FACTORS INFLUENCING SUCCESSFUL *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION OF MUSKMELON

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ABSTRACT

The development of a robust *Agrobacterium*-mediated transformation protocol for muskmelon (*Cucumis melo* L.) requires the identification and optimization of the factors affecting DNA delivery and plant regeneration. We have used pieces of mature cotyledon from cultivar Hales Best Jumbo and the *Agrobacterium tumefaciens* strain LBA4404, to investigate and optimize regeneration and transformation. *Agrobacterium* strain harboring the binary vectors which contains phosphino-thricin acetyl transferase gene (*bar*) as selectable marker for herbicide resistance (glufosinat ammonium) and β-glucuronidase gene (*uidA*) as reporter. Factors that produced differences in DNA delivery and regeneration included bacterial concentration, length of exposure, the time allowed for co-cultivation and antibiotic concentration (claforan, cefotaximum). These factors combined as a whole led to successful transformation. Bacterial concentration, time of exposure of the explants to the bacteria, co-cultivation period and antibiotic concentration were found to affect regeneration and transformation efficiency. Analysis of these parameters allowed the development of an optimized protocol for *Agrobacterium*-mediated transformation of *Cucumis melo* cv. Hales Best Jumbo cotyledon explants. We fully describe a protocol that allowed efficient DNA delivery and gave rise stable transgenic muskmelon plants. Selectable marker gene expression and reporter gene assay demonstrated that transgenes were integrated into the muskmelon genome. Genetic transformation of calli and regenerated plantlets was confirmed by histochemical β-glucuronidase assays.

Key words: Transformation, muskmelon, *Agrobacterium*-mediated, factors, herbicide, β-glucuronidase

INTRODUCTION

Plant genetic transformation has become an important tool for functional genomics and as an adjunct to conventional breeding programs. At the present time gene transfer by *Agrobacterium* is the established method of choice for the genetic transformation of most plant species. It is perceived to have several advantages over other forms of transformation (such as biolistics), including the ability to transfer large segments of DNA with minimal rearrangement and with fewer copies of inserted genes at higher efficiencies with lower cost **(Hiei *et al*., 1997; Hansen and Wright, 1999 and Shibata and Liu, 2000)**. Development of *A. tumefaciens*-based transformation procedures for muskmelon could contribute to the expansion

of the gene pool of this species by allowing the introduction of useful genes such as those for resistance to disease, insect pest and herbicides. *Agrobacterium*-mediated transformation is the main method used in the field of biotechnology **(Nadolska-Orczyk *et al.*, 2000)**. The application of *Agrobacterium*-based transformation systems for foreign gene transfer into muskmelon has been demonstrated in few studies **(Fang and Grumet, 1990; Dong *et al*., 1991; Vallés and Lasa, 1994; Gaba *et al*., 2000 and Guis *et al*., 2000)**. Cultivar specific differences exist in regeneration and transformation efficiency in muskmelon. Transformation of melon is still regarded as a difficult problem. It is well known that genotype limits the range of genetic transformation **(Mackay *et al*., 1989)**. The development of genetic transformation for muskmelon offers the potential of introducing valuable traits into this crop. To date, most of the research has been concentrated on the cultivar Hales Best. Therefore this cultivar was chosen as experimental material to carry out studies on the factors involved in *Agrobacterium*-mediated transformation.

The goal of the work presented here was to develop an improved protocol for *Agrobacterium*-mediated transformation of the muskmelon cultivar Hales Best Jumbo by determining the influence of some factors on regeneration and transformation efficiency. These factors were chosen because they had not been examined in previous work. Analysis of these parameters allowed the development of an optimized protocol for *Agrobacterium*-mediated transformation of *Cucumis melo* cv. Hales Best Jumbo cotyledon explants.

MATERIALS AND METHODS

Plant material and bacterial strain: Seeds from the *Cucumis melo* cultivar Hale's Best Jumbo (provided by the Vegetable Crop Research Institute, Budatétény, Hungary), were used in this study. Mature seeds coats were removed and surface-sterilized by treating in 15% chlorox for 15 min. They were rinsed three times with sterile distilled water and dried up with sterile filter paper. Seeds were germinated on hormone free MS medium, **Murashige and Skoog (1962)**, solidified with 2 g/l phytagel, pH 5.8 and germinated in thermostatic box at 32°C for two days. Seeds were germinated at 25°C under cool white fluorescent lights with a 16 hr light : 8 hr dark photoperiod. Cotyledon explants were excised from 4 day old seedlings grown under these conditions were used for co-cultivation experiments. Genetic transformation, has been performed using the LBA4404 *A. tumefaciens* strain containing the binary vector pRGG bar plasmid. Dr. Nagy I., Agricultural Biotechnology Center, Gödöllő, Hungary, provided the bacterial strain. This binary vector contains two genes, the β-glucuronidase gene (*uidA*) as reporter-coded β-glucuronidase, and phosphinothricin acetyl transferase gene (*bar*), as selectable marker for herbicide resistance, glufosinat ammonium.

Infection and co-cultivation procedure: *A. tumefaciens* was grew and maintained on AB medium, **Lichtenstein and Draper (1986)** with appropriate antibiotics to maintain the plasmid. An overnight bacterial culture were used for culturing the *Agrobacterium* strain on two different size petri dishes (8 and 12 cm in diameter, low and high amount of bacteria) on solid medium. Two petri dishes were used per each treatment, and harvesting the *Agrobacterium* in 30 ml sterile distilled water per each one. Excised 4 day

old cotyledons were cut on all around the edges with a dull scalpel blade, and cut into four segments per each cotyledon. Then, these segments were immersed in fresh overnight suspension culture of *Agrobacterium* strain for 20 and 60 min, blotted dry with sterile filter paper to remove the excess of bacteria. The tissues were then transferred to regeneration MS medium supplemented with 1.05 mg/l indole-3-acetic acid (IAA) + 0.6 mg/l 6-benzyladenine (BA) + 0.24 mg/l abscisic acid (ABA) and 3% sucrose, (pH 5.0). Co-cultivate explants were incubated in growth room at 25°C with a 16 hr light : 8 hr dark photoperiod for 3, 4, and 5 days.

Influence of bacterial concentration and time of exposure: Two bacterial concentrations were used. *Agrobacterium* growing on two petri dishes, 8 cm in diameter (low amount) and two petri dishes, 12 cm in diameter (high amount) on solid medium. The bacteria was harvested in 30 ml sterile distilled water for each case. Two different time of exposure of the explants to the bacteria (20 and 60 min) were compared.

Influence of antibiotic concentration: Various concentrations from the antibiotic (claforan, cefotaximum) were compared and tested to control the growth of *Agrobacterium* during shoot formation. Also, to investigate their effects on shoot regeneration and transformation. Cefotaximum concentrations ranged from 100 to 1000 mg/l (100, 200, 300, 500, 700 and 1000 mg/l).

Plant regeneration, selection: After co-cultivation with *Agrobacterium*, explants were rinsed in sterile distilled water to remove excess *Agrobacterium*. Then washed in 500 mg/l cefotaximum for 20 min in order to eliminate bacterial carry over. Then transferred into sterile distilled water to remove the rest of excess *Agrobacterium* on the surfaces. Finally, cotyledonary segments were blotted dry with sterile filter paper. The explants were transferred to selective regeneration MS medium supplemented with 1.05 mg/l IAA, 0.6 mg/l BA and 0.24 mg/l ABA, and containing 3 mg/l glufosinat ammonium (pH 5.8). Explants were kept on selective regeneration MS medium during shoot formations. Cotyledon explants without *Agrobacterium* infections and their shoots were used as control during all the experiments in order to confirm the effect of the selective medium and the genetic transformation. Shoots were excised from the explants and transferred into MS medium containing 0.3 mg/l indole-3-butyric acid (IBA) for rooting.

Assay for GUS activity: Transient GUS expression was determined on calli and shoots sampled using the histochemical GUS assay according the method of **Jefferson *et al*. (1987)**. Explants were incubated overnight at 37°C in buffer containing 1 mM X-Gluc (5-Bromo-4-Chloro-3-Indolyl-D-β-Glucuronide), 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1 % (v/v) Triton X-100. To assay for stable expression, we incubated calli, shoots and leaf fragments from regenerating plantlets overnight at 37°C.

RESULTS AND DISCUSSION

Effect of *Agrobacterium* concentration on callus and shoot formations: Histological observations under Electron microscope showed that a segment of cotyledon were infected by several cells of *Agrobacterium* after three day co-cultivation (Fig. 1). The results revealed that the number of callus and shoot formations following exposure to *Agrobacterium* were influenced by

some of the variables tested in this study. When two bacterial concentrations were compared, the number of callus and shoot formations ranged from 196 and 3 at high concentration to 339 and 27 at low concentration respectively (Table 1). Our results indicated that, callus and shoot formations were affected by the concentration of *Agrobacterium*. We found that the number of callus and shoot developments from muskmelon explants following *Agrobacterium* transformation were influenced by bacterial concentration and length of exposure to the bacteria. Similar findings have been reported for cucumber by **Sarmento *et al.* (1992)**. They indicated that the frequency of callus development from cucumber explants on kanamycin containing medium following *Agrobacterium* transformation were influenced by explant size, bacterial concentration, and length of exposure to the bacteria. On the other hand, the work of **Confalonieri *et al.* (1995)** showed some factors such as *Populus nigra* clone, strain of *A. tumefaciens* and addition of acetosyringone affect transformation efficiency of *P. nigra* by *A. tumefaciens*. Other factors examined were leaf wounding and bacterial concentration but they did not influence the transformation frequency. The frequency of leaf discs producing kanamycin resistant calli was not significantly different between the clones and bacteria concentration used. **Frary and Earle (1996)** evaluated the effects of other different factors on the efficiency of transformation in tomato such as type of explant (hypocotyls or cotyledons). Hypocotyls and cotyledons were found to give equivalent transformation efficiencies. Also, **Wu *et al.* (2003)** found that factors that produced significant differences in T-DNA delivery and regeneration in wheat included embryo size, duration of pre-culture, inoculation and co-cultivation, and the presence of acetosyringone and Silwet-L77 in the media. The results presented here showed at low concentration of *Agrobacterium*, callus and shoot formations were not effected while at high concentration, callus and shoot formations were strongly inhibited.

Effect of soaking time on callus and shoot formations: With regard to exposure time, at periods of 20 and 60 min, the callusing frequency was 346 and 42 respectively (Table 2). With prolonged exposure (1 hr), explants became necrotic and died. Soaking time also has an effect on cotyledon explants. We found that all explants were killed when they were soaked in *Agrobacterium* for one hr. However, 20 min soaking were effective for shoot regeneration, resulting in more shoots as shown in Table 2, so this exposure time was used in subsequent regeneration and transformation experiments. Our results are in comparable with the results obtained by **Sarmento *et al.* (1992)**. They showed that the callusing frequency in pickling cucumber was 35, 30, 24, 19 and 9% at exposure time, at periods of 5, 10, 30, 45 and 60 min respectively. They concluded that with prolonged exposures (>45 min), explants became necrotic and died.

Co-cultivation period and cefotaximum concentration: Several parameters were studied by **Chabaud *et al.* (1988)** in order to obtain the highest efficiency of regeneration and transformation in alfalfa. These were explant type, different *Agrobacterium* strains, number of days of co-cultivation and concentration of kanamycin used for selection. The results indicated that the number of days of co-cultivation had significant effect on regeneration and transformation efficiency in alfalfa. A 4 day co-cultivation resulted in a transformation frequency at least twice as high as that obtained after 2 or 3 days co-cultivation. Our results showed that three day co-cultivation period

resulted high number of shoot regeneration, while four or five day co-cultivation resulted low number. We suggest that duration of co-cultivation was an important parameter and differences observed could be due to several factors. Also, our results indicated that the control of *Agrobacterium* growth on the regeneration medium was affected by the previous co-cultivation period. Three day co-cultivation resulted high number of shoot regeneration, so this co-cultivation period was used in all subsequent experiments. It was difficult to eliminate the *Agrobacterium* after five day co-cultivation. The growing of *Agrobacterium* killed most of the treated explants. We found that after co-cultivation, the elimination of the bacteria was the key for successful regeneration and transformation. Currently, cefotaximum or carbenicillin was used to control the growth of *Agrobacterium*. In order to determine a suitable concentration of cefotaximum needed to eliminate the *Agrobacterium* and not affect on shoot regeneration. Explants were cultured on medium containing different concentrations from cefotaximum ranged from 100 to 1000 mg/l as shown in Table 3. However, if the antibiotic concentration is too high, it will affect the cotyledon growth, even killing the explants as shown in Table 3. Because of this reason, successful regeneration procedures of muskmelon by **Bársony *et al*. (1999)** might be ineffective when using high concentration of antibiotic in the medium. So, the antibiotic concentration as low as possible was used and this was an effective way to control *Agrobacterium*. On the basis of our experiments, 500 mg/l cefotaximum was used in subsequent regeneration and transformation experiments.

Table 1. Effects of *Agrobacterium* concentration on callus formation and shoot regeneration.

Agrobacterium conc. (30 ml/treat.)	Number of explants	Number of callus	Number of shoots
Two 8 cm petri dishes	350	339	27
Two12cm petri dishes	350	196	3

Table 2. Effects of exposure time on callus formation and shoot regeneration.

Exposure time (min)	Number of explants	Number of callus	Number of shoots
20	350	346	30
60	350	42	0

Our results showed that the antibiotic, cefotaximum at low concentration, 300 mg/l or below could not eliminate the *Agrobacterium* and more than 40% of explants were still infected. Otherwise at high concentration 700 mg/l, the *Agrobacterium* growing was not too much and less than 25% of explants were still infected, and regeneration of shoots were inhibited. At 1000 mg/l concentration, 30% of cotyledon explants were killed, while 5% only of the explants were still infected. Therefore, 500 mg/l cefotaximum concentration was used in subsequent subculture regeneration media and considered as the useful concentration for eliminating the *Agrobacterium*. Regenerated shoots were not affected by this antibiotic concentration (Table 3).

Table 3. Effects of claforan (cefotaximum) on callus formation and shoot regeneration.

Claforan concen. (mg/l)	Number of Explants	Number of callus	Number of shoots
100	350	15	0
200	350	14	0
300	350	26	0
500	350	345	26
700	350	336	6
1000	350	77	0

Plant regeneration and selection: Selection was carried out with 3 mg/l glufosinat ammonium. Plantlets were transferred onto MS selective regeneration medium supplemented with 3 mg/l glufosinat ammonium. Larger shoots survived very well during selection, while control died. The application of glufosinat ammonium selection during regeneration combined with GUS expression screening proved to be effective in identifying transgenic plants. Our results are in comparable with that obtained by **Wu *et al*. (2003)**, who used selective medium containing 2-4 mg/l L-phosphinothricin for selection transgenic wheat.

GUS transient expression: Callus and regenerated shoots were tested for GUS activity. Transient expression of the *uidA* (GUS) reporter gene was used to assess *Agrobacterium*-mediated DNA delivery in callus and regenerated shoots (Fig. 2). GUS-positive shoots were considered to be putative positive transgenic. Thereby confirming successful *uidA* gene integration and the development of some regenerative tissue. These results are similar to those obtained by **Confalonieri *et al*. (1995)** in several black poplar clones, and **Wu *et al*. (2003)** in wheat.

This work showed some factors such as bacterial concentration, length of exposure, the time allowed for co-cultivation and antibiotic concentration, cefotaximum that affect regeneration and transformation efficiency of muskmelon by *A. tumefaciens*. Although one strain (LBA4404) was used only in this study, the methodology should be suitable for genes that could improve agronomic traits.

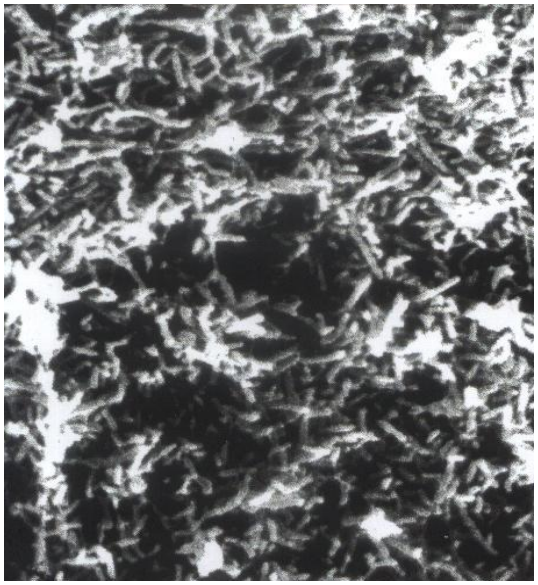
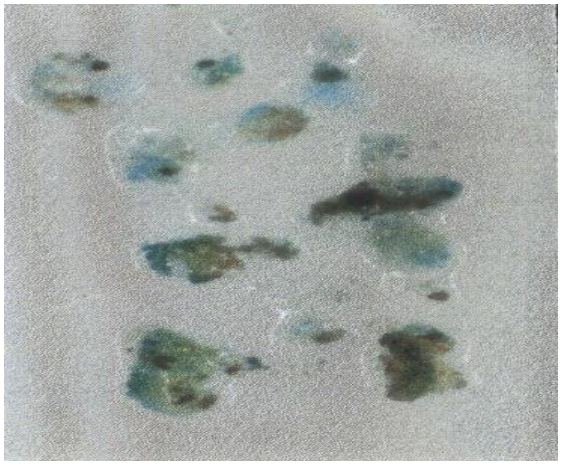
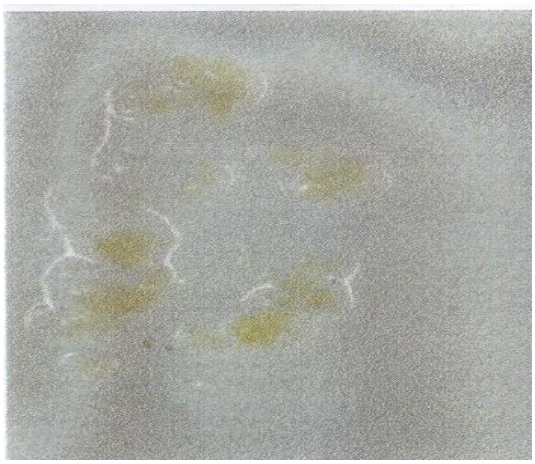


Fig. 1: *Agrobacterium*-infected a segment of cotyledon after 3 day co-cultivation under Electron microscope.





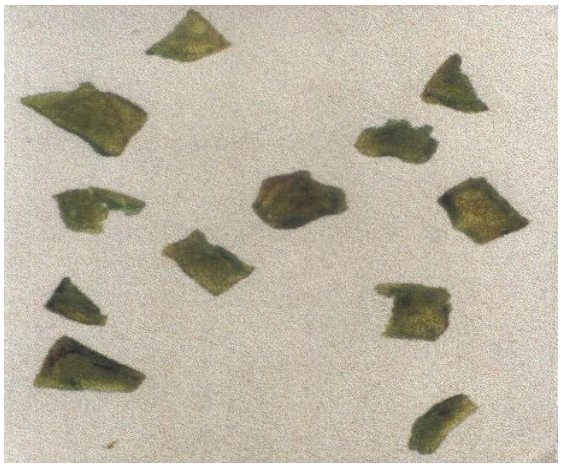


Fig. 2: From left to right, GUS expression in different tissues. Strong GUS expression in the cells of callus, while control callus was not showed positive reaction, GUS expression in the cells on cut surface of cotyledon explants and in young regenerated shoots.

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دراسة على العوامل التى تؤثر فى نجاح استخدام بكتيريا الاجروبكتيريم كوسيط لإحداث التحول الوراثى وإعادة تخليق وإكثار نباتات من القاوون الشبكى (الكنتلوب)

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***قسم الوراثة و تربية النبات - كلية علوم البساتين- جامعة سانت اشتيفان - بودابست - المجر**

إن التطور السريع فى بروتوكول التحول الوراثى باستخدام الاجروبكتيريم كوسيط لإحداث التحول الوراثى فى القاوون الشبكى (الكنتلوب) يتطلب تحديد وتعظيم العوامل التى تؤثر فى إدخال الـ DNA وإنتاج نباتات مهندسة وراثيا منه. تم استخدام أجزاء الفلقات الجنينية الناضجة من الصنف المنزرع عالمياً(هولز بست جامبو) وتم استخدام سلالة الاجروبكتيريم LBA4404 بهدف بحث وتعظيم عملية تحفيز إعادة تولد نباتات معدلة وراثيا. سلالة الاجروبكتيريم المستخدمة كانت محتوية على البلازميد الثنائى pRGG بار المتضمن جين (*bar*) كواسم انتخابي لمقاومة مبيد الحشائش جلوفوسينيت الامونيوم وجين (*uidA*) كمخبر ومقرر.

وكانت أهم النتائج المتحصل عليها ما يلي:

- العوامل التى أظهرت اختلاف فى إدخال ال DNA وإعادة تولد نباتات محولة وراثيا من القاوون الشبكى هى تركيز المعلق البكتيرى المستخدم، وقت التعريض فيه، فترة التحضين المشترك بين أجزاء الفلقات الجنينية والاجروبكتيريم، وتركيز المضاد الحيوى السيفوتاكسيمم المستخدم للتخلص من البكتيريا بعد المعاملة.
- كل هذه العوامل التى تم دراستها قد أدت إلى نجاح التحول الوراثى.
- تركيز المعلق البكتيرى، وقت تعريض الأجزاء النباتية للأجروبكتيريم، فترة التحضين المشترك وتركيز المضاد الحيوى المستخدم وجد أن لكل منها تأثير هام فى معدل تولد نباتات معدلة وراثيا.
- دراسة وتحليل العوامل سالفة الذكر معا قد أدى إلى تطور تعظيم بروتوكول التحول الوراثى فى الصنف (هولز بست جامبو) باستخدام الاجروبكتيريم كوسيط لنقل الجينات.

- لقد تم وصف البروتوكول الكامل الذى كان فعالا فى إدخال ال DNA وإعادة تكون وتولد نباتات مهندسة وراثيا من الكنتلوب.
- بدراسة تعبير كلا من الجين الواسم الانتخابي المستخدم (*bar*) والجين المقرر(*uidA*) قد بين انه قد تم انتقال تلك الجينات ودمجها داخل جينوم نبات الكنتلوب.

على الرغم من انه قد تم استخدام سلالة بكتيرية واحدة فقط فى هذه الدراسة فان هذه الطريقة محل الدراسة تعتبر مناسبة لنقل جينات هامة و التى يمكن بواسطتها تحسين صفات هذا المحصول الهام.