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APPLICATION OF LASER MICROBEAM CELL SURGERY AND *AGROBACTERIUM*–MEDIATED GENE TRANSFORMATION SYSTEMS IN MELON (*CUCUMIS MELO* L)

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ABSTRACT

In the current investigation, the application of laser microbeam cell surgery as a transformation system in melon has been studied and compared with transformation *via Agrobacterium tumefaciens* as well as a combine of the two systems. All these transformation treatments have been carried out on the hypocotyl proximal zone of melon *cv*. Shahd El-Dokki using the plasmid pISV2456 that harboring *gus*-intron and *bar* genes. The treated explants were regenerated on shoot regeneration medium supplemented with 250 µg/l bialaphos. Results showed that there was no significant difference of shoot regeneration between the laser and *Agrobacterium* treatments, however, the regeneration percentage varied among the treatments, the laser microbeam treatment showed highest percentage than the *Agrobacterium* and combined methods, (76, 72 and 68 % respectively). The transformation systems were evaluated by detecting the expression of the *gus* gene using histochemical assay, while the integration of the both genes (*gus* and *bar*) were confirmed by the PCR assays, indicating that the two genes were successfully transferred to the plants in the three methods. Seven shoots out of 30 tested shoots were PCR-positive, representing 23.33%, in both *Agrobacterium* and laser treatments, while, only 6 out of 30 tested shoots were PCR-positive, representing, 20%. This study demonstrates using laser tool as a new transformation method in melon *cv* Shahd El-DOkk, indicating that laser technique could be considered a competitive transformation method with the *Agrobacterium*-system.

INTRODUCTION

Melon is an important vegetable crop that is widely cultivated in South-East Asia, China, East Africa and throughout the tropical and subtropical regions (Yadav *et al.*, 1996). Plant biotechnology has the potential to genetically transform plants in order to transfer novel characteristics (Guis et al., 2000). Twenty-five years ago, the concept of using *A. tumefaciens* as a vector to create transgenic plants was viewed as a prospect and a "wish." Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium* mediated transformation seems to grow daily

using *Agrobacterium* and gun-particle bombardment methods, several transgenes, which provide different phenoltypic characteristics, has been transferred to the *Cucumis melo* explants. These have included different marker and reporter genes such as, *npt* II and *gus* (Fang and Grumet, 1990 and Valles and Lasa, 1994); *bar* and *gus* genes (El-Dossoky et al., 2006) and *dhfr* and the *gus* (Dong et al., 1991); virus resistance genes as ZYMV-*cp* gene (Fang and Grumet, 1993; Hosny, 1996 and 2003.) and ACC oxidase gene (Nunez-Palenius et al., 2005). On the other hand, the production of transgenic melon plants expressing the CMV-*cp* has been reported using the microprojectile-mediated gene transfer by Gonsalves et al., (1994).

Recently, the leaser microbeam has been studied as a new approach in the genetic manipulation of higher plants, Badr et al., (2004 and 2005) reported that a highly focused laser beam could be used as an "optical" microbeam to produce tiny submicrometer self-healing holes momentarily made in the cell membrane facilitating the exogenous DNA uptake into the cultured cells.

The laser microbeam has been utilized as a new approach in the genetic manipulation of plant cells has been reported for introducing a foreign DNA into a higher plant which leads to plant cell perforations that might facilitate transformation experiments (Weber et al., 1988 and 1990). They introduced bisbenzimid labeled DNA of the plasmid pBR322 in the cell wall of the pollen grains of *Brassica napus* L. *cv*. Lindora, this using 0.4 M sorbitol. They also reported that the cell wall of the plasmolyzed cell was perforated with a laser pulse, while a second pulse directed at the plasma membrane punctured a hole, which closed again. Moreover, Sanford (1993) demonstrated the feasibility of puncturing the cell wall of individual *Vinca minor* pollen grains without killing them by using the laser microbeam.

Guo et al., (1995) used laser microbeam under osmotic condition to establish an effective system for introducing foreign plasmid DNA's (pBI121, pB221, pACI 1-D and pRq6) into cells of embryonic calli of *Oryza sativa* L. cv. Japonica.

In addition, Buer et al*.,* (1998) reported a detailed protocol for precisely inserting microscopic objects into the periplasmic region of plant callus cells using laser microsurgery. The bacterium *A. rhizogenes* and the plant *Ginkgo biloba* were used as the model system for developing the optical tweezers and scalpel techniques using a single laser. Results showed that more than 95% survival after plasmolyzing *G. biloba* cells, ablating a 2-4 μm hole through the cell wall using a pulsed UV laser beam, trapping and translating bacteria into the periplasmic region using a pulsed infrared laser beam, and then deplasmolyzed the cells. Insertion of bacteria is also described.

A homemade UV excimer laser with two dimensional translation stages, a suitable computer program and a proper optical device has been modified by Badr et al., (2004) to introduce the plasmid pAB6 DNA that harboring the *gus* and *bar* genes into the embryogenic calli of the Egyptian wheat (*Triticum aestivum* L) cv. Giza 164. Osmotic treatment was performed

with 0.4 M manitol that mixed with the plasmid DNA. For irradiating the calli with laser microbeam focused for puncturing holes ~ 0.5 µm in the cell wall allowing DNA uptake. Results showed that this modified procedure of laser-mediated can be a successfully transformation system in wheat. Thereafter, in 2005 Badr et al., produced transgenic wheat cv. Giza 164 plants using the same homemade setup of the UV excimer laser. The obtained plants behaved bialaphos-postive in a selection and rooting stage, indicating the success of the laser system for introducing foreign DNA.

This study deal with the application of laser microbeam cell surgery (LMCS) for gene transfer in melon *cv* Shahd El-Dokki and compare it with the common transformation system *Agrobacterium*.

MATERIALS AND METHODS

Seeds: Seeds of the melon Shahd El-Dokki cultivar were obtained from the Horticulture Research Institute, ARC, Giza, Egypt and used in all regeneration and transformation experiments.

Plasmids: The plasmid pISV2678 (Unpublished data) harbors the *gus–intron* under the control of 35S promoter and *nos* terminator as well as the *bar* fused the AMV Leader gene under the control of *nos* promoter, pAg7 terminator (Becker et al., 1992) was used to optimize the transformation system in melon *via Agrobacterium* mediated transformation (Horch et al., 1985). The plasmid was kindly provided by Dr. P. Ratet, Institut des Sciences Vegetales (ISV), Centre National de la Recherche Scientifique (CNRS), Gif-Sur-Yvette, France.

Primers: Two sets of specific oligonucleotide for *bar* and *gus* genes were synthesized at AGERI, ARC, Giza, Egypt and used to detect the presence of the two genes in putatively transgenic plants.

Tissue culture and regeneration condition: In order to study the laser microbeam cell surgery system as a new transformation method in melon, following treatments have been carried out on the hypocotyl proximal zone explants of melon cv. Shahd El-Dokki with the DNA of the plasmid pISV2678 that harboring *gus*-intron and *bar* genes, i.e., the laser microbeam cell surgery system; *Agrobacterium tumefaciens* and a combine of laser microbeam cell surgery and *Agrobacterium tumefaciens* treatment. All treated explants were regenerated and rooted as described by El-Dessoky et al., (2006).

***Agrobacterium*-mediated transformation:** The *Agrobacterium* mediated transformation was carried out on melon with strain LBA4404 carrying the plasmid pISV2678 as described by El-Dessoky et al., 2006. The hypocotyl explants were soaked in the *Agrobacterium* culture for 10-15 min.

Laser microbeam cell surgery application: The proximal zone of hypocotyl as explants of melon were placed under osmotic condition by incubating the explants on MSBA1 that composed of MS (Murashige and Skoog 1962**)** medium and 1.0 mg/l 6-benzylaminopurine (BAP) supplemented with 0.4 manitol for 4 hours at 25^{o}C at dark. Subsequently, these explants were then exposed to the leaser microbeam irradiation under

aseptically conditions. The explants were then arranged on a specific slide, the plasmid pISV2678 DNA was dropped on the explants with a concentration of 1µg/ µl after mixing with the liquid medium MSBA1, then, the prepared slid was irradiated by Helium-cadmium (He-Cd) laser system. The optical and mechanical setup of the He-Cd leaser (Omni-chrome He-Cd laser) as described by Paterson et al., (2005) were utilized with following modified conditions. The optical system was adjusted with the wave-length 441.5 nm with power 40 mW, which transmitted through 60X objective lens. In the current study the continuous waves (CW) beam converted to pulsed waves using the chopper that placed in front of the device and the mechanical system was developed allowing to mobile the stage of the converted microscope in the X-Y directions by putting two Orile stepper motors beside the stage. These motors were adjusted as a lateral motion with 40microns/25min (Figure-1).The whole system was computerized control using the software that designed by Dr. Badr and his group (unpublished data).

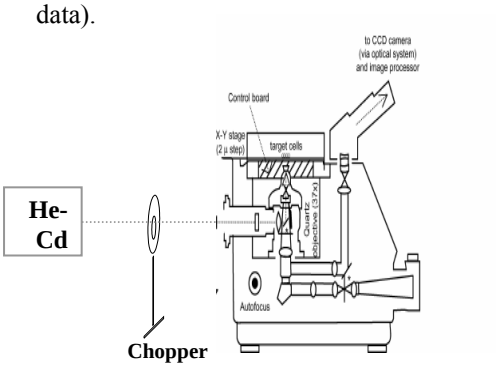

Fig.-1: The schematic diagram the visible He-Cd laser optical setup used for gene transfer.

The combined laser and *Agrobacterium*-mediated treatment:The melon explants were laser-bombardments as mentioned above, followed by soaking in a bacterial culture of *A. tumefaciens* strain LBA4404 for 10-15 min. All the previous steps of the laser and the *Agrobacterium* treatments were considered in the combined treatment.

Selection stage and regeneration: The transformed explants of the three transformation methods were transferred to the selection medium (MS medium and BAP (1mg/l) supplemented with 250 mg/l bialaphos) in addition to 300mg/l carbinicillin. Developed shoots were then subcultured on the elongation medium SE-3 (MS supplemented 100mg/l gibberellic acid (GA3) supplemented with carbinicillin and incubated for 4 weeks. Subsequently,elongated shoots were rooted on M2 containing MS medium and 20 mg/l NAA for 4 weeks. The obtained plant-lets were acclimatized when reaching 7-10cm in height and forming strong roots by transferring into pots containing peat-mosse: sand: clay (1:1:1 w:w:w), covered with plastic bags to increase the humidity and grown under a photoperiod of 16/8h (light /dark) in a controlled green house. Plantlets were hardened by removing the plastic bags after 7-10 days.

Data analysis: Each transformation experiment composed of 9 replicates each one had 10 explants with a total number of 90 explants. The obtained data were statistically analyzed using the analysis of variance (ANOVA) outlined by Gomez and Gomez (1984) with MSTATC program. The differences among means were compared using Duncan's multiple range test (Duncan, 1955).

Evaluation of transformed plant materials

GUS histochemical assay: In this experiment transformed explants and elongated shoots were subjected to detection of the GUS activity. Tissues were immersed in GUS buffer containing 1mg/ml X-gluc (5-bromo-4-chloro -3-indolyl glucuronide) (Clone tech) and incubated overnight at 37°C as described by Jefferson *et al*., (1987).

PCR assay: Genomic DNAs were isolated from young leaves of putative transgenic plants and analyzed by PCR (Dellaporta et al*.,* 1983). Two pairs of specific primers were used to identify the transformed plants, the first was designed to amplify 540 bp of the *bar* gene (PI, 5'AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG3' and P2, 5'AAG GAT CCT CAG ATC TCG GTG ACG G3') and the second pair was designed to amplify the full *gus-intron* gene (2070 bp) (P3, 5'CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C3' and P4, 5'CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC3'). The PCR temperature profile was as follows: initial denaturation of DNA at 94°C for 5.0 min, 35 cycles comprised of I.0min denaturation at 94°C, 1min annealing at 55°C for *gus* gene or 60°C for *bar* gene, 1.0min elongation step at 72^{o}C followed by a final extension step at 72^{o}C for 7min.

RESULTS AND DISCUSSION

There are many different approaches for plant genetic manipulation, some of which are more successful than others, and some have been superseded by more up to date methods. Two classes of plant transformation technology currently exist. These are non natural or *in vitro* methods, or natural methods. Non natural methods include microinjection and direct DNA uptake, whilst natural methods include technologies such as the use of viral vectors and *A*grobacterium *tumefaciens*. Transgenic melon plants expressing different genes have been produced using Agrobacterium (Fang and Grumet, 1990; Valles and Lasa, 1994; Nunez-Palenius et al., 2005) and gun-particle bombardment method (Gonsalves et al., 1994.)

In this study, the laser microbeam cell surgery as a new method in melon transformation has been adopted as well as compared with the *Agrobacterium*-mediated transformation method and combined treatment between the two methods, laser microbeam cell surgery and *Agrobacterium*. The *A. tumefaciences* transformation in melon cv. Shahd El-Dokki using the proximal zone of the hypocotyl explants protocol has been previously adopted by El-Dessoki et al., (2006).

Effects of different transformation methods **on melon:** The three methods used for melon transformation (Leaser beam surgery, *Agrobacterium* and the combined treatments) were carried out on cv. Shahd El-Dokki using the hypocotyl explants, the plasmid pISV2678; *A. tumefaciens* strain LBA4404 and Helium Cadmium (HeCd) laser. The treated explants were co-cultivated on MSBA1 medium for 2days and then transferred to selection medium (MSB A1 supplemented with 250µg/l bialaphos) to select putative transgenic shoots.

The first visible reaction of the treated proximal zone explants after several days of incubation on the selection medium was the enlargement

of these explants. The shoots were regenerated directly during 2-3 weeks of culture. It was also observed that there is no difference in the shoots performance among the treatments. Data in Table-1 showed that although the survived explants and shoot formation percentage varied among the three treatments but there was no significant difference detected between the laser and *Agrobacterium* treatments while the combined treatment showed the significant difference among them as it gave the lowest mean value. It was observed that the number of survived explants in the case of the laser treatment revealed the highest (87.7%) followed by the *Agrobacterium* treatment (78.8%) and the combined treatments (68.8%) as shown in Table-1. Furthermore, the laser microbeam treatment showed the highest shoot formation percentage among the treatments and the combined treatment revealed the lowest percentage, as it was 76, 72 and 68 for laser; *Agrobacterium* and combined treatments, respectively.

The average of shoots/explant obtained in the three treatments was approximately the same which varied from 4.08 to 4.50 among the treatments. This indicates that the transformation treatment did not affect on the number of shoot formation per explants. Result in Figure-2 illustrates the shoot formation stage in melon, cv. Shahd El-Dokki, 3 weeks post incubation on the selective medium obtained from the three treatments.

Fig. 2: Shoots of melon, cv. Shahd El-Dokki obtained after 3 weeks of transformation treatments and cultivation on selection medium

Selection stage and regeneration: The bialaphos-resistant shoots were then transferred to the elongation medium for approximately 3 weeks. Subsequently, the elongated shoots were rooted and acclimatizated (Figure 3). It was observed that there was no difference in shoots height of the three treatments.

Fig. 3: Shoots of melon, cv. Shahd El-Dokki obtained after 3 weeks of transformation treatments and cultivation on selection medium.

Evaluation of putative transgenic events:

Histochemical GUS assay: Endogenous GUS activity was detected in explants and tissues obtained from the three methods during different stages. The explants were randomly selected after incubating for one week on the selective medium and visually compared with non-transformed plant materials. The explants that developed blue color

were varied among the three treatments. All tested explants that obtained from the laser treatment behaved GUS-positive, representing a 100%, while, only 60% of the tested explants were positive. However, the combined treatment recorded a percentage of 80% (Fig. 4.)

Fig.-4: The bialaphos resistant shoots obtained from the laser transformation treatment on a) elongation medium b) rooting medium and c) acclimatization stage.

PCR assay: PCR assay was performed to confirm the genes presence on transformed melon plants. Results recorded positively impact of PCR to both genes (*gus* & *bar*) with different percentages in tested plants that obtained from all of the three transformation treatments, indicating that the two genes were successfully introduced into melon plants through the three methods. Similar results were observed in the two methods, i.e., laser and *Agrobacterium* transformation, as a number of 6 and 5 out of 30 tested shoots obtained from both treatments, representing 20% and 16.6% for *gus* and *bar* genes, respectively. A 13.3% and 16.6% PCR-positive for *gus* and *bar* genes, respectively, were recorded in a case of the plants obtained from the combined treatment, as 4 and 5 out of 30 tested shoots were positive (Fig.5).

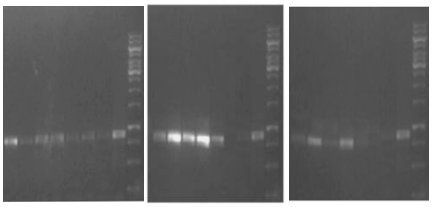

Fig.-5: PCR analysis using *gus*-specific Primers to amplify 900 bp of the putative transgenic melon plants obtained *via* a*) Agrobacterium* treatment (Lanes 1-6), b) laser micro beam treatment (Lanes 1-6) c) combined treat-ment (Lanes 1-6), M: 1kb DNA ladder. +: Positive control. –: Negative control, PCR mixture with no template.

It is of importance to mention that the current investigation is the first report using laser tool as a new transformation method in melon cv. Shahd El-Dokk., this technique could be considered competitive trans-formation method with the *Agrobacterium*-system. In addition, this study recorded that the osmotic treatment by incuba-ting the explants for 4hours at 25^0C with 0.4M mannitol and a visible laser at 441.5 nm, focused to less than 1µm proved to be an efficient for gene transformation in melon tissues. Moreover, the combined laser and *Agrobacterium* showed a successful modality as well.

In previous studies,laser-mediated transformation has been used in the pollen grains of *Brassica napus* L. cv. Lindora, by Weber et al., (1988 and 1990); rice (Gue et al., 1995) and on the embryogenic calli of the Egyptian wheat cv. Giza 164 (Badr et al., 2004 and 2005). It was also found that the osmotic treatment is an important factor for successfully DNA uptake into the plant cell. The mannitol and sorbitol at

concentration of 0.4M were utilized for performing the osmotic condition.

Table-2: Melon regeneration percentage resulted on the different transformation methods

Replicates	Total tested explants	Survived bialaphos resistant explants		Explants produced shoots		Shoots/explant	
		No.	%	No.	%	Total	Average
Laser treatment							
R1	10	7	70	5	71.4	33	6.6
R2	10	8	80	6	75	15	2.5
R3	10	8	80	4	50	28	7
R4	10	9	90	5	55.5	21	4.1
R5	10	9	90	5	55.5	22	4.4
R6	10	9	90	9	100	42	4.6
R7	10	10	100	8	80	21	2.6
R8	10	10	100	10	100	51	5.1
R9	10	9	90	8	89	29	3.6
Total	90	79	87.7	60	76	262	40.5 4.50
Mean	8.778^{A}		6.667^{A}				4.506^{A}
***Agrobacterium* treatment**							
R1	10	7	70	4	57.1	9	2.2
R2	10	5	50	4	80	17	4.2
R3	10	7	70	5	71.4	38	7.6
R4	10	8	80	5	62.5	11	2.2
R5	10	9	90	8	88.8	55	6.8
R6	10	8	80	5	62.5	22	4.4
R7	10	10	100	10	100	41	4.1
R8	10	8	80	6	75	15	2.5
R9	10	9	90	9	100	22	2.4
Total	90	71	78.8	51	71.8	230	36.8 4.08
Mean	7.889^{A}		6.222^{A}				4.068^{A}
Combined treatment							
R1	10	4	40	4	100	16	4
R2	10	8	80	4	50	17	4.2
R3	10	3	30	3	50	13	3.2
R4	10	7	70	5	71.4	28	5.6
R5	10	10	100	7	70	41	5.8
R6	10	7	70	4	57.1	12	3
R7	10	7	70	3	42.8	14	4.6
R8	10	7	70	6	85.7	21	3.5
R9	10	7	70	6	85.7	22	3.6
Total	90	62	68.8	42	67.7	184	37.7 4.1
Mean	6.667^{B}		4.667^{B}				4.197^{A}

Means followed by different letters in the same Column differ significantly at $P \leq 0.05$.
LSD at 0.05 =1.218, the number of survived explants. LSD at 0.05 =1.685, the number of explants produced shoots. LSD at 0.05 =1.557, the average of shoots per explant.

REFERENCES

Badr,Y.A., M.A.Kereim, M.A.Yehia, O. O.Fouad and A.Bahieldin, Production of fertile transgenic wheat plants by laser micropuncture. Photochem. Photobiol. Sci. **4**: 803-807(2005).

Badr,Y.A., A.Bahieldin, M.A.Aziz, M.A. Yehia Yehia, A. Abou-El-Magd and M.A. Madkour, A modified protocol for laser-mediated gene transfer in wheat. Arab J. Biotech. **7** (2): 299-304 (2004).

Becker, D., E. Kemper, J. Schell and R. Masterson, New plant binary vectors with selectable markers located proximal to the left T-DNA border. Plant Molecular Biology **20**: 1197 (1992).

Buer C.S.,T.Gahagank,G.A.Swartzlaander and J.Weathers, Insertion of micro scopic objects through plant cell walls using laser icrosurgery. Biotechnology and Bioengineering **60** (3): 348 -355(1998).

Dellaporta,S.L., J.Wood and J.B.Hicks, A plant DNA minipreparation. Version III. Plant Mol. Biol. **1**: 19-21(1983).

Dong, J.Z., M.Z. Yang, S.R. Jia and N.H. Chua, Transformation of melon (*Cucumis melo*) and expression from cauliflower mosaic virus 35S promoter in transgenic melon plants. Bio/Technology **9:** 858-863(1991).

Duncan, D. B., Multiple range and multiple F-tests. Biom. **11***:* 1-42 (1955).

EL-Dessoky,E.S., G. Hussien, A.S. Sadik and N.Abdallah, Efficient regeneration and *Agrobacterium* mediated transformation systems in melon (*Cucumis melo* L.). Egypt. J. Genet. Cytol. **35**: 35-47(2006).

Fang, G. and R. Grumet, *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Reports **9**: 106-164(1990).

Fang, G. and R. Grumet, Genetic engineering of potyvirus resistance using constructs derived from the zucchini yellow mosaic virus coat protein gene. Mol. Plant Microb. Interact. **6**: 358-367(1993).

Gomez,K.A. and A.A.Gomez, Statistical Procedures for Agriculture Research. (2nd ed.) J. Wiley and Sons Ltd., New York, Pp. 680, (1984).

Gonsalves, C., B. Xue, M. Yepes, M. Fuchs, K. Ling, S.Namba, P. Chee, J. L.Slightom and D.Gonsalves, Transferring cucumber mosaic virus-white leaf strain coat protein gene into *Cucumis melo* L. and evaluating transgenic plants for protection against infections. J. American Society for Horticultural Science **119:** 345-355(1994).

Guis, M, M.B. Amor, A. Latche, J.C. Peach and J.P. Roustan, A reliable system for the transformation of cantaloupe charentais melon (*Cucumis melo* L. var. *cantalupensis*) leading to a majority of diploid regenerants. Scientia Horticulturae **84**: 91-99(2000).

Guo, Y., H. Liang and M.W. Berns, Laser mediated gene transfer in rice. Hysiologia PLantarum **98**: 19-24 (1995).

Horsch,R.B., J.E.Fry, N.L.Hoffmann, S. G. Rogers and R.T. Fraley, A simple and general method for transferring genes into plants. Science **227**: 1229-1231(1985).

Hosny, G.M., Regeneration and transformation of muskmelon (*Cucumis melo* L.) for the development of zucchini yellow mosaic virus resistance. M.Sc. Thesis, Fac. Agric., Cairo Univ., Egypt (1996).

Hosny,G.M., Development of virus resistance in some plants via genetic engineering approaches. Ph.D. Thesis, Fac. Agric., Cairo Univ., Egypt (2003).

Jefferson,R.A., T.A. Kavanagh and M.W. Bevan, GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6:** 3901-3907(1987).

Murashige, T. and F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. **15**: 473-497 (1962).

Nunez-Palenius, H.G.P., J.C. Daniel, J.H. Don, C. Joseph, K.J Harry, Transformation of a muskmelon 'Galia' hybrid parental line (*Cucumis melo* L. var. *reticulates* Ser.) with an antisense ACC oxidase gene. Plant Cell Rep.**25**:198-205(2005).

Paterson, L., B. Agate, M. Comrie, R. Ferguson, T.K. Lake, J.E. Morris, A.E. Carruthers, C.T.A. Brown, P.E. Bryant, F. Gunn-Moore, A.C. Riches and K. Dholakia, Photoporation and cell transfection using a violet diode laser. Optics Express **13:** 595-600 (2005).

Sanford, J.C., Pollen studies using a laser microbeam, Pollen biology and implications for plant breeding, Proceedings of the symposium, Villa feltrinelli, Lake Garda, Italy, 23-26 June, 1982 (edited by mulcahy, D.L. and Ottaviana, E.) New York, USA, Elsevier Science Publishing Company, Inc. Pp. 107-115 (1983).

Valles,M.P. and J.M.Lasa, *Agrobacterium* mediated transformation of commercial *Cucumis melo* L. (*cv*. Amarillo Oro). Plant Cell Reports **13:** 145-148 (1994).

Weber, G., S. Monajembashi, K.O. Greulich and J. Wolfrum, Genetic manipulation of plant cells and organelles with a laser microbeam, Plant Cell Tissue Organ Culture **12**: 219-222 (1988).

Weber, G., S.Monajembashi, J.Wolfrum and K.O. Greulich, Genetic changes induced in higher plants by UV laser microbeam. Physilogia Plantarum **79:** 190:193 (1990).

Yadav, R.C., M.T. Salah and R. Grumet, High frequency shoot regeneration from leaf explant of muskmelon. Plant Cell Tissue and Organ Culture **45:** 207-214 (1996).