiole	1.43 ± 1.07	Soft, wet, light brown
B	Catawba	Internode	1.09 ± 1.35	Soft, wet, white
B	Catawba	Leaf	1.43 ± 1.37	Soft, wet, white
B	Catawba	Petiole	1.94 ± 1.63	Soft, firm, gray
B	Riesling	Internode	0.11 ± 0.15	Soft, dry, white
B	Riesling	Leaf	0.10 ± 0.10	Very little callus, white
B	Riesling	Petiole	0.07 ± 0.16	Most explants dead
B	Vanessa	Internode	0.01 ± 0.00	All explants dead
B	Vanessa	Leaf	2.14 ± 1.31	Soft, wet, brown
B	Vanessa	Petiole	1.00 ± 0.96	Firm, dry, white

It can be inferred from this data that Riesling calluses slightly better on B5 medium with kinetin and NAA, but neither condition really optimizes callus production for this particular cultivar. Vanessa leaves callus slightly better on MS with BA and NAA, but Vanessa internodes seem damaged on the MS medium - all died and did only tolerably on B5. Probably Vanessa internodes are a poor choice of tissue for callus production. In most cases, the differences attributable to medium were slight, with tremendous variation within a single treatment. Overall, Catawba petioles and Vanessa leaves, both on MS, produced the greatest amount of callus.

The type of callus is a very important characteristic for future use in genetic engineering. Friable or soft callus is generally considered superior, and callus that does not brown is more likely to be embryogenic. However, none of the treatments in this experiment produced exceptional callus quality or quantity. Previous experiments in our lab, using other growth factors, notably the conjugates of IAA, have been more successful in producing green, rapidly growing, friable callus.

2. Medium/Light/Temperature Effects

In this experiment, we applied this basic concept to grape explants. We grew tissue from 2 grape cultivars for 4 weeks on MS medium with 2 μ M BA, 5 μ M NAA in the dark, followed by 4 weeks on the same medium under lights. After that time, the tissue was subcultured to hormone free medium for 4 weeks and was held at 4°C in a standard refrigerator. Then the tissue was subcultured a fourth time to MS medium containing 13 μ M BA and 1 μ M GA. The cultures were evaluated after 8 weeks, at the end of the first light treatment, and then again at 16 weeks.

Table 10. Medium/Light/Temperature Effects on Callus Production

<u>Cultivar</u>	<u>Explant</u>	<u>Callus characteristics after 8 weeks</u>
Catawba	Leaf	Considerable callus, soft, white-brown, moderate rooting, no shoot formation
Catawba	Petiole	Considerable callus, hard, green-white, slight rooting, shoot formation on 4% explants
Cabernet	Leaf	Moderate callus, variable texture, white-brown, no rooting or shoot formation
Cabernet	Petiole	Considerable callus, hard, green-white, much rooting, no shoot formation

After the 8 week evaluation, the cultures were then subjected to the 4°C treatment. During this time, most of the calli browned and did not grow any further. No additional growth occurred after the subculture onto the GA-containing medium.

From this experiment, we conclude that this regime of varying light, temperature, and medium hormone level does not result in yields of superior quality callus suitable for our needs.

Chapter 4. a portion of the Ph.D. thesis research of Zong-Ming Cheng:

***Agrobacterium*-mediated Genetic Transformation of *Vitis* Tissues**

Abstract

Genetic transformation of grapevine with *Agrobacterium tumefaciens* was attempted. Petiole explants of 'Catawba' (*Vitis X labruscana*) and 'Vanessa' (interspecific hybrid), infected by strain LBA4404 with a binary vector pBI121 or strain 3850 with either pBI121 or pGA472, did not produce calli in media with 100 or 10 mg/l kanamycin. 'Catawba' petioles produced kanamycin resistant calli after infection by strain A281 with a wild type Ti plasmid pTiBo542 and a binary vector pGA472 in media with 100 or 10 mg/l kanamycin, but the same strain with the disarmed plasmid pTiBo542 did not. Strains A348, 328, 3004, and A3025 with the plasmid pGA472 were infectious to 'Catawba' and 'Vanessa' and infected petioles produced kanamycin (100 or 10 mg/l) resistant calli. 'Catawba' petioles and 'Cabernet Sauvignon' leaves infected by strain CUG0027 with pGA472 produced kanamycin resistant calli in medium with 10 mg/l kanamycin. Grape petioles were very sensitive to kanamycin, and a concentration of 10 mg/l kanamycin was sufficient to completely inhibit callus formation and shoot regeneration. 'Catawba' petioles infected by *Agrobacterium* strain 3025 with pGA472 produced kanamycin resistant calli via two different selection schemes: selection in kanamycin containing medium for a period of days followed by transfer to kanamycin free medium (pre-selection), or culture in kanamycin free medium for a period of days, then transfer to kanamycin containing medium (post selection). But these kanamycin resistant calli failed to give rise to kanamycin resistant shoots or plants.

Introduction

Plant genetic transformation technology has advanced in recent years and now provides breeders with promising techniques for the direct and highly specific manipulation of plant genomes. This technology is particularly beneficial for the improvement of vegetatively propagated perennial woody plants which have long generation cycles, highly heterozygous genomes and where relatively little genetic information has been available. This technology has broadened the possibilities for transferring genes between unrelated organisms, and creating novel genetic materials by the specific alteration of cloned genes. Using *Agrobacterium*-mediated transformation techniques, many agriculturally important genes have been transferred to several annual crops, including genes for viral resistance (e.g., Stark and Beachy, 1989; Golemboski et al., 1990), insect resistance (Barton et al., 1987; Fischhoff et al., 1987; Hilder et al., 1987) and herbicide resistances, such as to glyphosate (Shah et al., 1986; Filatti et al., 1987a) and 2,4-D (Streber and Willmitzer, 1989). Transformable plant species now include several perennial woody species, such as walnut (Dandekar et al., 1988), apple (James et al., 1989), *Populus* (Filatti et al., 1987b; De Block, 1990), and loblolly pine (Senderoff et al., 1986).

Regeneration of grapevine shoots from somatic tissues has been achieved by both organogenesis and somatic embryogenesis (Barlass and Skene, 1980; Rajasekaran and Mullins, 1981; Krul and Worley, 1977). Regeneration system has recently been improved such that the frequency of plant regeneration per explant has increased to about 70% (Cheng and Reisch, 1989), and this technology has been extended to other important cultivars (Stamp and Meredith, 1988). These regeneration systems and the fact that grapevines are a natural pathogenic host of *Agrobacterium* suggest that grapevines are amenable to the insertion of foreign genes via *Agrobacterium*-mediated transformation. Baribault et al. (1989) have obtained transgenic grape calli by cocultivation of grape suspension cells with *Agrobacterium* strain LBA4404 harboring a disarmed Ti plasmid. Cheng (1988) also obtained kanamycin resistant (Km^R) calli with a wild type strain A281 harboring a binary vector. Recently, transgenic chimeric grapevines expressing β -glucuronidase have been developed by infection of fragmented shoot apex cultures (Baribault et al., 1990).

Our objective in this work was to combine the regeneration system developed previously in our laboratory with the transformation technology utilizing *Agrobacterium* Ti plasmid vectors to develop a transformation system for grapevines.

Materials and Methods

Plant materials. Four grape cultivars used in transformation experiments were *Vitis X labruscana* 'Catawba', *V. vinifera* 'Cabernet Sauvignon' ('C.S.') and 'Riesling', and an interspecific hybrid 'Vanessa'. All cultivars were maintained in Murashige and Skoog (Murashige and Skoog, 1962; MS) medium with 3.0 μ M benzyladenine (BA) and 0.6% agar except 'Vanessa' which was grown in C₂D medium (Chee and Pool, 1985) with 3.0 μ M BA and 0.6% agar. The regeneration system for 'Catawba' has been previously reported (Cheng and Reisch, 1989). The regeneration system for 'C.S.' and 'Riesling' was essentially the same as that of 'Catawba' (Cheng and Reisch, unpublished). The medium was Nitsch and Nitsch (1969; NN69) with 10.0 μ M BA and 0.5 μ M indole-3-butyric acid (IBA). The regeneration medium for 'Vanessa' was also described (Reisch et al., 1990). The medium was NN69 with 4.0 μ M BA and 2.0 μ M thidiazuron.

***Agrobacterium* strains.** The strains of *A. tumefaciens* used in this work are listed in Table 1. *Agrobacterium* strains with binary vectors were designated as host strain/binary vector, for example, LBA4404/pBI121 means that the host *Agrobacterium* strain LBA4404 with a Ti plasmid harbors a binary vector pBI121 (Jefferson et al., 1987). Strain LBA4404 (Hoekema et al., 1983), 3850 (Zambryski et al., 1983), EAH101 (Hood et al., 1986), and CUG0027 (J. Norelli, unpublished) contained disabled Ti plasmids. Strain A348 is a wild type strain, strain 328 contains a Tn5 insertion in locus tms, strain 3004 contains a deletion covering loci tms and tmr and A3025 contains the same deletion as strain 3004 with an additional insertion in locus tml. Locus tms encodes two genes for auxin synthesis; locus tmr encodes a gene for cytokinin synthesis. Mutations in locus tml cause a larger tumor (Ream et al., 1983). A binary vector, pGA472, was developed by G. An (An et al., 1985).

Table 1 *Agrobacterium* strains and plasmids used

host strain	chromosome	binary plasmid	antibiotic resistance*	source
LBA4404	Ach5	pBI121	Str/spec, Km	M. Hanson
3850	3850	--	Amp	J. Schell
3850	3850	pBI121	Amp, Km	this work
3850	3850	pGA472	Amp, Tc	this work
EAH101	C58	--	Km	E. Hood
EAH101	C58	pGA472	Km, Tc	this work
3004	C58	--	--	E. Nester
A3025	C58	--	Km	E. Nester
328	C58	--	Km	E. Nester
A348	C58	--	--	E. Nester
3004	C58	pGA472	Tc	this work
A3025	C58	pGA472	Km, Tc	this work
328	C58	pGA472	Km, Tc	this work
A348	C58	pGA472	Km, Tc	this work
CUG0027	CG935	--	Km	J. Norelli
CUG0027	CG935	pBI121	Km	J. Norelli
CUG0027	CG935	pGA472	Km, Tc	this work

Km: kanamycin; Tc: tetracycline; Amp: ampicillin; Str: streptomycin; Spec: spectinomycin.

Triparental mating. The method of transferring binary vectors from *Escherichia coli* into *A. tumefaciens* strains was basically described by Rogers et al. (1987). Briefly, *Agrobacterium* host strains were cultured in minimal T medium and *E. coli* strains with binary vectors and the *E. coli* strain RK2013 with helper plasmid pRK2013 were cultured in LB broth. The antibiotics and their concentrations used for selection of strains or plasmids were: strain LBA4404-- streptomycin (100 mg/l); 3850-- ampicillin (100 mg/l); EAH101, 328, 3025, *E. coli* with plasmid pBI121, and *E. coli* with pRK2013-- kanamycin (50 mg/l); CUG0027--kanamycin (150 mg/l); *E. coli* with pGA472--tetracycline (25 mg/l). After culture for 24 hours in a shaker at 30 °C (for *Agrobacterium*) and 37°C (for *E. coli*), all strains were harvested by centrifugation and resuspended in LB broth without antibiotics. Ten µl each of all possible combinations of one, two or three strains were mixed and deposited on the LB agar plates. After 2 days of "mating" at 28 °C, the mixtures were eluted from the agar plates and suspended in LB broth and plated on minimal T media with appropriate antibiotics for selection. The resistant colonies were selected one more time.

Suspension cultures of *A. tumefaciens* strains for infection of plant materials. *A. tumefaciens* strains were cultured in minimal T medium (5 ml of medium in 15 ml-tube) with appropriate antibiotics in a shaker at 28 °C for 24 hours. The cultures were centrifuged and then resuspended in minimal T medium without antibiotics for infecting plant tissues.

Infection of grape materials. Petiole materials were collected from in vitro grown shoots of 'Catawba' and 'Vanessa'. Leaf blades were collected from 'C.S', since regeneration was possible from leaf tissues (data not shown). The edges of leaf blades were cut, thus creating wound sites for *Agrobacterium*. The petioles or leaves were dipped into *Agrobacterium* suspensions after excision for about 1 minute. They were then blotted on sterile filter papers to absorb excess liquid medium and were placed onto regeneration media without antibiotic. Unless specified, plant tissues were transferred three days later to the same medium with carbenicillin (500 mg/l) and vancomycin (200 mg/l) to eliminate *Agrobacterium* cells. The concentrations of Km are specified in the text. In one experiment, 'Catawba' petioles were dipped in the *Agrobacterium* suspension for 24 hours and then transferred directly to selection medium. In experiments designed to infect basal internode segments, 'Catawba' and 'Riesling' shoots with 3-4 nodes were excised just above a node so shoots had an entire internode without an axillary bud in the base. The basal internodes were dipped into suspensions of *Agrobacterium* strains and then were blotted onto filter papers to absorb excess liquid medium. They were then inserted into medium without antibiotics in baby food jars. After three days of cocultivation, the shoots were transferred to the same medium with carbenicillin (500 mg/l) and vancomycin (200 mg/l) and Km (5 or 25 mg/l).

β-Glucuronidase activity assay. The small leaves from shoots regenerated from leaf explants infected by strain CUG0027/pBI121 were transferred to small vials into which 1-1.5 ml of GUS substrate mixture was added. The substrate mixture consisted of potassium ferricyanide (5.0 mM), potassium ferrocyanide (5.0 mM), 5-bromo-4-chloro-3-indoyl B-D-glucuronic acid (X-Glu, 0.3% wt/vol), and Triton X-100 (0.3%, vol/vol). The tissues with solution were incubated for 24 h at 37 °C. GUS-expressing cells were detected by the appearance of distinct blue spots.

Results

The effect of infection by *Agrobacterium* strains on callus production of 'Catawba' petioles in medium with 100 mg/l kanamycin. The control treatment without *Agrobacterium* produced calli from all petioles and regenerated shoots from 50% of petioles in medium without Km. Infection by strain LBA4404/pBI121 and strain 3850 with either plasmid pBI121 or pGA472 did not result in any callus in medium containing 100 mg/l Km, which was shown to be inhibitory to callus production (Table 2). 'Catawba' petioles infected by strain A281

(an A136 with wild type Ti plasmid pTiBo542) containing pGA472 produced calli, but shoot regeneration was not obtained. On the other hand, petioles infected by EAH101 (A136 with a disarmed Ti plasmid pTiBo542) harboring the same binary vector did not produce callus in the medium with 100 mg/l Km (Table 2).

Infection with strain A348 harboring a wild type Ti plasmid and a binary vector produced calli in 70 out of 80 petioles. Strains with single, double or triple mutations in three T-DNA loci, tmr, tms, and tml (Ream et al., 1983) also produced calli from 50 to 78.6% petioles. However, no regeneration was obtained.

To test if these strains were functional, they were used to infect tobacco (*Nicotiana tabacum* cv. Havana) leaf discs. Leaf discs infected with all strains produced calli resistant to 300 mg/l Km (data not shown).

Table 2 The effect of infections with *Agrobacterium* strains containing binary vectors on callus production of 'Catawba' petioles selected in medium with 100 mg/l kanamycin

Infected with strain/plasmid	Km (mg/l)	No. petioles producing callus per number of explants ¹ (%)	Explants producing shoots (%)
none	0	80/80 (100)	50
none	100	0/70 (0)	0
LBA4404/pBI121	100	0/180 (0)	0
3850/pBI121	100	0/170 (0)	0
3850/pGA472	100	0/180 (0)	0
A281/pGA472	100	70/80 (87.5)	0
EAH101/pGA472	100	0/70 (0)	0
A348/pGA472	100	70/80 (87.5)	0
328/pGA472	100	55/70 (78.6)	0
3004/pGA472	100	45/70 (64.3)	0
A3025/pGA472	100	40/80 (50.0)	0

1: The data were pooled from two independent experiments, each with 40 petioles except in three strains with 100 petioles per experiment (LBA4404/pBI121, 3850/pBI121 and 3850/pGA472). Contamination reduce replications in several cases. Each Petri plate had 10 petioles.

The effect of infections with *Agrobacterium* strains on callus production of 'Vanessa' petioles in medium with 100 mg/l kanamycin. Non infected petioles grown on Km-free medium gave 100% callus production and regeneration from 45% of petioles (Table 3). No callus was produced from petioles infected by strains LBA4404/pBI121 and 3850 with either plasmid pBI121 or pGA472. Infection with strain A348 harboring a wild type Ti plasmid and a binary vector produced calli from 70% of all explants. Petioles infected by strains 328, 3004, and A3025 also produced calli from 55-67.5% of petioles. However, no regeneration was obtained (Table 3).

Attempt to regenerate shoots from Km^R calli of 'Catawba' petioles infected with strain A3025/pGA472. Calli produced from 'Catawba' petioles infected by strain A3025/pGA472 in NN69 medium with 10.0 µM BA and 0.5 µM IBA were transferred to 4 different media with or without 50 mg/l Km: MS medium with 0.5 µM BA and 1.0 or 5.0 µM

NOA, 5 μ M BA and 1.0 or 5.0 μ M NOA. The calli (15 in each medium) proliferated further on all media, but shoot regeneration was not obtained.

Table 3. Effect of *Agrobacterium* infection of 'Vanessa' petioles on callus production in medium with 100 mg/l kanamycin¹

Infected with strain/plasmid	Km mg/l	% of explants producing callus	% of explants producing shoots
none	0	100.0	45
none	100	0	0
LBA4404/pBI121	100	0	0
3850/pBI121	100	0	0
3850/pGA472	100	0	0
A348/pGA472	100	75.0	0
328/pGA472	100	67.5	0
3004/pGA472	100	58.8	0
A3025/pGA472	100	55.0	0

¹: Data were pooled from 2 independent experiments, each with 40 petioles.

The effect of kanamycin on callus formation of 'Catawba' petioles. Since 'Catawba' and 'Vanessa' petioles infected by several different strains did not produce calli or did not regenerate shoots in the medium with 100 mg/l Km (Table 2, 3), it was speculated that 100 mg/l Km was inhibitory to callus formation or shoot regeneration. A further test of Km sensitivity was conducted (Table 4). 'Catawba' petioles did not produce callus in the medium with Km in levels of 10 mg/l or above, 25% of petioles produced a little callus in medium with 7.5 mg/l Km. If the Km concentrations were lowered down to 5 or 2.5 mg/l, no inhibition was observed.

Table 4. The effect of kanamycin concentration on callus formation from 'Catawba' petioles¹

Km (mg/l)	Explants forming callus (%)	Km (mg/l)	Explants forming callus (%)
100	0	7.5	25
50	0	5.0	100
25	0	2.5	100
20	0	0.0	100
10	0		

¹ Each treatment had 40 explants

The effect of infection by *Agrobacterium* strains on callus production of 'Catawba' and 'Vanessa' petioles by selection in medium with 10 mg/l kanamycin. Since 'Catawba' and 'Vanessa' petioles infected by certain strains did not produce calli or shoots in the medium with 100 mg/l Km, further experiments were conducted in medium with 10 mg/l Km.

In the absence of Km, petioles formed calli from almost all 'Catawba' petioles and regenerated shoots at high frequency (45%), but did not produce calli in the medium with 10 mg/l Km without infection (Table 5). Petioles infected by strains LBA4404/pBI121 and 3850 with

either pBI121 or pGA472 did not form callus. Strain A281 with pGA472 incited calli from all petioles, but EAH101/pGA472 did not (Table 5).

Table 5 also shows that 93.8% of control 'Vanessa' petioles formed calli and regenerated shoots from 30% of petioles in medium without Km, but did not produce calli in the medium with 10 mg/l Km. Strains LBA4404/pBI121 and 3850 with either pBI121 or pGA472 did not form callus (Table 5).

Table 5 The effect of infections by *Agrobacterium* strains on callus production of 'Catawba' and 'Vanessa' petioles selected in medium with 10 mg/l kanamycin1

Cultivar	Infected by strain/plasmid	Km (mg/l)	% of petioles producing callus	% explants producing shoots
'Catawba'	none	0	97.5	45
	none	10	0	0
	LBA4404/pBI121	10	0	0
	3850/pBI121	10	0	0
	3850/pGA472	10	0	0
	A281/pGA472	10	100	0
	EAH101/pGA472	10	0	0
<hr/>				
'Vanessa'	none	0	93.8	30
	none	10	0	0
	LBA4404/pBI121	10	0	0
	3850/pBI121	10	0	0
	3850/pGA472	10	0	0

1: The data were pooled from 2 independent experiments, each with 40 petioles. Each plate had 10 explants.

The effects of pre-selection and post-selection on callus formation of 'Catawba' petioles. Since 'Catawba' and 'Vanessa' petioles infected by strains LBA4404/pBI121 and 3850 with either pBI121 and pGA472 did not produce calli, and Km^R calli incited by strain 3025/pGA472 did not regenerate shoots in the medium with 100 or 10 mg/l Km, further experiments were conducted with either pre-selection or post-selection. The pre-selection technique was to culture the *Agrobacterium*-infected petioles in medium with 10 mg/l Km, followed by transfer to medium without Km to induce shoot formation. The post-selection technique was to culture the *Agrobacterium*-infected petioles in medium without Km to induce shoot formation, followed by transfer to medium with 10 mg/l Km to select for possible Km^R shoots.

Petioles without selection (0 days) produced calli and regenerated from 42.5% petioles. These regenerated shoots did not survive after transfer to medium with 10 mg/l Km (Table 6). Pre-selection for 7 to 21 days in medium with 10 mg/l Km did not permit callus production of petioles infected with LBA4404 (Table 6). When petioles were infected with A3025/pGA472, all petioles produced calli and regenerated from 55% petioles without Km. These regenerated shoots all died upon transfer to medium with 10 mg/l Km (Table 6). When petioles were preselected for 7 to 21 days in medium with 10 mg/l Km, 10 to 15 of 40 petioles (25-40%) produced calli. Explants, infected with A3025/pGA472 and grown on Km-containing medium for 7 and 14 days, regenerated from 5 and 1 petioles, respectively. But none of these shoots survived when transferred back to selection medium with 10 mg/l Km (Table 6).

Table 6. The effect of pre-selection on callus formation of 'Catawba' petioles¹

Selection in Km (10 mg/l) for No. of days	infected by strain/plasmid	move to nonselection medium	
		% of petioles producing callus	% of explants producing shoots
0	none	100	47.5
0	LBA4404/pBI121	100	42.5
7	LBA4404/pBI121	0	0
14	LBA4404/pBI121	0	0
21	LBA4404/pBI121	0	0
0	A3025/pGA472	100	55.0
7	A3025/pGA472	37.5	12.5
14	A3025/pGA472	35.0	2.5
21	A3025/pGA472	25.0	0

¹: Each treatment had 40 explants.

In the post-selection experiment, all petioles without selection produced calli and 47.5% of petioles regenerated shoots (Table 7). When petioles were infected by LBA4404/pBI121, non selection for 0 or 7 days did not permit petioles to produce calli after transfer to selection medium. After 14 or 21 days in non selection medium, 25% and 50% of petioles, respectively, produced calli after transfer to medium with 10 mg/l Km (Table 7). Five petioles regenerated shoots within 21-days in Km-free medium. These shoots started to bleach in 3-5 days after the transfer to selection medium and did not survive more than 3 weeks.

When petioles were infected with strain A3025/pGA472, 45% to 70% formed calli following transfer to selection medium with 10 mg/l Km after 0 to 21 days in non selection medium (Table 7). Calli started to form in the medium about 14 days after initial culture. Five petioles regenerated shoots within 21 days in Km-free medium. These shoots started to bleach in 3-5 days after transfer to selection medium and did not survive more than 3 weeks.

The effect of infections with strain CUG0027/pGA472 on callus production of 'Catawba' petioles. Non-infected 'Catawba' petioles swelled, formed calli and regenerated shoots in Km-free medium, but did not swell, produce calli or regenerate shoots when 10 mg/l Km was added to the medium (Table 8). Five mg/l Km did not affect callus production, but inhibited regeneration. Petioles infected with method I formed calli in 27.5% of petioles in medium with 10 mg/l Km, but no regeneration occurred (Table 8); 70% of petioles infected by method I callused in the medium with 5 mg/l Km and 5 of them regenerated shoots which did not survive when transferred to medium with 20 mg/l Km. Petioles, infected by dipping in *Agrobacterium* suspension for 24 hours followed by direct transfer to selection medium, did not swell or produce callus in medium with 10 mg/l Km, but produced calli in medium with 5 mg/l Km (Table 8).

Table 7. The effect of post-selection on callus formation of 'Catawba' petioles¹

No selection first for No. of days	infected by strain/plasmid-----	move to selection medium (10 mg/l Km)	% petioles producing callus	% petioles producing shoots
control: no infection & no selection			100	47.5
0	LBA4404/pBI121		0	0
7	LBA4404/pBI121		0	0
14	LBA4404/pBI121		25.0	0
21	LBA4404/pBI121		50.0	12.5
0	A3025/pGA472		50.0	0
7	A3025/pGA472		47.5	0
14	A3025/pGA472		45.0	0
21	A3025/pGA472		70.0	0

¹: Each treatment had 40 explants.

Table 8. The effect of infection by *Agrobacterium* strain CUG0027/ pGA472 on swelling, callus formation and regeneration of 'Catawba' petioles

infection method ¹	Km (mg/l)	swelling	% petioles producing calli ²	% petioles producing shoots ²
no infection	0	++	100	70.0
no infection	5	++	85	0.0
no infection	10	--	0	0.0
method I	5	++	72.5	6.3
method I	10	++	27.5	0.0
method II	5	++	56.3	0.0
method II	10	--	0.0	0.0

¹: method I: Petioles were dipped in *Agrobacterium* suspension for 1 minute, then transferred to solid non-selection medium for 3 days before transfer to selection medium;

method II: Petioles were dipped in *Agrobacterium* suspension for 24 hours then transferred to selection medium.

²: Forty petioles were used in non infected controls and 80 petioles used in "method I" and "method II".

The effect of infections with strain CUG0027/pBI121 on callus production of 'Cabernet Sauvignon' leaf segments. 'Cabernet Sauvignon' leaves produced calli from all 80 leaves when they were cultured in Km free medium and 6.3% regenerated shoots (Table 9). An addition of 5 mg/l Km inhibited callus production and prevented shoot regeneration and 10 mg/l Km prevented both callus production and shoot formation. When the leaf segments were infected

with strain CUG0027/pBI121, 5 to 37.5% leaf segments produced calli in media with 5-20 mg/l Km. But shoot regeneration only occurred from 2.5% leaf segments in the medium with 5 mg/l Km. Assay for GUS production was all negative and shoots did not survive when transfer to medium with 10 mg/l Km (Table 9).

Table 9 The effect of infection by strain CUG0027/pBI121 on callus formation and shoot regeneration of 'Cabernet Sauvignon' leaves¹

infection by	Km (mg/l)	% explants producing calli	% of explants producing shoots
none	0	100	6.3
none	5	25	0
none	10	0	0
CUG0027/pBI121	5	37.5	2.5
CUG0027/pBI121	10	15.0	0
CUG0027/pBI121	20	5.0	0

¹: Each treatment had 80 leaf segments.

The effect of infection with strain A3025/pGA472 on callus formation of 'Catawba' and 'Riesling' intact shoot bases. Calli were produced at all intact shoot bases (12 in each treatment) of both cultivars in media with 0, 5 or 25 mg/l Km with or without infection by *Agrobacterium*. Calli were produced about 10 days later in medium with 25 mg/l Km than those in media with 0 or 5 mg/l Km, and shoots grew slower. So it was not possible to determine the effect of infection and selection.

Discussion

A reliable, easy, relatively high frequency regeneration system has been developed for the grape genotype 'Catawba' (Cheng and Reisch, 1989), and Km^R calli have been obtained previously for this genotype (Cheng, 1988), yet attempts described here to combine the regeneration system with a transformation system to develop transgenic plants so far have not been successful. Several factors discussed below may affect the development of the system.

Host range of *Agrobacterium* The variability of host range of *Agrobacterium* has been well documented (De Cleene and De Ley, 1976; Anderson and Moore, 1979). Different strains have different host ranges, and responses may vary among plant species, genotypes or tissue types (Knauf et al., 1982). The strain LBA4404 with binary vectors has been very widely used in plant transformation (e.g., Jefferson et al., 1987), but this strain does not infect grape genotype 'Catawba' (*Vitis X labruscana*) and 'Vanessa' (interspecific hybrid of *V. vinifera* X *V. labrusca*) (Table 2, 3, 5, 6, 7). This strain has been reported to infect suspension cells of 'Cabernet Sauvignon' (*V. vinifera*) and produced Km^R calli (Baribault et al., 1989). So this strain has a differential ability to infect *V. vinifera*, *V. X labruscana*, or the interspecific hybrid. Differences in resistance to crown gall disease has been found among the species and the hybrids of grapevines (Szegedi et al., 1984). The difference in ability to infect plant tissues may also be due to the different tissues infected (cells vs. petioles). Different responses of tissues to *Agrobacterium* have been observed (Knauf et al., 1982). Strain 3850 with two different binary vectors was also non-infectious to 'Catawba' and 'Vanessa' under the conditions tested (Table 2, 3, 5).

Strain A281 with the wild type Ti plasmid pTiBo542 was a supervirulent strain (Jin et al., 1987). A281 containing the binary vector pGA472 was infectious to 'Catawba' but the same strain with disarmed pTiBo542 (Hood et al., 1986) was non-infectious to 'Catawba' (Table 3, 6). This suggests that the T-DNA genes are somehow involved in the determination of the host range, or tumor development. The involvement of T-DNA genes in the host range, based on tumor formation, has been observed in some cases (Facciotti et al., 1985; Yanofsky et al., 1985). In the latter situation, the limited host range (LHR) strains that form tumors on grapevines are avirulent on common *Agrobacterium* hosts including tomato. Genetic analysis has revealed that the LHR Ti plasmids pTiAg162 and pTiAg63 do not have an active cytokinin gene. Thus, neoplastic growth of tomato cells requires both the auxin and cytokinin genes (Yanofsky et al., 1985). In our case, however, it is unknown how the T-DNA genes were involved, since both cytokinin and auxin were present in the medium.

A set of strains with wild type or partially mutated T-DNA genes (Ream et al., 1983) did not affect the overall host range, since they are all infectious to 'Catawba' (Table 2, 3, 6, 7) and 'Vanessa' (Table 3). The biovar 1 strain CUG0027 (J. Norelli, unpublished) with pGA472 and pBI121 was also infectious to 'Catawba' and 'Cabernet Sauvignon' as determined by the formation of Km^R calli (Table 8, 9). With the same plant genotype, different strains of *Agrobacterium* gave rise to different responses (Table 2, 5, 8).

In nature, grapevines are usually infected by biovar 3 strains of *A. tumefaciens* (Burr et al., 1983, 1987). In vitro, at least some biovar 1 and 2 strains can infect grape leaf and petiole tissues (Table 2, 3, 8, 9), but the frequency of Km^R calli was not very high, about 50% in strain A3025/pGA472 (Table 2, 3) and 70% in strain CUG0027/pGA472 (Table 8). Baribault et al. (1990) obtained a low frequency of transformation with a biovar 1 strain. It may be beneficial to develop a biovar 3 vector with a very high frequency of transformation.

The inhibition of callus formation and shoot regeneration by kanamycin. Kanamycin resistance has been widely used as a marker gene in plant transformation (Horsch et al., 1985; Filatti et al., 1987; Dandekar et al., 1988). The sensitivity to kanamycin may vary among different species or tissues. Up to 800 mg/l was required to inhibit the growth of rice cell suspension (Hauptmann et al., 1988); while as low as 35 mg/l was enough to select transformants in *Brassica napus* (Charest et al., 1988). Grape tissues are very sensitive to kanamycin (Table 4). A concentration of 10 mg/l could completely inhibit callus formation and shoot regeneration from petiole tissues (Table 5, 8, 9). This level of kanamycin was also recently reported to completely inhibit the adventitious shoot formation of grapevines (Colby and Meredith, 1990) and for selection of transgenic shoots (Baribault et al., 1990). To overcome this problem, two other strategies were applied, a pre-selection (Table 5) and a post selection (Table 6). These two strategies have been applied in apple to recover a very low frequency of transgenic shoots (James et al., 1989). The *Agrobacterium* infected 'Catawba' petioles were first selected on Km for a period of 7 to 21 days, then transferred to Km-free medium. Pre-selection for 7 and 14 days allowed shoot regeneration, but these shoots did not survive when they were transferred back to selection medium (Table 6). On the other hand, post selection had a similar effect to that of direct selection, no shoots regenerated in non-selection medium for 7 or 14 days (Table 7). Non selection for 21 days allowed 5 shoots to regenerate, but they bleached and did not survive when they were transferred to selection medium.

The lack of a callus regeneration system for grapevines. Although a regeneration system with a relatively high frequency for 'Catawba' has been developed (Cheng and Reisch, 1989), shoots seemed to regenerate directly with very little callus formation. When petioles were infected with *Agrobacterium*, Km^R calli formed (Table 2, 3, 5, 8, 9). Regeneration of shoots from these calli has been successful in the regular regeneration medium (Table 2, 3, 5, 7). The callus regeneration systems in the literature had low frequencies of regeneration (Krul and Worley,

1977; Favre, 1977; Rajasekaran and Mullins, 1981). Using one of these systems (Rajasekaran and Mullins, 1981) did not enable us to obtain regeneration from these Km^R calli of 'Catawba' petioles even in the medium without Km (Table 4). The recently developed transformation system (Baribault et al., 1990) used the fragmented shoot apex regeneration system which is based on adventitious shoot formation (Barlass and Skene, 1980). The lack of a regeneration system from callus has been a major obstacle for the development of a high frequency transformation system.

Chapter 5. a portion of the Ph.D. thesis research of Zong-Ming Cheng:

Transient Gene Expression of β -Glucuronidase in Grapevine Leaf Tissues After Biolistic Bombardment

Abstract

The biolistic transformation system for delivery of foreign genes into grapevine leaf tissues was tested. The β -glucuronidase (GUS) gene gave transient expression in leaf tissues as determined by the development of blue color after staining. Several factors were examined for their effect upon the frequency of DNA delivery. These included plant genotypes, DNA loading methods, bombardment pressures, osmotic concentration, number of bombardments, and distances between the microprojectiles and target tissues. The most important parameter that affects the efficiency of delivery could not be determined because of the low frequencies of transformation in all cases. Up to 30 blue spots could be detected in a single grape leaf blade, but this is unlikely to be high enough to achieve stable transformation. Further tests are needed to optimize the delivery system for grapevines with the biolistic transformation system.

Introduction

Since Sanford and coworkers first developed the biolistic transformation system (Klein et al., 1987; Sanford et al., 1987), research has flourished in developing genetic transformation systems with the biolistic process. In the last two to three years, biolistic gene delivery has been used to transform nuclear genomes in a very diverse range of organisms and organelle genomes (for review, see Sanford, 1990a, b). This includes bacteria, yeast and other fungi, algae such as *Chlamydomonas*, animal cells, intact animal organs and higher plants. In plants, reporter genes have been delivered and transiently expressed in onion (Klein et al., 1987), corn (Klein et al., 1988 a, b), rice, wheat, soybean (Wang et al., 1988) and barley (Creissen et al., 1990). Stable transformation and regeneration of plants were obtained by culturing bombarded materials in selective media, from tobacco (Klein et al., 1988c), soybean (McCabe et al., 1988), cotton (Finer and McMullen, 1990), maize (Gordon-Kamm et al., 1990) and papaya (Fitch et al., 1990).

Biolistic transformation in plants is a method most suitable for those species which are not hosts for *Agrobacterium tumefaciens* or *A. rhizogenes* and for those which do not have a regeneration system from protoplasts or are recalcitrant to regeneration. These include most cereal food crops, many dicotyledonous species and many perennial woody species. Grapevines have been very difficult to transform with the *Agrobacterium* Ti plasmid system. Kanamycin resistant calli can be obtained relatively easily by infection with *Agrobacterium* strains harboring plasmids with a kanamycin resistance gene, but regeneration from these calli has not been successful (Cheng, 1988; Baribault et al., 1989; Martin et al., 1989).

The purpose of this work was to examine the feasibility of utilizing the biolistic transformation system in grapevines and to determine the most important parameters which affect the delivery of DNAs into grape leaf tissues.

Materials and Methods

Plant Materials. Grape leaves of *Vitis vinifera* cultivar 'Cabernet Sauvignon' and *V. X labruscana* 'Catawba' from in vitro grown plantlets were used as materials for bombardment. Shoot cultures were maintained in MS medium (Murashige and Skoog, 1962) with 3.0 μ M benzyladenine (BA) and 0.6% agar in baby food jars. Unless specified in the results section, leaf materials were collected from cultivar 'Cabernet Sauvignon'. The leaf tissues from this cultivar were shown to be regenerable (unpublished) based on the method developed for 'Catawba' (Cheng and Reisch, 1989).

Osmotic Conditions. Molarities were adjusted by adding equal amounts of sorbitol and mannitol to liquid NN69 medium (Nitsch and Nitsch, 1969) with 10.0 μM BA and 0.5 μM indole-3-butyric acid (IBA). The added osmotic concentrations were: 0.0, 0.3, 0.6, 0.9, 1.2 M.

Preparation of tungsten microprojectiles coated with plasmid DNA. Leaf samples were bombarded with tungsten particles coated with DNA as described by Boynton et al. (1988). To absorb DNA to the microprojectiles, 2.5 μl of DNA (1.0 $\mu\text{g}/\mu\text{l}$ plasmid pBI505 in 10 mM Tris.HCl and 1 mM EDTA, pH 7.7) was added to 25 μl of a suspension of tungsten particles (0.05 mg/ml of 50% glycerol) in a 1.5-ml Eppendorf tube. After the addition of DNA, CaCl_2 (25 μl of 2.5 M solution) and spermidine free base (5.0 μl of a 1.0 M solution) were added to the suspension. After 10 minutes of incubation, the particles were pelleted by centrifugation in a microfuge for 30 seconds, and a portion of the supernatant (25 μl) was removed. The final microprojectile preparation therefore contained 39 μg of tungsten per μl of suspension and 2.0 μg of DNA per μg of tungsten. The clumps of particles were dispersed by briefly (1 second each of three times) touching the outside of the Eppendorf tube to the probe of a sonicator. After 3 one-second sonications, the microprojectiles were bombarded into grape leaf materials.

Bombardments. Young leaf blades were cut at the junction with the petiole, and placed in NN69 medium with 10.0 μM BA and 0.5 μM IBA just before bombardment. In the experiment with different added osmotic molarities, leaf blades were placed in different osmotic solutions for 2 hours to reach equilibrium. Just before bombardments, they were placed on top of 3-layer Whatman 3 filter papers in Petri plates with 5 ml of the same osmotic solutions. The leaves were placed adaxial side down.

Five μl of microprojectiles coated with DNA were loaded on yellow flying discs or in nylon membranes. The distance was 9.0 mm between the rupture membranes and DNA in all treatments. After bombardments with the Helium-Charged Biolistic Device, the plant tissues were incubated for 48 hours. All bombarded tissues were transferred to small vials into which 1-1.5 ml of GUS substrate mixture was added. The substrate mixture consisted of potassium ferricyanide (5.0 mM), potassium ferrocyanide (5.0 mM), 5-bromo-4-chloro-3-indoyl β -D-glucuronic acid (X-Glu, 0.3% w/v), and Triton X-100 (0.3%, vol/vol). The tissues with solution were incubated for 24 hours at 37 $^{\circ}\text{C}$. GUS-expressing cells were detected microscopically by the appearance of distinct blue spots.

Statistical Analysis. The statistical analysis was done with Duncan's multiple range test using the SAS program. The significance level was 5%.

Results

Effect of loading methods on expression of β -glucuronidase. The DNA loaded on flying discs did not result in expression of β -glucuronidase in leaf tissues whether they were bombarded with a pressure of 1200 or 1500 psi, or bombarded once or twice with a distance of 14.8 cm between DNA and tissue samples (Table 1). When DNA were loaded onto nylon membranes, averages of 0.067 with one bombardment and 0.03 blue spots per leaf with two bombardments were detected when they were bombarded in 1200 psi; while averages of 0.27 with one bombardment and 1.0 blue spots with 2 bombardments were detected when they were bombarded at 1500 psi (Table 1).

Effect of bombardment pressures on expression of β -glucuronidase. The result in Table 1 also showed the effect of different pressures of bombardment. When DNAs were bombarded at 1200 psi, only 3 blue spots were detected when they were loaded in nylon membranes, while 38 blue spots were detected when they were bombarded at 1500 psi.

Table 1 Influences of loading methods, pressures and times of bombardment on expression of β -glucuronidase in leaf tissues*

microprojectiles loaded on	bombardment pressure	times	% survival	total blue spots	blue spots /leaf
Flying discs	1200	1	100	0	0.0
Flying discs	1200	2	100	0	0.0
Flying discs	1500	1	100	0	0.0
Flying discs	1500	2	100	0	0.0
nylon membrane	1200	1	100	2	0.07
nylon membrane	1200	2	100	1	0.03
nylon membrane	1500	1	100	8	0.27
nylon membrane	1500	2	100	30	1.0

*: Petri plates with tissues were placed 14.8 cm to the DNA.

Distance between rupture membrane and DNA was 9.0 mm.

**: Each treatment had two plates each with 15 leaves in the center of the Petri dishes.

Table 2 Influences of added osmotic concentrations on expression of β -glucuronidase in leaf tissues*

osmotic molarity (M)	No. of leaves	% survival	% of surviving leaves with blue spots	Total No.	blue spots per surviving leaf
0.0	45	100	15.6	17	0.4
0.3	45	100	4.5	9	0.2
0.6	45	55.6	16.0	16	0.6
0.9	45	40.0	11.1	7	0.4
1.2	45	42.3	15.8	7	0.4

*: Three Petri plates with tissues of each treatment were placed 14.8 cm to microprojectiles. The microprojectiles were loaded on nylon membrane discs. The pressure was 1500 psi.

Table 3 Influences of different genotypes on expression of β -glucuronidase in leaf tissues*

cultivar	No of leaves	% survival	% of surviving leaves with blue spots	blue spots /leaf
'Cabernet Sauvignon'	45	100	8.8	0.11
'Catawba'	45	100	11.1	0.16

*: Three Petri plates with tissues of each cultivar were placed 14.8 cm from the microprojectiles. The microprojectiles were loaded on nylon membrane discs. The pressure was 1500 psi.

The effect of osmotic concentration on survival of leaf tissues and expression of β -glucuronidase. Osmotic concentrations did not affect the frequencies of blue spots in the condition tested (Table 2). Tissue death occurred at molarity concentrations equal to or greater than 0.6 M (Table 2).

Effect of genotypes on expression of β -glucuronidase in leaf tissues. Two cultivars were tested for the efficiency of transient expression. There was no apparent difference in transient expression frequency which was low in both cases (Table 3).

The effect of distances between microprojectiles and plant tissues on expression of β -glucuronidase in leaf tissues. When tissues were placed 7.0 cm from the DNA, a total of 5 blue spots were detected in a total of 26 leaves (Table 4). When the tissue was placed 9 cm or 14.8 cm from the DNA, a total of 14 or 22 blue spots were detected, respectively. There was no statistically significant difference among the treatments in the average number of blue spots per leaf (Table 4).

Table 4 Influences of distances between microprojectiles and plant tissues on expression of β -glucuronidase*

cm between DNA & sample	# of leaves	% of survival	% surviving leaves with blue spots	Total No.	blue spots per surviving leaves
7.0	26	100	19.2	5	0.2
10.9	26	100	38.4	14	0.5
14.8	26	73	68.4	22	1.2

*: Each treatment had 2 Petri-plates with 13 leaf blades. The microprojectiles were loaded on nylon membrane discs. The pressure used was 1500 psi.

Table 5 Influence of number of bombardments on expression of β -glucuronidase in leaf tissues*

No. of bombardments	No. of leaves	% of survival	% of surviving leaves with blue spots	total No.	blue spots per surviving leaf
one	26	100	69.2	57	2.2
two	26	100	57.7	51	2.0
three	26	100	84.6	134	5.2
four	26	50	100.0	25	1.9

* Each treatment had two Petri plates each with 13 leaves. The plates were placed 7.0 cm to the DNA. The microprojectiles were loaded on nylon membrane discs. The pressure used was 1500 psi.

The effect of bombardment times on expression of β -glucuronidase in 'Cabernet Sauvignon' leaf tissues. Table 5 showed that a total of 57 blue spots were detected in 26 leaves when tissues were bombarded one time, 51 blue spots when they were done twice. The total blue spots increased to 134 in 26 leaves when they were bombarded three times

ranging from 0 to 30 per leaf. Among these, 3 leaves had over 25 blue spots each. The number dropped to 25 when they were bombarded four times because 13 leaves were killed. There was no statistically significant difference among the treatments in the average number of blue spots per leaf.

Discussion

Although genetic transformation by the biolistic process is very new, it has proven to have great potential in delivering genetic materials to plants (Klein et al., 1988c; Finer and McMullen, 1990; Gordon-Kamm et al., 1990), fungi (Armaleo et al., 1990), bacteria (Smith et al., in preparation), algae (Boynton et al., 1988; Blowers et al., 1989), animal cells (Johnston et al., 1989; Foote and Hough, 1990) and uniquely to organelles, yeast mitochondria (Johnston et al., 1988; Fox et al., 1988) and chloroplasts of tobacco (Daniell et al., 1990) and *Chlamydomonas* (Boynton et al., 1988; Blowers et al., 1989). It may prove to be a 'universal' system for genetic transformation. In higher plants it has been shown to be especially significant for those species which are not susceptible to the *Agrobacterium* Ti plasmid system, such as most food cereal crops and many woody species which are recalcitrant to regeneration.

Grapevine has been very difficult to transform and regenerate (Baribault et al., 1989; Cheng, 1988; Martin et al., 1989). The results shown in this work indicate that the biolistic process might be feasible for grapevines. The β -glucuronidase gene can be delivered and expressed transiently in grape leaf tissues. The highest number of blue spots in a single leaf was 30 after being bombarded three times when the leaves were placed at the highest level in the chamber. However, this number is not high enough for stable transformation. In corn, about 2-5% of transient transformants became stably transformed, which yielded one in $0.2-5 \times 10^5$ cells, or about 50-60 stable transformants per Petri plate (Klein et al., 1989). These rates have been considered only marginally acceptable (Sanford, 1990a).

There are many variables affecting frequencies of transformation (Klein et al., 1988b; Armaleo et al., 1990; Sanford, 1990a). Examples are the DNA loading method, osmotic condition of the bombarded material, forces (pressures) used in bombardment, distance between the microprojectiles and plant tissues, times of bombardment, and others. These factors may influence both cell survival as well as transformation frequencies. The preliminary experiments in this work indicate that all these factors may play a role, but the most important variable has not been elucidated yet, since expression of β -glucuronidase in all experiments was quite low or too highly variable with the given sample sizes. It is not possible to conclude which factors were the most important based on these data, though the data suggest that number of times of bombardment as well as distance from the DNA source may play major roles in success. Increasing the number of bombardments from 1 to 3 may improve the frequency of surviving leaves with blue spots as well as the blue spots per leaf (compare Tables 1-4 with Table 5). The use of nylon membranes as DNA carriers was preferable to the use of flying discs. Results of biolistic transformation have also varied from day to day, and experiment to experiment due to undetermined factors (Gradziel, pers. comm.), so the results from any given day must be interpreted cautiously. Much more work is needed to determine the most important variables, so the frequency can be increased to maximize the chances for stable transformation.

Chapter 6. Other particle gun transformation experiments, M.H. Martens, M.S. Strefeler and B.I. Reisch:

Particle Gun Transformation of Regenerable Grapevine Petioles

The first experiment was carried out to ascertain the efficiency and feasibility of using the biolistic process (microprojectile bombardment) to genetically transform grape tissues. Leaf petiole segments (3-5 mm in length) from in vitro grown plantlets of *V. X labruscana* 'Catawba' were placed with basal ends facing upward on Nitsch and Nitsch 1969 media and cultured for 1 or 3 days prior to bombardment. Petioles were bombarded 1, 2 or 3 times with tungsten particles, M-10, (0.4 to 1.0 μ) coated with the plasmid pBI221.1 which contains the gene for β -galacturonidase (GUS). After bombardment petioles were incubated for 48 h and then assayed for transient expression of GUS. The control treatment involved bombarding petioles with non-coated tungsten particles. The highest frequency of GUS expressing petioles (5%) was observed on those precultured for 1 day and bombarded 3 times (See Table below). These results demonstrated it was feasible to transform grape cells using the biolistic process but that more work was needed to optimize the system for grapes.

The second experiment examined the effect of particle size and length of preculture time on the frequency of transformation of grape petiole cells. The plant material and DNA used were the same as in Experiment 1. Petioles were precultured prior to bombardment for 9, 15 or 21 days and all were bombarded 3 times. The size of the tungsten particles used were M-10 (median size: 0.64 μ) or M-20 (median size: 1.36 μ). The frequency of transformation was higher using the M-20 particles than that observed for M-10 particles, regardless of length of preculture (See Table below). Transformation frequency decreased as the duration of culture increase when using M-10 particles. Transformation frequency peaked at 15 days of preculture but decreased after 21 days of preculture. These results indicate that preculture for 15 days in conjunction with M-20 particles was the most effective treatment for transformation of grape petioles. Also, these results indicate that the biolistic process is a very effective method for obtaining transformation of grape petiole tissue.

Table 1. Initial results in biolistic transformation of grapevine petioles

Days precultured	Particle size (μ)	# bombardments	% petioles transformed	Avg. # blue spots /petiole
<i>Experiment 1</i>				
1	M-10	1	2.0	1.0
3	M-10	1	2.0	1.0
1	M-10	2	3.3	1.0
1	M-10	3	5.0	1.0
Control	M-10	1, 2 or 3	0.0	0.0
<i>Experiment 2</i>				
9	M-10	3	40.0	2.5
15	M-10	3	42.0	3.2
21	M-10	3	22.0	2.2
9	M-20	3	61.7	3.7
15	M-20	3	75.0	3.2
21	M-20	3	54.0	2.3
Control	M-10 or M-20	3	0.0	0.0
(9, 15 or 21)				

Over the past 3 years, we have conducted many experiments to identify some of the parameters that might influence transformation, such as particle size(M-5 through M-20 tungsten),

explant source, distance of explant from blast (10 vs 13 cm), length of preculture time prior to bombardment (10 days to 6 weeks), and particle composition (gold vs. tungsten). (Most of these experiments are summarized below). During the same time, our collaborators in Dr. Sanford's lab have continually improved the biolistic process, developing new techniques for handling the DNA-coated particles and enhancing the operation of the gun. From our experiments, many variables have been determined and the level of transient transformation has been greatly increased, but to date, we have not recovered any stably transformed regenerated plants. We have noticed a continuing level of variation in transformation efficiency between experiments, perhaps due to health and age of petioles, working condition of the gun, quality of the DNA, or other unknown factors. Recently, we have tested several different plasmid DNA constructs, with the greatest success coming from pBI505 which carries the GUS marker gene along with a particularly powerful promoter region. Plasmid pBI426 which carries both the GUS gene and a gene coding for kanamycin resistance has also been used. This second trait permits the use of kanamycin-containing selective medium following bombardment. It is suspected that many of the transformed cells are so damaged by the impact of the particle and the trauma of the process that they are unable to regenerate, or that they are weakened and are rapidly overgrown by healthy, non-transformed cells. The use of a selective medium will give transformed cells a critical advantage in regeneration unless they are damaged beyond repair.

Following investigation of numerous factors affecting transient expression, we have settled at present on a system utilizing the following conditions: A high pressure helium blast (at 1200 psi) is used to accelerate tungsten particles into in vitro cultured grapevine petiole tissues. Target tissues are held 10 cm from the origin of the helium shock wave. Petioles are grown on regeneration medium for 10-14 days prior to bombardment. Basal cut edges are oriented edge up on the agar medium to expose the regenerable surface to the particle stream. The tissues are then assayed for GUS gene expression to determine the extent of successful transient transformation. Plants are then regenerated from the bombarded tissue and assayed for stable transformation. Plasmid pBI505 has given the highest rate of transient GUS expression (Table 1), but pBI426 is in current use because it is more resistant to methylation and provides NPTII along with the GUS gene.

Table 2. Factors Affecting Transformation with the Particle Gun*

Cultivar	Plasmid DNA	# Times Shot	Variable	% Petioles with blue spots	Avg # Blue spots per petiole
Catawba	pBI505	1	Distance =13 cm	22	0.67
Catawba	pBI221	1	Distance =13 cm	17	0.43
Catawba	pBI505	3	Distance = 13 cm	88	5.62
Catawba	pBI505	3	Distance = 10 cm	93	10.97
Merlot	pBI505	3	Distance = 10 cm	75	2.10
Catawba	pBI505	3	Tungsten particles, Dist=13 cm	22	0.67
Catawba	pBI221	3	Tungsten particles, Dist=13 cm	16	0.43
Catawba	pBI505	3	Gold particles, Dist=13 cm	0	0
Catawba	pBI221	3	Gold particles, Dist=13 cm	0	0
Catawba	pBI505	3	6 week pre-trt, Dist=13 cm	0	0
Catawba	pBI505	3	2 week pre-trt, Dist=13 cm	3.7	0.04
Catawba	pBI121	3	6 week pre-trt, Dist=13 cm	0	0
Catawba	pBI121	3	2 week pre-trt, Dist=13 cm	0	0
Catawba	pBI121	4	6 week pre-trt, Dist=13 cm	0	0
Catawba	pBI121	4	2 week pre-trt, Dist=13 cm	0	0
Catawba	pBI426	3	Post-trt= No Km, Dist=13 cm	50	1.7
Catawba	pBI426	3	Post-trt=5mg/l Km, Dist=13 cm	45	1.45
Catawba	pBI426	3	Post-trt=10 mg/l Km, Dist=13 cm	33	0.53
Catawba	pBI426	2	Distance=10 cm, 1M Sorbitol	13	0.16
Catawba	pBI426	2	Distance=10 cm, no Sorbitol	20	0.33

* - This table is a compilation of results from several experiments. Most experiments were small, consisting of 2-3 plates of 10, 30 or 50 petioles each per treatment.

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Description of Cooperation:

In March of 1988, Bruce Reisch traveled to Bet Dagan. During this visit, he consulted with co-PIs Aliza Vardi and P. Spiegel-Roy concerning research progress and future plans. Early results from each lab were shared. Because of the promising results with petiole culture of several varieties at Geneva, Drs. Vardi and Spiegel-Roy were able to extend this technique for use on varieties of importance in Israel. During the course of this project, there has been ongoing correspondence between Geneva and Bet Dagan concerning project progress and plans. Results were shared at an early stage and we were fortunate that techniques which worked well in New York also were successful in Israel. Chemicals in short supply in Israel have been sent to Bet Dagan.

Evaluation of Research Achievements:

The most significant feature of this project was the development and improvement of techniques to regenerate plants from leaf, petiole, and root cultures of *Vitis*. The system was originally defined with 'Catawba' and extended by this project to other *labruscana*, hybrid and *vinifera* varieties. Following reports of our progress, other labs reported on the use of similar techniques, based on high cytokinin media, for the regeneration of *vinifera* and rootstock varieties from leaf and petiole explants. Unfortunately, though a great deal of effort was made, plans to obtain genetically engineered plants fell short. Though efforts to genetically engineer grapevines are taking place in at least five labs around the world, only two labs have recently reported some measure of success, with only a very low frequency of success being reported in one of these cases. This attests to the difficulty of the project. Future efforts should focus on defining the barriers to the transformation of regenerable tissues.

We have refined the petiole regeneration system so as to obtain more consistency of results. Because of our experience with this system, we focussed gene transfer efforts on petiole explants. Great progress has been made using the particle gun to shoot DNA into the cut edges of petioles. Though chimerism may be a problem using this technique, as well as with *Agrobacterium* based gene transfer systems, it should be resolvable by the further regeneration of shoots from transformed sectors, or the propagations of axillary buds from these sectors. Recent reports suggest that *Agrobacterium* plasmids are not transforming tissues within petioles and leaf stubs which can be induced to regenerate. This should not be a concern with particle gun work. Transient expression is easily obtained and further experiments should lead to the definition of conditions conducive to stable plant transformation. The BARD funded project has laid the ground work for efforts in this area.

List of Publications:

- Cheng, Z.M. and B.I. Reisch. 1989. Shoot regeneration from leaf and petiole cultures of *Vitis labruscana* cv. Catawba. Plant Cell Reports 8:403-406.
- Reisch, B.I., M.H. Martens and Z.M. Cheng. 1990. High frequency regeneration from grapevine petioles: Extension to new genotypes. *Vitis* (special issue) Proc. 5th Int. Symp. Grape Breeding 419-422.
- Cheng, Z.M. and B.I. Reisch. 1988. High frequency shoot regeneration from *Vitis labruscana*. HortScience 23:803. (abstr.)
- Reisch, B.I. and M.R. Martens. 1988. Shoot regeneration from grape leaf, petiole, and tendril tissues. HortScience 23:807. (abstr.)
- Reisch, B.I., M.H. Martens and Z.M. Cheng. 1989. High frequency shoot regeneration from grape leaves, petioles and tendrils. V International Symposium on Grape Breeding, St. Martin, Federal Republic of Germany. (abstr.)
- Bruce I. Reisch, Mary-Howell R. Martens and Z.M. Cheng. 1989. Shoot regeneration from grape leaf, petiole and tendril tissues. Meeting of Am. Soc. Enol. Vitic. - Eastern Section, July 1989. (abstract)
- Cheng, Z.M. 1990. Strategies for genetic engineering of grapevines for crown gall disease resistance and attempted transformation via *Agrobacterium* Ti plasmid vectors and the biolistic process. Ph.D. Thesis, Cornell University, Ithaca, NY.