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**Genotype, Explant and Growth Regulator Effects in
the Determination of Adventitious Regeneration in
Cucurbits, in Aid of Genetic Transformation**

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Genotype, explant and growth regulator effects in the determination of adventitious regeneration in cucurbits, in aid of genetic transformation.

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TABLE OF CONTENTS

Cover Page.....	1
Table of Contents	2
Abstract	3
Objectives of the Original Proposal	4
Relevant Data, Methods, Results and Discussion	5
In Vitro Plant Regeneration Systems	5
Melon	5
Anatomy & morphology of bud regeneration in melon	6
Direct regeneration from the petiole of melon cotyledons	15
Genetics of rapid regeneration from cotyledons with petiole fragments	17
Improving the tissue culture procedure for melon	20
Watermelon	26
Development, identification & testing of in vitro-derived tetraploid plants	27
Molecular Genetics and Vectors - Particle Guns	28
Gene constructs for transformation	28
Particle gun development	29
Experiments to optimize and study particle bombardment	30
Watermelon	30
Effects of culture, particles and gun settings on GUS expression	30
Melon	33
Histology of particle bombardment of melon cotyledons	33
Genetic Transformation	39
Transformation overview	39
Growth of plants in the greenhouse	39
Particle Bombardment Experiments	40
Melon	40
Transformation of melon cotyledons by particle bombardment	40
<i>Agrobacterium</i> Experiments	41
Watermelon	44
Effects culture and transformation methods	44
Stable transformation of watermelon and the recovery of transformed progeny	46
Melon	48
Transformation of 'Galia' melon	48
Apical Meristems for Transformation	49
Apical meristems for melon a target for transformation	49
Transformation of melon apices and selection of shoots	51
Description of Cooperation	53
Evaluation of Research Achievement	54
Conclusions	55
List of Publications	56

ABSTRACT

Genotype, explant and growth regulator effects in the determination of adventitious regeneration in cucurbits, in aid of genetic transformation.

The objective of this study was to gain an understanding of the in vitro regeneration process in watermelon and melon to enable the development of genetic transformation systems. The objectives were met and additional progress, unplanned during the original proposal, was made. Organogenic regeneration in vitro was studied in both melon and watermelon. Genotype played a significant role in regeneration. In melon, epidermal cells were responsible for most regeneration. Methods to obtain in vitro-derived watermelon tetraploids, needed for seedless varieties, were developed. The culture systems were refined so that they could be routinely used for transformation. Particle guns were constructed and *Agrobacterium* strains were obtained to study the effect of transformation procedures on culture system performance, allowing refinement of transformation protocols. The culture systems were shown to enable the stable transformation of both crops, allowing their future use for insertion of agriculturally-important genes. In addition, we showed that shoot apical meristems might be suitable target tissue for transformation and allow a wider range of genotypes to be used, which is needed for crops as diverse as cucurbits.

OBJECTIVES OF THE ORIGINAL PROPOSAL

To gain an understanding of the regeneration process in vitro, especially direct organogenesis, in watermelon and melon. This will enable the development of genetic transformation systems. Additionally, we intend to exploit two methodologies not previously reported with cucurbits: the enhanced regeneration ability of immature seeds, and Thin Cell Layer technology.

Sub-objectives to achieve the above aim:

- (1) to study the direct regeneration capacity of cotyledon and embryo explants of certain important varieties of melon and watermelon during seed development;
- (2) to optimize the embryogenic capacity of watermelon seed-derived explants during seed development, and to improve the organogenesis method on with watermelon;
- (3) to develop the method of regeneration from Thin Cell Layers (TCL) for mature and immature melon and watermelon seeds, and seedlings;
- (4) determination if the mode(s) of regeneration will permit the use of *Agrobacterium tumefaciens* or the particle gun as a transformation method, and regeneration of suitable material.

During this project, we achieved all of the objectives, with the exception of developing thin cell layer technology, which was determined to be of limited use compared to other methods. Major additions to the project occurred with the decision to study shoot apical meristems as a target for transformation and with the development of two advanced particle guns. In addition, the obtention of watermelon tetraploid plants (very useful in triploid - seedless watermelon production) from in vitro organogenic cultures previous to the start of this project led to an analysis of tetraploid identification and subsequent field performance. The overall objective of understanding the

regeneration processes in melon and watermelon, leading to systems suitable for genetic transformation was achieved.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

During this project, we developed and studied plant regeneration systems in both melon and watermelon as a basis for subsequent transformation. We developed devices for particle bombardment and then studied the behavior of particles in recipient tissues. We studied the effects of *Agrobacterium* as a transformation vector for both crops and evaluated apical meristem tissue as an alternate target for transgenes. From this experimentation has come regeneration and transformation systems for both melon and watermelon. This report is arranged to discuss the development of regeneration systems for each crop first. Next, the plasmids used, the development of particle guns and study of transformation systems are presented, as well as the methodology to obtain stable transformation in both crops. Finally, experiments evaluating the use of apical meristem tissue for transformation are presented; this was an unanticipated project that was suggested both by our results and by research conducted elsewhere. Certain parts of each project were variously accomplished in the US and/or Israel in very close collaboration. The results and progress reported should illustrate the fine collaborative spirit and friendship that this project has facilitated between the PI's.

IN VITRO PLANT REGENERATION SYSTEMS

IN VITRO CULTURE OF MELON

Prior to the start of this project, a culture system was developed that enabled high-frequency somatic embryogenesis in melon (see Plate 6C) (Gray et al., 1993, J Amer Soc Hort Sci, 1993, 118:425-432). This system was used successfully in some of the subsequent transformation studies. During this project, an organogenic shoot

regeneration system was developed and refined. Experiments that led to development of the organogenic system are described below. Many of these experiments also are contained in publications or submitted manuscripts listed at the end of this report. Three sets of reprints and abstracts (also preprints) are provided separately. New publications stemming from the project will be submitted later as they become available.

Anatomy and Morphology of Bud Regeneration in Melon Cotyledons

Certain cultivars of melon (*Cucumis melo* L.), such as cv. Galia, regenerate only by direct organogenesis from the cotyledon explant. We studied the anatomy and morphology of bud regeneration in such a melon cultivar (Galia). Explants were cut from three-day-old in vitro seedlings. After 3 days on a medium containing benzyladenine the start of cell division can be observed in the epidermal layer on the adaxial side in the center of the explant, near the most proximal cut edge. Over the next week the area of the meristem spreads laterally. Additional layers are added to the area of the meristem by apparent cell division in the epidermis. In many places the epidermis remains active in cell division. Alongside those active places there are zones where the epidermis has become inactive, although the sub-epidermal layers remain in cell division. In transverse section the explant now has small protrusions on the adaxial surface. Now, after 10 days on cytokinin-containing medium, the first morphological signs of development are visible on the adaxial surface adjacent to the proximal cut edge. However, the primordia that appear now do not develop further, as none have been observed to produce an apex by either stereoscopic or histological observation. The first shoot buds are not observed histologically until after 15 days, by which time the surface has many small protrusions and primordia. The first shoot is only found by histology after 22 days. By this time the surface is covered with leaves, mostly without accompanying buds. The leaves are produced from the protrusions visible after 10 days on the cytokinin-containing medium.

Development of the regeneration process in 'Galia' melon

The melon cv. Galia is recalcitrant to transformation by *Agrobacterium tumefaciens* (Gaba et al, 1995, Cucurbitaceae '94, pp. 188-190), possibly due to poor regeneration in vitro. To understand why this process is problematic, and to seek solutions, we are studying the anatomy and morphology of the regeneration process.

After several attempts we have found a preparation protocol to enable us to prepare explants of 'Galia' melon for histological examination. A major stumbling block was that the cells of the cotyledon explants were filled with fat and protein storage bodies. It was therefore impossible to make accurate observations on the cell division activities of these cells if the explants were placed immediately on to regeneration medium (with cytokinin). However, if pregerminated seeds (i.e. 4 days old) were used for explant preparation, the internal structures of the cells were not obscured by the storage bodies, as the storage lipids had already been partially metabolized (as reported for cucumber by Davies and Chapman, 1981) by further staining with a hematoxylin stain, which permits us to identify cells in the process of division. We can also observe areas where buds have been induced to develop, well before any bud regeneration is visible to the naked eye. As the kinetics of regeneration (see separate section) are also precisely defined we can intercept defined stages of regeneration at the histological level at will. For good results with the histology of regeneration we have noted that the location of the regeneration on the explant is very important.

Seeds of the Israeli melon cv. Galia had the seed coats removed, and were sterilized for 20 min in a solution of 1% sodium hypochlorite, with 2 drops of Tween 20 per 100 ml solution. After washing with sterile distilled water, seeds were germinated on Murashige and Skoog (MS) medium with 3% sucrose, vitamins and 5 g liter⁻¹ Micro agar (Duchefa) (MS0 medium), in a growth room at 26C, 16h photoperiod, 100 (mol.m⁻²s⁻¹ cool white fluorescent light. After 4 days the hypocotyl, apical bud and adjoining 2-3 mm

of the cotyledons were excised from seedlings and discarded, and the remaining cotyledons halved in a transverse manner. Only experiments using explants derived from the part proximal to the apex of the seedling are reported here. Explants were placed in 9 cm diameter petri dishes with the abaxial side on Murashige and Skoog (1962) medium with 3% sucrose, vitamins and 8 g liter⁻¹ TC agar (JRH Biochemicals) with 1 mg liter⁻¹ benzyladenine (MSBA1 medium), in a growth room at 26°C, 16h photoperiod, 30-40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ cool white fluorescent light, 4 explants per petri dish. Explants were observed daily by dissecting microscope, and samples taken every 3 or 4 days for histology, and every 2-3 days for morphology. Results were consistent, and the developmental sequence was always similar, but the timing of the responses even within the each experiment was very variable. For histology all explants were fixed in FAA, dehydrated in tertiary butyl alcohol, embedded in Paraplast, and stained with hematoxylin iron. Sections were cut 8 μm thick by microtome.

Morphology of development of melon explants

Proximal cotyledon explants were incubated on MS medium with 1 mg/L benzyladenine (BA). The original explant is fairly featureless, the surface being gently rippled (Plate 1.1), but grows and greens, and within 5 days a yellow band appears at the most proximal edge (Plate 1. 2). By 7 days (Plate 1.3) the yellow band is more pronounced and has become a ridge. After 10 days (Plate 1. 4) there are "buds" visible along the proximal surface of the cotyledon, adjacent to the cut edge of the explant. On closer inspection the "buds" (Plate 1. 5 by stereomicroscope) never progress beyond primordia. The primordia are mixed with leaves.

Anatomy of development of primordia on explants

The cotyledon explants show no meristematic differentiation when first prepared (Plate 1.6). After 3 days on cytokinin-containing medium there is meristematic activity in the center of the cotyledon at the adaxial surface in the most proximal region (Plate 1.7), always opposite the developing bundle of xylem and phloem in what will become the midrib of the cotyledon. It can be seen that the small palisade parenchyma cells packed with storage materials (protein and lipid bodies) (Plate 1.6), has become a area of large vacuolated cells (Plate 1.7) between the developing meristems of the adaxial surface and the xylem and phloem area that will be the midrib. By 7 days (Plate 1.8) there is much increased meristematic activity in the central zone - spreading out wider and deeper. By 10 days (Plate 1.9) the central area has become very active, with large protuberances, and an intense region of meristematic activity beneath this. No buds are visible. A section (Plate 1.10) through a primordium (as in Plate 1.5) again shows that there are no buds visible, only finger-like protuberances.

Where do buds come from in melon?

Because we can regenerate plants from such melon explants, we know that there must be buds. What is the origin of these buds? We suggest that the buds develop from the epidermis. The epidermis (arrow - e) is first seen thin and compact on top of columnar cells when the explants are prepared (Plate 2.11). At this stage the explant is full of fat- and protein-storage bodies (arrow - f), which obscure the internal details. After 3 days cell division visibly starts in the epidermis (Plate 2.12), in the central proximal region. At higher power it is noticeable that only the epidermis is undergoing cell division at this stage (Plate 2.13). It is possible to find epidermal cells dividing (Plate 2.14), producing both further epidermal cells and a new sub-epidermal layer. At this stage it is also possible to find areas where the cell division in the epidermis (and also

perhaps the layer beneath the epidermis) has ceased to divide actively (as these cells are less intensely stained), while cell division proceeds actively in a group of cells slightly deeper in the explant (Plate 2.15). By 7-10 days a multiple layered epidermis can be found in many places (Plate 2.16), interspersed with areas of finger-like protuberances (Plate 2.17).

These multiple-layered epidermal structures sit on a ground of vacuolated cells. No buds are visible, although there are areas of finger-like protrusions that are meristematic to the epidermal layer. The protuberances grow without much further development (Plate 2.18). Among such protrusions on day 15 we find the first buds (Plate 3.19), which are difficult to visualize, and which are not visible morphologically (Plate 3.20). Many small leaves are visible now. By day 22 microscopic shoots can now be found (Plate 3.21), although not visible on the explant (Plate 3.22).

This is the first report on the histology of the development of regeneration in a cucurbit crop in vitro, to our knowledge. We show that regeneration actually develops from the epidermal cell layer, and that this process is not connected to the primordia and nodules observed developing on the surface of the explant. Reports in other crops also demonstrate regeneration from epidermal-derived meristems on cotyledonary explants e.g. mung bean (Mendoza et al Plant Cell Org Tissue Cult 32:137, 1993), and Frazer Fir (Saravitz et al., J Amer Soc Hort Sci 118: 163-167, 1993). There are few reports of the in vitro development of leaves without associated buds, and as far as we know this is the first developmental study of this type. Knowledge of the source of the regenerating buds is useful for the development of a transformation system.

Legends to Plates 1-3. (following 3 pages)

Note on orientation of photographs on Plates 1-3: all morphological photographs (nos. 1-4, 20, 22) are presented adaxial surface upwards, with the most proximal part of the explant towards the top of the page. Photographs 6-18 are oriented with the adaxial surface of the cotyledon towards the top of the page.

Plate 1

1.1. explant freshly prepared from 4-day-old melon seedling, x 30; 1. 2. explant after 5 days x 15; 1. 3. explant after 7 days x 15; 1. 4. explant after 10 days x 15; 1.5. stereomicrograph of primordia on 10 day old explant, x 100; 1.6. section of explant freshly prepared from 4-day-old melon seedling, x 15; 1.7. section of explant after 3 days in culture, x 80; 1.8. section of explant after 7 days in culture, x 80; 1.9. section of explant after 10 days in culture, x 15; 1.10. A section through a primordium (as in 1.5); x 150.

Plate 2

2.11. Section of explant freshly prepared from 4-day-old melon seedling, the epidermis (arrow - e) fat- and protein-storage bodies (arrow - f), x 500; 2.12. section of explant after 3 days in culture, x 200; 2.13. section of explant after 3 days in culture, x 400; 2.14. section of explant after 3 days in culture, x 1000; 2.15. section of explant after 3 days in culture, x 400; 2.16. section from 10 day old explant, x 200; 2.17. section from 10 day old explant, x 200; 2.18. section from 22 day old explant, x 200;

Plate 3

3.19. Longitudinal section through a microscopic bud, x 400; 3.20. explant after 15 days, x 5; 3.21. Longitudinal section through a microscopic shoot, x 250; 3.22. explant after 22 days, (x 2).

PLATE 1

PLATE 2

PLATE 3

Direct Regeneration From the Petiole of Melon Cotyledon Explants

Occasionally we have observed very rapid shoot development on melon explants *in vitro*. This rapid response has always confused us and has led to problems in evaluating experiments. Eventually, we realized that this rapid response is only from explants that were prepared so that part of the area that will be the petiole of the cotyledon is still attached. If the part of the cotyledons that will eventually develop into the cotyledonary petiole remains attached to the explant during the regeneration process, then a large single bud often (in up to 50% of samples) develops very rapidly (sometimes in only 1 or 2 days). Around this major bud will develop later several other smaller independent buds. If the petiole is completely excised from the cotyledon explant there is no single major bud produced, but the cut edge proximal to the petiole is covered with many small "buds". There are no visible differences between the petiole and the cotyledon prior to 4 days after germination, at which point the area destined to be the petiole appears a slightly lighter green color (i.e. the petiole greens more slowly than the blade of the cotyledon). We surmise that the very rapid regeneration occurs opposite the preformed meristem in the conductive tissue.

Seeds were sterilized and germinated as in the above. Seedlings were selected when 3-4 days old, when hook was still at least partially closed, and the cotyledons were slightly green. Explants were cut in a variety of ways seedlings. The most consistent was to cut off the hypocotyl as close to the cotyledon as possible, then cut the cotyledon across in half, discarding the distal parts. Next explant is stood on its hypocotyl stub, and a scalpel blade forced flat between the cotyledon remnants. The 2 proximal parts of the cotyledon are then cut apart, giving a unit as Plate 4A. Care has to be exercised to remove the apical bud of the seedling, using a stereomicroscope and lateral illumination. Regeneration then proceeds on MS medium with 3% sucrose, vitamins and 8 g liter⁻¹ TC agar (JRH Biochemicals), with 1 mg liter⁻¹ benzyladenine.

Morphology and anatomy

The development of regeneration from cotyledon explants that include the petioles shown in Plate 4. The initial explant from the cotyledon of a 4-day-old germinating seedling is shown in Plate 4A. The solid arrow marks the top part of the hypocotyl left over from explant preparation. The open arrow marks the light colored, moon-shaped, area at the most proximal area of the cotyledon. This area stays a lighter color for several days while the cotyledon grows and develops, and will become the petiole. The explant is oriented adaxial side up, the proximal end to top of the page (x 16 magnification). After 2-4 days primordia become visible on the pale colored part of the explant (Plate 4B). The explant is orientated adaxial side up, the proximal end to top of the page (x 10 magnification). After another 3-4 days a bud becomes visible on the petiole part of the explant (Plate C; x 25 magnification). The development of the first bud may be followed by subsidiary buds around the first. After several more days a stem may become visible (Plate 4D; x 3.3 magnification). The stem shown here after about 25 days in culture has a massive base, and is quite short. A subsidiary bud is visible at the base of the stem. The most proximal part of the petiole is situated at the right of the photograph. Less frequently the shoot has a more normal appearance (Plate 4E; x 2.8 magnification). The proximal end of the petiole points to the lower right hand corner. Such a plant can develop into a perfect in vitro plant, including tendrils, within 4 weeks (Plate 4F; x 2.5). A examination of the histology of such developments was undertaken. In a sample, in which no development was visible externally by dissecting microscope (Plate 4G ;x 60), there is a small, meristematic bulge on the adaxial side (at the top of the picture), opposite the vasculature of the midrib. Another sample shows a bud structure developing in the same position on the explant (Plate H; x 120).

Physiology of regeneration from cotyledon explants with petiole fragments

Besides the morphological and anatomical study above, we have also begun to determine various physiological parameters of the rapid regeneration response.

Light. A requirement for light has been noted previously for regeneration from melon cotyledon explants in culture (Neidz et al, 1989, Plant Cell Tiss Org Cult, 18:313-319; elsewhere in this report). Cotyledon explants were incubated on MS medium with 1 mg/l BA, and after 2 weeks in darkness 0/16 explants had regenerated primordia. However, when similar explants with petiole fragments attached, or just the petiole itself, were incubated on a similar medium in darkness, after 1 week 8/8 explants had regenerated buds. When incubated for 2 weeks in darkness shoots were found regenerated in 11/12 explants in a parallel experiment.

Explant. The basic explant type is shown in Plate 4A. The rapid regeneration (or stem production in darkness) can occur if the entire green part (the cotyledon) is excised, or even if the excision removes part of the lighter-colored petiole section. However, if the part of the petiole closest to the cotyledon remains on the cotyledon, then there is an increase in the rapid and dark responses of the cotyledon (to 10-20%).

Age. The above responses were found in explants taken from 3-4 day old seedlings. However, for explants removed from plants 7-8 days old (cotyledons expanded, and the petiole a definite separate organ) the rapid response is greatly decreased (to 10 - 20%).

Genetics of the rapid Regeneration from Cotyledon Explants with Petiole Fragments

We have observed the rapid regeneration from petiole-containing explants from all types of melon from which we have attempted regeneration i.e. Galia types (especially cvs. Galia and Revigal/C8), American cantaloupe types (cvs. Hales Best Jumbo, Topmark, Perlita), Spanish types (cvs. Amarillo Oro, Pinonet Piel de Sapo). As these

types of melon are regarded as diverse among cultivated melons, this might permit the argument that the rapid response observed is in fact quite a general response of melon in culture.

The rapidity of the regeneration response is quite surprising. There is obviously a great difference between the response of the future petiole and in the response of the cotyledon blades themselves. Previously rapid differentiation of shoot meristems has been noted on the petioles of Brassica cotyledons (Sharma and Bhojwani, Plant Sci. 69: 207, 1990). The ability to regenerate in darkness is most surprising, suggesting that the regeneration process is controlled in a manner somewhat different from regeneration from cotyledons. Perhaps the response from the petiole is more similar to that of the embryo itself, than to the cotyledons. Various authors have discussed regeneration from

Plate 4. (next page)

Development of regeneration from cotyledon explants that include a fragment of the petiole. A. Initial explant from the cotyledon of a 4-day-old germinating seedling. The solid arrow marks the top part of the hypocotyl left over from explant preparation. The open arrow marks the light colored moon-shaped area at the most proximal part of the cotyledon that will be the petiole. The explant is orientated adaxial side up, the proximal end to top of the page (x 16 magnification). B. Primordia on the petiole. The explant is orientated adaxial side up, the proximal end to top of the page (x 10 magnification). C. Bud on the petiole part of the explant (x 25 magnification). D. a short stem, with a massive base (x 3.3 magnification). The most proximal part of the petiole is situated at the right of the photograph. E. a normal shoot on a petiole (x 2.8 magnification). The proximal end of the petiole points to the lower right hand corner. F. perfect in vitro plant (x 2.5). G. Young meristem developing on petiole (x 60). H. A bud structure developing on top the petiole explant (x 120).

PLATE 4

the basal parts of cucurbit cotyledon explants. Gambley and Dodds (1990, *Plant Cell Tiss Org Cult*, 20:177-183) especially found that part of the explant gave very rapid regeneration in cucumber (*Cucumis sativus* L). Additionally, in watermelon (Compton and Gray, 1993b, *J Amer Soc Hort Sci*, 118:151-157) found that only the most proximal part of the explant would give efficient regeneration.

Improving the Tissue Culture Procedure For Melon

Interaction between ancymidol and benzyladenine

We found a positive interaction (synergism) between low concentrations of benzyladenine and ancymidol (an anti-gibberellin) in the stimulation of primordia development. the response to ancymidol is counteracted by gibberellic acid, which delays primordia production significantly. The results of the study of the interaction between benzyladenine and ancymidol have now been accepted for publication (manuscript enclosed).

Use of immature seeds

A key part of the original proposal was the investigation of the regeneration response of explants derived from cotyledonary explants from immature melon seeds. We examined the parental varieties of cv Galia, and 'Galia' itself, and the latest commercially important 'Galia'-type melon C8 - also known as Revigal, and its parental lines (6 different genotypes in all). In this series of experiments, hand pollinated melon fruits were removed from the mother plants at 10, 20 and 30 days after pollination. Time points were replicated. Twenty proximal and twenty distal explants were prepared per treatment. Fruit were surface sterilized, and the seeds extracted axenically. Explants were cut from the seeds, and plated on MS medium with 1 mg/L BA, or the PGR combination of Neidz et al., 1989, *Plant Cell Tiss Org Cult*, 18:313-319), in vented magenta boxes, for

30 days. In brief, although most of the ages of most of the 6 varieties tested gave 100% regeneration, there was no observable advantage of the use of immature organs when compared to explants derived from mature seeds in terms of bud and shoot production. Additionally, the use of the medium with the Neidz PGR's (5 μ M BA and IAA) led to a great increase of hyperhydration (vitrification). This approach was therefore not continued.

Regeneration from callus

We have made efforts to regenerate plants from callus tissues of 'Galia' melon. This would be a good route to obtain transformed plants if it were successful. We have found it possible to generate green centers in the calluses of this variety, but no treatment we have tried to date permits these green centers to progress further. It would appear that Galia-type varieties lack the genetic ability to regenerate from callus.

Control of hyperhydricity

We have tried many treatments to reduce hyperhydration (formerly called vitrification), one of the major technical problems that we have found in vitro tissue culture of melon. Most of the treatments described in the literature against hyperhydration, a physiological disease that results in poor plant quality, do not appear to be very helpful in our case. The best treatments involve (a) reducing the concentration of plant hormones involved in the regeneration process (although this reduces overall productivity), and (b) a much more rapid transfer of the plant material to a medium designed to promote the growth of buds produced during the first treatment. The treatments described above can be combined (c) with the use of "vented" covers for boxes, which is also an effective method for reducing hyperhydration in melon.

The vents in the lids consist of an inserted microporous membrane, which permits the continuous loss of water vapour from the culture box, thereby reducing water condensation (the basis of much of the problem). Most recently we have found that (d) abscisic acid (ABA) can reduce the rate of hyperhydricity in melon regeneration (see Table 1 below), without apparent side effects on regeneration.

This experiment was performed during mid-summer, when hyperhydricity is always at its worst at Bet Dagan, due to the very high humidity. This is the first report of ABA having an effect on hyperhydricity. This has been found too recently to be as yet employed in practice.

Table 1. Hyperhydricity response of proximal melon explants incubated on MS medium supplemented with 1 mg/l BA (control), or BA with 0.050mg/l ABA, or BA with 0.25mg/l ABA. n = 48 explants per treatment.

Treatment	Hyperhydricity rating (No. plants)			Useable plants
	Full	Partial	None	
Control	39	7	2	9
ABA 0.05 mg/l	39	7	2	9
ABA 0.25 mg/l	17	18	18	31

The rate of hyperhydration is now approximately 20%, using the vented covers on magenta boxes. In general, almost all other treatments that reduce hyperhydration do so by limiting growth and development very greatly. The treatments we tried included: the use of inhibitors of ethylene action, the addition of salicylic acid, the use of a range of

agar concentrations (0.8% to 1.5%), the use of media without the nitrate ion, etc. have not given any advantage.

The effect of light

We found that the light intensity (photon fluence rate) plays a significant role in regeneration. At a relatively high fluence rate of cool white fluorescent light ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) very few shoots are produced. Reducing the photon fluence rate to 30 or even $10 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ greatly increases the number of shoots recovered from each sample. Additionally, it is clear that the area of the cotyledon producing apparent "regeneration activity" or "buds"(see discussion above) increases with photon fluence rate, and is not related to the number of stems recovered. Therefore, we have a system where a high photon fluence rate of white fluorescent light pushes the development of primordia to leaves, the apical meristem region apparently not developing. The photomorphogenetic effect is visible at gross morphological levels (number of shoots formed), and also at the histological level. We have tested the effect of different wavebands of light, and found little difference in effect on regeneration between red and blue light at $10 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, both being similar to white fluorescent light.

In Table 2, the results from Cycle 1 report the production from the original explants: at $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ only 6 explants in each of the proximal and distal treatments produced material useful enough to transfer to the next cycle. At 10 and $30 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ all explants regenerated usable material, but only about 10 were transferred to the next stage. At the end of each cycle shoots were removed from cultures, and usable blocks of cotyledon material transferred to fresh medium. If the cotyledon material had grown enough, it was sub-divided. The first cycle was the growth of the original explants (cut from 4-day-old seedlings) on petri dishes with MS medium and $1\text{mg}\cdot\text{l}^{-1}$ BA. Transfers after the first cycle were to MS medium with $0.1\text{mg}\cdot\text{l}^{-1}$ BA and

1 mg l⁻¹ gibberellic acid (GA3) in test tubes, maintained at the original photon fluence rate for that treatment. after the first cycle of 30 days, subsequent transfers were at intervals of about 6 weeks.

Table 2. Effect of light on the production of shoots by 'Galia' melon. Each treatment started with 20 explants from the proximal or distal parts of the cotyledon.

Explant & light ¹	Cycle 1		Cycle 2		Cycle 3		Cycle 4		Total plants
	bl	sh	bl	sh	bl	sh	bl	sh	
Prox 100	0	6	2	5	5	6	0	6	7
Dist 100	0	6	2	14	14	2	0	7	16
Prox 30	0	10	6	7	8	7	1	5	15
Dist 30	0	10	1	7	5	10	0	3	6
Prox 10	2	11	11	11	14	10	5	32	32
Dist 10	0	10	7	6	3	10	0	10	10

¹Prox = proximal, dist = distal; 100, 30, 10 are photon fluence rates of white fluorescent light in $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. sh = shoots produced; bl = blocks of cotyledon material.

This result is very interesting: at a low photon fluence rate (10 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) a larger number of stems might be produced than at higher light fluxes, despite the initial area of the cotyledon that is activated being much smaller - another example of the misleading appearances of regeneration in melon. The production of shoots by distal segments can also be quite high (Table 2). It is notable that regeneration of shoots is not an immediate process and that the blocks of cotyledon material can be

maintained in vitro producing new shoots for several cycles, although the peak is apparently after 3 cycles. We have maintained cotyledon material of both cvs. Galia and Revigal (C8) in culture for nearly 2 years, shoots being harvested every few weeks. Obviously, losses due to hyperhydration and contamination eventually become limiting.

Quality of regenerated plants

An important question relates to whether any somaclonal variation is detectable in the regenerated plants from the above system. On a first determination 5 out of 13 plants were found to be tetraploid, the remaining 8 being apparently normal, flowering and setting fruit with seeds. A group of about 120 plants were regenerated from different cycles from in vitro production from a variety of different experiments. The plants were removed from culture for quality testing, but had to be destroyed in the greenhouse as they became infected by a pathogen from another source.

Timing of explant production

The standard method in our laboratory for the preparation of explants has been to excise the explants directly from seeds. However, while preparing techniques for histology we found that if the cotyledons are excised from germinating seeds the regeneration response was quite different (Table 3). As germination proceeds the percentage of explants regenerating buds increases with time for both proximally derived explants (Table 3) and those distally derived also (data not shown). Not only is the number of responding explants increased, but the time to response is also reduced as the source material ages. The rapidity in changes of response can be seen in a decrease in the times to a specific response level.

The experiments examining the responses at 5 days have to be repeated for greater accuracy for publication. there is also a great increase in the apparent number of

"buds" regenerated between excision from seeds and 4-day-old explants (data not shown), but whether this is real, or apparent, regeneration has to be determined.

Table 3. Effect of proximal explant age on regeneration and rate of regeneration¹.

Explant age (d)	Replicate No.	Max. regeneration (%)	Days to max. reg. level in indicated % of population			
			10%	50%	90%	100%
0	5	86	5.2	9.8	15	19.2
2	2	95	3.5	6.8	11	16.5
3	4	100	3.8	7.3	12	16
4	4	100	4.4	7.8	9.4	10.5
5	2	91	3.5	8.3	14.4	16.5
7+8	5	100	2.2	4.3	6.4	7.6

¹Seeds were peeled, sterilized, washed and then germinated on growth regulator-free Murashige and Skoog (MS) medium. Explants, consisting of the proximal halves of cotyledons were excised after the above sample times. Explants were placed on MS medium, with the addition of 1 mg/l BA. Regeneration was recorded daily.

IN VITRO CULTURE OF WATERMELON

Three distinct watermelon culture systems had already been developed prior to the start of this project. This included systems for somatic embryogenesis (Compton and Gray, 1993a, Plant Cell Rep, 12:61-65), organogenesis (Compton and Gray, 1993b, J Amer Soc Hort Sci, 118:151-157), and axillary shoot micropropagation (Gray and

Elmstrom, 1991, US Patent No. 5,007,198; Compton et al., 1993c, *Plant Cell Tiss Org Cult*, 33:211-217). The embryogenic and organogenic systems utilized cotyledon explants excised from germinated seeds, whereas, the micropropagation system utilized apical meristem explants obtained from any number of sources (in vitro shoots from organogenesis, seedlings or fruiting plants in the field). For the subsequent studies supported by this project, only the organogenic system was used because it was more reliable than the embryogenic system. The micropropagation system was used continually to manage in vitro shoots derived from organogenesis and to prepare plants for greenhouse or field testing.

Development, Identification and Testing of In Vitro-Derived Tetraploid Plants

An outcome of our previously developed watermelon organogenic culture system is that some of the shoots regenerated from diploid germplasm were found to be tetraploid. This abnormal development, which would be undesirable in most crops, would be of great use in watermelon, since tetraploid lines are needed to produce triploid seedless fruit ($4n \times 2n > 3n$). Previously, tetraploid lines were created by treating the plumule of diploid seedlings with colchicine. This resulted in a low recovery of seemingly tetraploid plants. However, most were found to be chimeric and did not transmit tetraploidy to progeny. The problem of producing stable tetraploids was so great as to have stifled improvement of seedless varieties. Simple recovery of non-chimeric tetraploids in vitro will allow more rapid improvement of seedless watermelon, since many more useful tetraploids can be produced and screened.

During the present project, we confirmed that our methods of rapidly detecting tetraploids was accurate and that the field performance of the plants was as expected (Compton et al., 1996, *Euphytica*, 87:165-172). In our system, tetraploid shoots were identified by examining the number of chloroplasts per leaf guard cell pair. The number

of chloroplasts was found to be a direct reflection of ploidy level. Diploids had an average of 11 - 12 chloroplasts per guard cell pair, whereas, tetraploids possessed an average of 18 - 21 chloroplasts (Compton et al., 1996, *Euphytica*, 87:165-172). The fact that the selected plants were in fact tetraploid was confirmed by morphometric analysis of leaves and flowers and by actual breeding. When selected tetraploids were crossed with diploids, the resulting progeny gave rise to seedless melons. This research confirms that useful tetraploids are produced from organogenic cultures and that our detection methods are accurate.

Development of the tetraploid program is important to the project, since, ultimately, useful genes must be expressed in seedless varieties. We need to eventually study the effect of ploidy level on transgene expression, especially as it relates to gene dosage. For example, an important question is whether a transgene will still function when doubled in a tetraploid. Since plants with multiple transgene inserts are common and have occasionally been shown to not express the transgenic trait due to the phenomenon of "co-suppression", it is possible that doubled transgenes will similarly be inactivated.

MOLECULAR GENETICS & VECTORS - DEVELOPMENT OF PARTICLE GUNS *GENE CONSTRUCTIONS FOR TRANSFORMATION*

During the first 1½ years, we had difficulty in obtaining stably-transformed plants. We believe that the difficulty was partially related to poor selection pressure due to a mutation to the kanamycin selection gene that occurs in the vector pBI121, which renders it inefficient for selection. This plasmid-marker system had been used in all of our experiments. We determined the mutant gene to be present in our constructs. To overcome this obstacle, we obtained the new set of vectors from E. Hiebert (Plant Pathology Dept., Univ. of Florida) that enhanced transformation and expression

efficiencies. These new vectors have allowed us to make significant progress. Two vectors originated from Dr. A. G. Hunt (Dept. of Agronomy, Univ. of Kentucky). The pKYLX71:35S² contains a doubled cauliflower mosaic virus (CaMV) 35S enhancer element as well as enhancer elements from *Agrobacterium tumefaciens*, a kanamycin selection gene and other elements necessary for binary vector function. This vector with the doubled 35S enhancer was shown to improve the level of gene expression. The pKYLX80:35S² vector contains a doubled 35S enhancer and kanamycin selection gene in a bacterial vector. This vector has a high copy number, which enables us to obtain very high levels of recombinant DNA for particle bombardment experiments. A third vector, pGA472, originated from Dr. Gloria Moore (Horticulture Dept., Univ. of Florida). These plasmids were utilized in transformation experiments.

PARTICLE GUN DEVELOPMENT

Particle guns designed and constructed under this project in both the US and Israel are based on the non-commercially-available particle inflow gun (PIG), since it can be fabricated with readily-available parts and offers performance on par with the Biolistic-R PDS 1000/He. After testing a series of five prototype particle guns, we settled on a final version constructed from stainless steel in the US that has highly automated operating features, which now is in routine operation (Plate 6E). We found that it was faster and less expensive to operate than the commercially-available Biolistic-R PDS-1000/He sold by Biorad, which consumes several dollars of consumable items for each "shot". Recently, a study found no difference in transformation frequencies when comparing the PIG with the Biolistic-R PDS-1000/He (Brown et al, 1994, Plant Cell Tiss. Org. Cult 37:47-53); thus, our particle guns should offer advantages and no disadvantages over the commercial device. As mentioned in the last report, we developed an additional version of the PIG that is much easier to construct (it can be built in 40 min. with simple

hand tools) and, as judged by assays of transient GUS activity, was equal in performance to the previous versions of the PIG (see year 1 report). A publication describing construction of the new version (Gray et al, 1994, *Plant Cell Tiss Org Cult*, 37:179-184) is appended. Results of experiments conducted that utilized the guns are presented throughout this report. A most recent outcome of this project is the development of another version of this gun, which does not require a vacuum chamber and, thus, can be used on larger plants, even in a greenhouse setting. This gun has been shown to be indispensable for transmitting certain viruses (see Gaba et al., manuscript attached).

EXPERIMENTS TO OPTIMIZE AND STUDY PARTICLE BOMBARDMENT

Watermelon

Effects of Culture, Particles and Gun Settings on GUS Expression

Effect of particle size on GUS expression

Examples of transient GUS expression in watermelon cotyledons are presented in Plate 6G & H. Tungsten particle size dramatically affected GUS expression in watermelon cells (Table 4). The number of watermelon cells expressing GUS was highest following bombardment with 1.1 μm DNA-coated tungsten particles. GUS expression was reduced 2.8- and 2.2-fold when the 0.4 and 1.7 μm tungsten particle sizes, respectively, were used.

Effects of helium pressure and target distance on GUS expression

Optimum GUS expression was obtained when watermelon cotyledons were bombarded with DNA-coated tungsten particles propelled using 620 kPa or 830 kPa helium blasts (Table 5). GUS expression was 3.3-fold higher at 620 kPa than at 410 kPa. The 620 kPa pressure was chosen for further experiments because the degree of tissue damage was less than at 830 kPa (data not shown). The number of blue foci per bombardment was 1.2-fold greater when watermelon cotyledons were positioned 10 cm

from the particle support screen compared to 20 cm (Table 6). No difference in GUS expression was observed among explants positioned at either 10 and 15 cm, or 15 and 20 cm distances. The 15 cm distance was chosen for further experiments because tissue damage was less than at 10 cm (data not shown).

Effects of the explant preculture before bombardment

Regression and LOF analysis revealed that optimum GUS expression for watermelon cells was obtained by culturing cotyledon explants on regeneration medium for 2 days before bombardment. GUS expression was 28% less when explants were not cultured before bombardment and 51% less when explants were precultured for six days.

Effects of particle type and number of bombardments

A comparison between gold and tungsten particles with one, two or three recurrent bombardments per explant demonstrated that three bombardments with tungsten was distinctly better than other treatments (Table 7). Explant age (ie. the age of imbibed seeds from which cotyledon explants were taken) showed that four day-old seeds were best (Table 8).

Particle-mediated transformation experiments were designed to select stably transformed tissue and plants. Cotyledons bombarded with plasmids containing both GUS and kanamycin resistance genes, or only the kanamycin resistance gene were selected by culture on medium that contained kanamycin. Only transformed tissue can proliferate in the presence of kanamycin so that stably-transformed tissue and plants can be selected with this method, even if the frequency of stable transformation is very low. GUS staining was used as a convenient early screen of putative transformed plants (i.e. those plants that can develop and root on kanamycin-containing medium). Confirmation of stably transformed plants was accomplished with a battery of analytical tests, including

PCR, northern blotting and ELISA. ELISA cannot be used to detect antisense constructs, which will not produce proteins. This phase of the project subsequently was put into place (see below).

Table 4. Effects of tungsten particle size on transient expression of GUS in watermelon cotyledons following particle bombardment.^a

Particle size (μm)	Number of blue foci per explant	
0.4	5.2	b
1.1	14.7	a
1.7	6.5	b

^aA helium pressure of 410 kPa (60 psi) and a specimen distance of 10 cm from the particle support screen were used.

Table 5. Effect of helium pressure on transient expression of GUS in watermelon cotyledons following particle bombardment.^a

Helium pressure (kPa) ^b	Number of blue foci per explant	
410	9.9	c
620	33.2	a
830	25.5	b

^aThe tungsten particle size used was 1.1 μm . Specimens were positioned 10 cm from the particle support screen. ^bPressures were 60, 90 and 120 in psi units.

Table 6. Effect of target distance on transient expression of GUS following particle bombardment.^a

Specimen distance ^b (cm)	Number of blue foci per explant	
10	24.2	a
15	23.0	ab
20	19.6	b

^aThe tungsten particle size was 1.1 μm . The helium pressure used was 620 kPa.

^bThe distance between the DNA/particle support unit and the target specimens.

Melon

The Histology of Particle Bombardment of Melon Cotyledons

The goal of this experiment was to determine histologically the effectiveness of the Particle Inflow Gun (PIG) bombardment system of Finer et al. (Plant Cell Rep. 11: 323, 1992), and to compare the PIG to the commercial device of Biorad (PDS), designed by Sanford. Histology is an important tool, as it is critical to determine that the particles enter the cells destined to regenerate plants.

Table 7. Effect of particle composition and the number of bombardments per explant on the ability of watermelon cotyledons to express GUS.¹

Particle composition	Number of bombardments	Number of areas per explant expressing GUS	
Gold	1	7.6	d
	2	14.4	b
	3	12.9	bc
Tungsten	1	16.5	ab
	2	12.3	c
	3	19.9	a

¹The parameters for bombardments were: a) helium pressure = 60 psi and b) a tissue distance of 10 cm. Watermelon cotyledons were two days old at the time of bombardment.

Seeds of the Israeli melon cv. Galia had the seed coats removed, and were sterilized for 20 min in a solution of 1% sodium hypochlorite, with 2 drops of Tween 20 per 100 ml solution. After washing with sterile distilled water, seeds were germinated on MS medium with 3% sucrose, vitamins and 5 g liter⁻¹ Micro agar (Duchefa), in a growth room at 26C, 16h photoperiod, 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ cool white fluorescent light. After 4 days the hypocotyl, apical bud and adjoining 2-3 mm of the cotyledons were excised from seedlings and discarded, and the remaining cotyledons halved in a transverse manner. Only experiments using explants derived from the part proximal to the apex of the

Table 8. Effect of explant age on the ability to express GUS after particle bombardment.¹

Explant age (days)	Number of areas per explant expressing GUS
0	7.5 c
2	9.9 b
4	14.3 a
6	6.5 c

¹The parameters for bombardments were: a) helium pressure = 60 psi and b) a 10 cm. tissue distance.

²Means in the same column with identical letters are not significantly different.

seedling are reported here. For bombardment explants were placed adaxial side up in 5 cm diameter petri dishes upon MS medium with 3% sucrose, vitamins and 8 g liter⁻¹ TC agar (JRH Biochemicals) with 1 mg l⁻¹ benzyladenine. Bombardment was with a Particle Inflow Gun (PIG), which we fabricated ourselves. Particle preparation of M20 tungsten particle was according to Perl et al. (1992, Molec Gen Genet, 235:279-284). Our optimum bombardment condition were very similar to those of Gray et al. (1994, Plant Cell Tiss Org Cult, 37:179-184): 4.2 bars driving pressure helium gas for a 50 ms burst, 14 cm below the Leur-lok connector, 73 cm Hg vacuum in the chamber. Alternatively, explants were bombarded with the PDS device (Biorad), following the manufacturer's instructions. After bombardment the dishes with the explants were returned to the

growth room mentioned already. After 24 hours in the above mentioned growth room conditions explants were incubated at 37C overnight for the (-glucuronidase (GUS) reaction in 5-bromo-4-chloro-3-indolyl-(-D-glucuronic acid (X-gluc) solution. After the (-glucuronidase reaction explants were fixed in FAA, dehydrated in tertiary butyl alcohol, embedded in Paraplast, and stained with safranin. Sections were cut 8 μ m thick by microtome).

The distribution of GUS expression and microprojectiles following bombardment with the PDS of Biorad can be seen in Plate 5.1. The particles and stain are located almost exclusively in the epidermal and subepidermal layers of the cotyledon. By contrast, following bombardment with the PIG, although we find also GUS staining in the epidermis accompanied by microprojectiles (data not shown), we find a much wider dispersion of both microprojectiles and the GUS stain through the tissues. In Plate 5.2, a GUS reactive region is centered 3 to 5 cell layers deep within the tissue. In Plate 5.3 GUS staining is seen actually within protoxylem, while in Plate 5.7 microprojectiles can be seen in and around protoxylem. GUS staining can be seen sometimes very deeply (Plate 5.6), beside protoxylem elements. Additionally we have observed a field of microprojectiles spread in a band 20 to 30 cell layers deep (Plate 5.4). A similar band of microprojectiles penetrating through a cotyledon can be observed in Plate 5.5, where the microprojectiles are visible in groups, apparently suspended in the cell.

In our comparison between the two particle bombardment devices we found that both are effective in transient expression in melon cotyledons. However, the PIG is capable of much deeper penetration of a variety of cotyledon tissues, whereas the Biorad PDS projects particles mainly into the 2 outer layers of the explant.

Similar experiments have been described previously in the literature with the Biorad PDS. This implies that the PIG can be used to transform tissues where deep penetration is essential. Additionally, we have found that the PIG and a hand held

version are very useful for the infection of intact plants with a cloned potyvirus under the control of the 35S promotor (see Gal-On et al, ms submitted). This idea was generated from the histological data presented here. Sato et al (Plant Cell Rep 12: 408, 1993) noted a penetration into soya shoot similar to that reported here. Taylor and Vasil (Plant Cell Rep 10: 120, 1991) report microprojectiles in cells up to 6 layers deep in pearl millet embryos, although they also report that the microprojectiles were concentrated in the epidermal and first subepidermal layers. As did Klein et al. (Bio/Technology 6: 559, 1988), we generally found that the deeper microprojectile penetration was generally not accompanied with a successful GUS reaction. This could be due to inadequate penetration of the GUS substrate, or perhaps the DNA is stripped off the deep penetrating particles.

It is notable that both models of particle guns can transform the epidermal cells in melon explants destined to regenerate buds.

Plate 5. (next page)

1. Section of "Galia" cotyledon bombarded by the PDS (Biorad system), showing GUS reaction (stained with X-gluc) in epidermal and sub-epidermal layers. 2. Section of melon cotyledon bombarded by PIG, showing GUS stain centered 3-5 cell layers deep. 3. Section of melon cotyledon bombarded by PIG, showing GUS stain in protoxylem (arrow). 4. Section of melon cotyledon bombarded by PIG, showing a field of microprojectiles (some of which are marked by arrows) in a band 20-30 cell layers deep. 5. Transverse section of melon cotyledon bombarded by PIG, showing groups of microprojectiles (some of which are marked by arrows). Bombardment was from the adaxial side, topmost in this picture. 6. GUS expression beside protoxylem elements, deep inside melon tissue. 7. Microprojectiles (marked with arrows) adjacent to and within protoxylem.

GENETIC TRANSFORMATION

Transformation Overview

To date, we have produced putative transformed plants of: 1) watermelon by organogenic regeneration and treatment with *Agrobacterium*, with or without particle wounding, and 2) muskmelon by embryogenic regeneration from cotyledons or adventitious shoot regeneration from shoot apical meristems, each by particle bombardment or *Agrobacterium* with particle wounding (see below). Transformation has been confirmed by several means, including PCR, Southern dot blots and antibody assays for the NPTII (kanamycin resistance-conferring) gene; however, accurate screening has been more difficult and unreliable than expected and confirming Southern hybridization analyses and analysis of progeny have not been accomplished. These plants and their progeny will be used in part of the proposed project to refine screening and selection procedures for detecting transformation and virus resistance.

Growth of Plants in the Greenhouse

We have developed the expertise to establish plants from all of the in vitro regeneration systems described above (organogenic, embryogenic and shoot apex-derived adventitious shoot) in greenhouse pots. We are able to grow the plants to maturity, cause them to flower and set fruit (Plate 6K). Typically, two fruit can be produced on each plant (Plate 6L). Initially the fruit are allowed to develop without support (Plate 6M), but are placed in netted support bags as they mature (Plate 6N). Typically, 200 viable seeds are produced from each melon. Currently, progeny tests of muskmelon plants that were obtained from each of the regeneration/transformation approaches described above and that tested positive for presence of the NPTII gene, based Southern dot blots, are underway. Plate 6O shows 576 progeny reared from 16 self-pollinated melons. Each of these progeny were subjected to NPTII Elisa analysis on November 11,

1994 and eleven positive plants were selected. This number of positive selections was unexpectedly low, strongly indicating the need for optimization of the protocols used for regeneration/transformation/selection.

Despite our early assumption that the GUS gene and high resistance to kanamycin would be useful in detecting stable transformation, this has not turned out to be the case. While GUS is detected early at the transient level, it soon fades. Similarly, early resistance to kanamycin allows the selection of embryos or shoots from treated tissue, but the expected rooting of transformed shoots into kanamycin-containing medium does not occur. This led to us prematurely discard many plants. However, cursory examination of plants with Southern dot blot analysis showed that many were transformed. Our experience in this regard parallels that of others. The GUS gene is known not to be reliable in many crops (H. Matthews, Agritope, Inc., pers. comm.) and transformed melon frequently does not root on selection medium (R. Grumet, Michigan State Univ., pers. comm.). Therefore, our current selection procedure is to rely on GUS only as an indicator of early transient expression and *Agrobacterium* contamination. Kanamycin is utilized for early selection, but plants that do not root in its presence are not discarded.

PARTICLE BOMBARDMENT EXPERIMENTS

Melon

Transformation of Melon Cotyledons by Particle Bombardment

After a series of optimization experiments utilizing embryogenic melon cotyledons (Plate 6C) and transient analysis of GUS (eg. Plate 6F), which were described in a previous report, we began experiments to achieve stable transformation. We have obtained transformed plants from somatic embryos by bombardment of embryogenic cotyledon explants (based upon Southern dot blot analysis of the NPTII gene). We also have utilized the commercially-available NPTII antibody kit to conduct ELISA tests of these plants, obtaining similar results. These analytical procedures do not absolutely

confirm that stable transformation has occurred, rather, they detect the presence of transgenic DNA in the host tissue whether or not it is integrated into the host genome. More detailed Southern hybridization is underway to confirm stable integration. Table 9 shows that dot blot-positive plants were obtained with both plasmids. Although more plants were obtained with pKYLX80:35S² than pGA472 (5 vs. 3), the relatively low response per explant precludes a decision on whether the former is better than the latter plasmid.

Table 9. Particle bombardment of embryogenic melon cotyledons with two plasmids. Somatic embryos were obtained during selection on medium containing 25 mg/l kanamycin and Southern dot blots tested for the presence of the NPTII gene.

Plasmids	No. explants	Somatic embryos	Plants	Dot blot +
pKYLX80:35S ²	121	443	11	5
pGA472	120	441	11	3

AGROBACTERIUM EXPERIMENTS

Previously, we reported the preliminary use of tobacco as a model transformation system in order to test plasmids and refine protocols. In 1993, A series of experiments designed to improve transient GUS activity resulted in the best GUS assays to date. As mentioned above, GUS was used only in preliminary experiments due to subsequent difficulties in obtaining stable expression. In particular, use of particle bombardment to pre-wound cotyledonary tissue prior to *Agrobacterium* infection has resulted in superior results as described below.

Plate 6. (next page)

Regeneration systems (Figs. A-D), transformation (Figs. E-J) and greenhouse manipulation (Figs. K-O) of watermelon and muskmelon. A. Watermelon cotyledon explants cultured on regeneration medium. B. Adventitious shoot development from watermelon cotyledon via organogenic regenerative pathway. C. Somatic embryos of muskmelon. D. Adventitious shoot development from a cultured apical meristem of muskmelon. E. Particle inflow gun (PIG) developed by our program to accomplish particle bombardment and particle wounding. F. Transient GUS reaction following particle bombardment of muskmelon cotyledons. G. Transient GUS reaction following particle bombardment of watermelon cotyledons. H. Transient GUS reaction following particle wounding and Agrobacterium infection of watermelon cotyledons. I. Transient GUS reaction following particle bombardment of muskmelon shoot apical meristem and hypocotyl. J. Selection of a green shoot on medium containing kanamycin following particle wounding and Agrobacterium infection of a muskmelon shoot apical meristem. Note bleached white main shoot and green axillary shoot and bud, suggestive of selection from chimerically-transformed tissue. K. Growth and breeding of kanamycin-selected muskmelon plants under greenhouse conditions. Note melon (arrow). L. Melons (arrows) on selected greenhouse-grown muskmelon. M. Young melon developing on selected muskmelon plant after controlled self-fertilization. N. As melons mature, they are placed in netted supports. Such melons typically produce 200 viable seeds. O. Progeny derived from various kanamycin-selected plants after self-pollination.

PLATE 6

Watermelon

Effects of *Agrobacterium* Concentration, Culture and Transformation Methods

Effects of *Agrobacterium* concentration and co-cultivation period on GUS expression

An interaction was observed between the *Agrobacterium* inoculum concentration and the number of days that explants were co-cultivated with *Agrobacterium*. Transient expression of GUS was greatest when cotyledons were co-cultivated with *Agrobacterium* for 5 days when the starting inoculum density was 10^8 cells per ml. Fewer GUS expressing foci were observed when the initial bacterium titer was 10^7 , 5×10^7 , 5×10^8 or 10^9 cells per ml, or when the co-cultivation period was shorter than 5 days.

Effects of explant preculture period before inoculation with *Agrobacterium*

Optimum GUS expression was obtained when watermelon cotyledons were infected with *Agrobacterium* on the day of explant preparation (0 days preculture). Transient expression of GUS was less when explants were precultured before infection with *Agrobacterium*.

Effects of transformation method on transient expression of GUS in watermelon cotyledons

Wounding watermelon cotyledons with tungsten microprojectiles prior to infection with *Agrobacterium* resulted in a 1.7- to 2.7-fold increase in the number of GUS expressing foci per cotyledon when compared to *Agrobacterium* or particle bombardment alone, respectively.

Agrobacterium-mediated transformation of watermelon

Due to initial poor transient response following particle bombardment of watermelon, *Agrobacterium*-mediated transformation is also being tested. Several

abstracts have been published that describe this work (see list of publications at the end of this report).

Transformation of watermelon tissue pieces with *Agrobacterium* are currently underway. Coincubation experiments with *Agrobacterium* and watermelon are being conducted to optimize transformation parameters. Early results showing the interaction between *Agrobacterium* strains with various plasmid constructs and bacterial concentration show that lower bacterial inoculants resulted in the most GUS expression. The highest concentration tested overgrew the cotyledon explants and discrete GUS expression did not occur. We have selected several shoots on kanamycin-containing medium from coincubated cotyledons. One of the shoots had a leaf that expressed intense GUS staining on one longitudinal half, suggesting that the shoot may have been stably, but incompletely, transformed (i.e. it was a chimeric transformant).

A series of experiments designed to improve transient GUS activity resulted in the best GUS assays to date. In particular, use of particle bombardment to pre-wound cotyledonary tissue prior to *Agrobacterium* infection has resulted in superior results, as described below.

Effects of Agrobacterium concentration and co-cultivation period on GUS expression. Transient expression of GUS was greatest when cotyledons were co-cultivated with *Agrobacterium* for 5 days when the starting inoculum density was 10^8 cells per ml. Fewer GUS expressing foci were observed when the initial bacterium titer was 10^7 , 5×10^7 , 5×10^8 or 10^9 cells per ml, or when the co-cultivation period was shorter than 5 days.

Effects of explant preculture period before inoculation with Agrobacterium. Optimum GUS expression was obtained when watermelon cotyledons were infected with *Agrobacterium* on the day of explant preparation (0 days preculture; Table 10). Transient expression of GUS was less when explants were precultured before infection with *Agrobacterium*.

Effect of transformation method on transient GUS expression in watermelon.

Wounding watermelon cotyledons with tungsten microprojectiles prior to infection with *Agrobacterium* resulted in a 1.7- to 2.7-fold increase in the number of GUS expressing foci per cotyledon when compared to *Agrobacterium* or particle bombardment alone, respectively (Table 11).

Table 10. Effect of preculture period on GUS expression following infection with *Agrobacterium*.^a

Preculture period (days)	Number of explants	Number of blue foci per explant	
0	48	76.4	a
2	44	22.0	b
4	48	14.9	b

^aThe *Agrobacterium* concentration was 10^8 cells per ml. Explants were co-cultivated with *Agrobacterium* for 5 days.

Stable transformation of watermelon and recovery of transformed progeny

An experiment was conducted using *Agrobacterium* strain LBA4404 with pKYLX71:35S. The pKYLX71:35S construct contained only the NPT-II gene as a selectable marker. Out of 37 plants recovered from organogenic cotyledons, only two showed the presence of the NPT-II gene with PCR analysis (Table 12). These plants also were strongly ELISA-positive and, when placed back into in vitro micropropagation, grew vigorously on medium containing 100 μ M kanamycin. Selfed progeny obtained from one plant showed presence of the NPT-II gene in 75% of plants tested with PCR,

Table 11. Effect of transformation method on transient expression of GUS in watermelon cotyledons.^a

Transformation method	Number of explants	Number of blue foci per explant	
Bombardment/ <i>Agrobacterium</i>	42	69.0	a
<i>Agrobacterium</i> alone	44	40.9	b
Bombardment alone	50	25.0	c

^aThe *Agrobacterium* concentration was 10^8 cells per ml. Explants were co-cultivated with *Agrobacterium* for 5 days.

presence of the NPT-II gene in 75% of plants tested with PCR, suggesting that a single gene insertion even had occurred. All of the progeny that grew vigorously on medium containing $100\mu\text{M}$ kan were found to be PCR - positive. The fact that the gene was detected in progeny and segregated in a predictable manner strongly, if not irrefutably, indicates stable transformation. The presence of the gene in all progeny that survived on kanamycin-containing medium, shows that the selection method was effective.

Table 12. Selection of transformed watermelon plants and their progeny from organogenic cotyledons with pKYLX71:35S².

No. Plants Selected	ELISA+	PCR+	PCR+ progeny
37	6	2	yes ¹

¹Progeny from only one of the PCR+ plants have been tested to date.

Melon

Transformation of 'Galia' Melon

Transformation of the Galia melon type was a major objective of this project. We have previously reported on the production of transformed vitrified shoots produced from cultures of 'Galia' melon (Gaba et al, 1995, Cucurbitaceae '94 eds. G. Lester and J. Dunlap. Gateway Publishing pp. 188-190). We have discussed the problems of transformation of melon in relation to the response of various genotypes to *Agrobacterium*, in a recent publication (Gaba et al, 1996, Cucurbits Towards 200, Proc 5th Eucarpia Meetings on Cucurbit Genet Breed, pp. 172-178). We have tried all the published methods for transformation of melon (listed in Gaba et al, 1996), and have to date not been able to repeat them, either Galia-type melon, or any of the other varieties reported in those publications. Therefore we have worked on a method (wounding by particle bombardment, followed by the use of *Agrobacterium tumefaciens*) that depends only on the standard responses that we can observe with most melon types in culture.

In brief, "Galia" melon seeds were peeled, sterilized and germinated on MS0 medium and explants prepared as described above in the Anatomy and Morphology section. Proximal and distal explants were separated and placed in 5 cm diameter petri dishes, filling a 3 cm circle in the center of the dish. The abaxial side of the explants were placed on MSBA1 medium.

Explants were then bombarded as described in "Histology of Particle Bombardment", however without DNA, incubated 15 min with *Agrobacterium* EHA105 hosting pBI 121. Explants were then blotted dry, and returned to the original petri dishes for 3 days co-cultivation. After 3 days explants were washed 3 times with sterile water, blotted dry and transferred to MSBA1 medium, supplemented with 300 mg l⁻¹ kanamycin and 500 mg l⁻¹ carbenicillin. After about 5 weeks surviving explants displaying regenerating "clumps" were subcultured to tubes containing Murashige and Skoog (1962) medium with 3% sucrose, vitamins and 8 g liter⁻¹ TC agar (JRH Biochemicals) with 0.1

mg^l⁻¹ BA, 1 mg^l⁻¹ GA3 with 300 mg^l⁻¹ kanamycin and 500 mg^l⁻¹ carbenicillin. After about 6 weeks emerging shoots were excised and placed in MS0 medium for rooting, subsequently hardened, and transferred to an insect-proof greenhouse. The presence of the NPTII gene was detected by PCR (Gaba et al, 1995, Cucurbitaceae '94 eds. G. Lester and J. Dunlap. Gateway Publishing pp. 188-190). With this method we have now regenerated transgenic shoots of "Galia" melon flowering in the greenhouse. About 1 explant per 100 produces such transgenic shoots.

Changes in the conditions of the experiment, such as delayed transformation after explant preparation, prevent the production of transgenic shoots.

This is the first time that a recalcitrant melon such as cv. Galia has been transformed. To date the range of transformable genotypes has been rather narrow (e.g. discussion in Gaba et al., 1996, Cucurbits Towards 200, Proc 5th Eucarpia Meetings on Cucurbit Genet Breed, pp. 172-178). It is possible that such a method will be useful more widely in other melon varieties, and perhaps other cucurbit crops that regenerate directly from the explant.

APICAL MERISTEMS FOR TRANSFORMATION

Apical Meristems of Melon as a Target for Transformation

Based on research reported by Bidney et al (1992, Plant Mol. Biol. 18:301-313) and Christou (1993, In Vitro Cell. Dev. Biol. 29P:119-124) and following discussions with Bidney at Pioneer HiBred, who disclosed routine use for sunflower, we decided to evaluate the use of apical meristems as a target for transformation.

Due to its simplicity and possible lack of genotypic effects, direct transformation of apices is a very attractive idea. The theoretical feasibility of this approach to transformation relates to the growth dynamics of shoot apical meristems and our ability to perturb normal development both chemically, by application of plant growth

regulators, and physically, by wounding. It is known that the fate of individual cells in apical meristems normally is predetermined. The meristem is composed of zones of related cells, the specific arrangement of which is distinct for a given species. The zones make-up specific layers of cells. Often, the most superficial layers in the center of the meristem are relatively quiescent. However, rapid cell division and differentiation occurs at locations progressively away from the center. Likewise, species-specific differences in cellular activity varies among the layers. While there is a population of cells that are not differentiated, the fate of many individual cells often is predetermined, such that young leaf, floral and axillary bud primordia are differentiating across the surface of and within the meristem in a generative spiral arrangement. The actual pattern of differentiation is species-specific. Since the fate of many of the individual cells may already be set, transformation of such individual cells would tend to result in, at best, the recovery of chimeric plants. The conclusion that can be reached is that, in intact apical meristems, the population of cells that are not predetermined and, thus, could give rise to non-chimeric plants if transformed, is relatively small.

However, it is very easy to manipulate the normal developmental pattern of shoot apical meristems by application of plant growth regulators, physical disruption, or both (eg. Plate 6D). This can be seen by the ease with which isolated apical meristems are induced to produce profuse numbers of adventitious shoots (personal observations) by such relatively simple treatments.

The implications to development of such treatments, with regard to our interest in transformation, are significant. Our ability to induce rapid multiple adventitious shoot production (Plate 6D) indicates that a change in cell determination has occurred. Predetermined cells, such as those, for example, that might normally exist only as a component of a developing shoot, instead become dedifferentiated into shoot initials. Such individual cells are totipotent, capable of developing into a new shoot. Thus,

following proper treatment, the population of undifferentiated totipotent cells temporarily becomes greatly increased. Transformation procedures applied at such a time have a much greater chance of success, since the number of potential targets is significantly increased.

Transformation of Melon Apices and Selection of Shoots

We have, thus far, conducted two experiments designed to test the effect of plasmids, plasmid delivery system (bombardment vs. *Agrobacterium*) and pretreatments on recovery of transformed plants from apical meristems. Plate 6I shows transient GUS reaction of a germinated embryo apex-hypocotyl explant that was subjected at an earlier stage (one week previously) to particle bombardment. Utilizing particle bombardment and the protocol described below (Table 13), treatment with the pKYLX80:35S² plasmid resulted in more Southern dot blot-positive plants.

In the second experiment, the pGA472 plasmid in *Agrobacterium* was used and a series of pretreatments consisting of placing dissected apices on medium containing 5 μ M BA were tested (Table 14). The choice of BA over kinetin (kinetin used in Table 13) was based on a series of experiments (data not shown) that demonstrated the superiority of BA in inducing multiple shoot formation. Before *Agrobacterium* infection, the apices were first wounded by particle bombardment. Table 5 shows that more Southern dot blot-positive plants were obtained utilizing the 8-day pretreatment than longer or shorter times. Again the response per explant is relatively low, such that a firm conclusion as to the optimum pretreatment time cannot be made and additional experiments are ongoing.

We have obtained putative apical meristem-derived transformants but confirming genetic analysis still is underway. However, positive results have been achieved. For example, in vitro selection for kanamycin resistance results in bleaching of tissue and the

Table 13. Bombardment of muskmelon apices pretreated for 3 days on medium with 20 μ M kinetin with two plasmids and gold particles. Shoots were obtained during selection on medium containing 50 mg/l kanamycin for two 2-week culture cycles, followed a third culture cycle with 75 mg/l kanamycin. The second and third culture cycle medium also contained 0.25 μ M NAA to encourage rooting. Shoots subsequently were rooted on medium with NAA, but lacking kanamycin.

Plasmids	No. explants	Plants	Dot blot +
pKYLX80:35S ²	104	18	4
pGA472	111	6	1

outgrowth of green (presumably transformed) shoots (Plate 6J), which would be expected if the treated material was highly chimeric (see "note concerning chimeric plants" below). Recent experiments have produced plants that root well on medium containing kanamycin, a response that is suggestive of transformation. Southern dot blots have indicated the presence of probed DNA sequences in approximately 10% of selected plants. We now are in the process of producing a large number of transformed muskmelon and watermelon plants in order to further selection research and finalize project objectives. Our preliminary success with transforming muskmelon apices, when viewed in light of routine usage of this approach for sunflower and other crops (Bidney, pers. comm.) as discussed below, demonstrates a high degree of feasibility for the proposed project.

Table 14. *Agrobacterium*-mediated transformation with plasmid pGA472 of muskmelon apices that had been wounded with tungsten particles. Apical meristem explants were pretreated for various time periods on medium containing 5 μ M BA before bombardment-wounding and *Agrobacterium* infection. After 4 days, explants were transferred to medium with 0.5 g/l mefoxin and after an additional 3 days, the explants were transferred to medium with both mefoxin and 50 mg/l kanamycin. The explants were subcultured to fresh medium biweekly. Rooting was accomplished as in Table 2.

Days of pretreatment	No. apices	Shoots	Plants	Dot blot +
0	114	18	6	1
1	109	15	8	0
2	83	9	4	1
4	85	19	8	2
8	112	49	37	3
12	101	46	26	1

DESCRIPTION OF COOPERATION

Cooperation between the two PI's has been outstanding. Both communicate regularly by E-mail and a free exchange of ideas occur. The use of E-mail allowed large files to be passed from computer-to-computer, which facilitates editing. Gaba attended the 90th annual meeting of the American Society for Horticultural Sciences in Nashville, Tennessee July 1993, where he interacted with Gray. Gray visited Gaba at the Volcani Center in June of 1994 and also met with Gaba the previous week at the IAPTC meeting in Italy. These visits enabled useful and creative discussions, which continue today.

EVALUATION OF RESEARCH ACHIEVEMENT

With sole exception of our early abandonment of thin layer culture technology, we have substantially met our objectives, including the following: 1) development of improved regeneration systems, bolstered by a fundamental anatomical study of regeneration of melon in vitro, 2) development of particle gun and *Agrobacterium* technology, 3) a study of whether particle bombardment can transform those regenerating sites and comparisons of particle bombardment vs. *Agrobacterium* as transformation methods, 4) transformation of both watermelon and melon, including Galia-type melon and the clear demonstration of transgene transmission to progeny. In addition to the planned objectives, we accomplished a number of previously unanticipated objectives, including the following: 1) Development of new types of particle guns. 2) We incorporated the discovery made before the start of this project - that tetraploid plants can be produced from in vitro watermelon cultures - by performing a study of methods for tetraploid plant identification. Since tetraploid watermelon lines are difficult to produce by conventional means and are indispensable for triploid (seedless) watermelon production, the development of in vitro technology in light of genetic transformation is very important. For example, we now can conveniently study the behavior of transgenes in plants of various ploidy levels and can determine dosage effects on phenotypic responses. 3) We pioneered the use of apical meristems as a target tissue for transformation of cucurbits in general, in anticipation of this technique eliminating the genotypic block to transformation seen in conventional systems. Considering the hundreds of distinct cultivars present in a single species like *Cucumis pepo* (squash and pumpkin), we believe that this approach is potentially of great importance.

A number of abstracts and both refereed and non-refereed publications, as well as some submitted manuscripts, have arisen from this project and are attached.

CONCLUSIONS

This project met all of its objectives and achieved additional progress that was not anticipated in the original proposal, including the refinement and testing of *in vitro* regeneration systems for melon and watermelon, including tetraploid lines of the latter. The most significant outcome of the project is the development and current availability of transformation systems for both melon and watermelon that now enables the insertion of useful genes. This will allow more rapid improvement of both crops, including the addition of certain traits, such as various types of disease resistance, that could not be accessed by conventional breeding technology. The development of apical meristem-based transformation systems enables a wider range of *curcubit* germplasm (including the squashes) to be transformed than heretofore possible with existing regeneration systems.

Both PI's have obtained a wealth of knowledge and experience through completion of this project. They now are well-practiced in regeneration systems and transformation methodology for both crops and hope to make contributions to the genetic improvement of melon and watermelon. Finally, this project enabled a close, lasting collaborative relationship to develop between the PI's.

LIST OF PUBLICATIONS

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- Gaba, V., Elman, E., Perl Treves, R. and Gray, D.J. A theoretical investigation of the genetic variability in the ability of *Agrobacterium* to transform *Cucumis melo* L. pp. 172-178. In: *Cucurbits Towards 2000*. Proc. 6th Eucarpia meeting on Cucurbit Genetics and Breeding, Malaga, Spain, 1996. Eds. Gomez-Guillamon, Soria, Cuartero, Fernandez-Munoz. ISBN 605-5163 6.
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- Compton, M. E., D. J. Gray, E. Hiebert, C. M. Lin, Expression of the B-glucuronidase gene in watermelon explants following particle bombardment or infection with *Agrobacterium tumefaciens*, 90th Annual Meeting ASHS, HortScience 28, 1993, 498.
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Currently in preparation

- The interaction/synergism between BA and ancymidol in the regeneration of primordia in melon explants. Gaba, Elman, Watad and Gray.
- The effect of explant age on the kinetics of primordia development induced by cytokinin in melon explants. Gaba, Elman, Watad and Gray.