The Effects of Some Parameters on Agrobacterium-Mediated Transformation in Muskmelon

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Summary: Some parameters involved in Agrobacterium-mediated transformation in muskmelon Hales best (HBS) were studied. Cotyledon explants excised from 3.5-day-old seedlings were co-cultivated with *Agrobacterium tumefaciens* harbouring binary vectors which contained GUS and BAR genes. After co-cultivation on a low pH medium, explants were transferred to selective medium, with higher pH, containing Claforan and Finale. The medium was changed every two weeks till shoots were induced. All shoots rooted on MS medium supplemented with 0.3 mg/L IBA. These parameters combined as a whole led to successful transformation. The expression of the introduced gene construct was confirmed by GUS staining of shoot segments.

Introduction

Muskmelon is a kind of important crop cultivated all over the world. With the increase of people's consume level, existing cultivars cannot meet the requirements. The development of genetic transformation for muskmelon offers the potential of introducing valuable traits into this crop, e.g. disease resistance, high sugar content and high protein content, to improve its productivity and quality beyond the limits of conventional breeding. There have been a few reports on successful muskmelon transformation since 1990 (*Fang* et al, 1990, 1993.; *Gonsalves* et al, 1994). On the basis of research by other investigators (e.g. *Orts* et al, 1987. *Dirks & Van Buggenum* 1989) and observations, cultivar specific differences exist in regeneration and transformation efficiency in muskmelon. To date, most of the research has been concentrated on the cultivar Hale's Best. Therefore this cultivar was chosen as experimental material to carry out studies on the parameters involved in Agrobacterium-mediated transformation. Our objective was to develop a more efficient transformation procedure by which useful genes, such as herbicide resistance, can be introduced into the cultivars.

Material and methods

Plant material. HBS, a muskmelon cultivar was used in all the experiments described. Seeds were peeled and surface-disinfected in 15% Chloral (sodium hypochlorite), 20% H_2O_2 for 10 min, 15 min, 30 min, respectively. They were rinsed three times with sterile distilled water and dried up with sterile paper. The seeds were sown on MS medium (*Murashige & Skoog*, 1962) without hormones, solidified with 2g/L PHYTAGEL (Sigma), pH 5.8 and germinated in thermostatic box at 32 °C for two days, then transferred to the tissue culture room with a temperature of 25C and 16h photoperiod provided by cool white fluorescent lamps. Cotyledon explants from 4-day-old seedlings grown under these conditions were used for co-cultivation experiments.

Agrobacteria. *Agrobacterium tumefaciens* strain LBA 4404 was used, which contained binary vector PRG (Nagy et al). The plasmid carried GUS gene and BAR gene, a transferable selectable marker for herbicide resistance. *Agrobacterium tumefaciens* was grown and maintained on AB medium (*Chilton* et al, 1984).

Co-cultivation. Four-day-old cotyledons were excised, all around the edges were cut away, and cut into four segments. Then, these explants were soaked in *Agrobacterium tumefaciens* suspension for 20–60 minute and blotted dry with sterile paper to remove excess bacteria. Finally, they were placed onto solid medium containing MS salts and vitamins supplemented with 0.9 mg/L IAA, 0.6 mg/L BA and 0.24 mg/L ABA (pH 5.0). Explants were incubated in a growth room at 25°C with a photoperiod of 16 hour light for 3, 4, 5 days, respectively.

Regeneration and selection. After co-cultivation, explants were rinsed in water and than washed in 500 mg/L Claforan solution (HUMAN) or 100–200 mg/L Carbenacillian for 10–30 minutes, respectively, and then put into

sterile distilled water to remove the rest of excess bacteria on the surface. Finally, cotyledonary segments were blotted dry with sterile paper. In order to induce shoot formation, explants were transferred onto MS medium plus 0.9, 1.05 mg/L IAA, 0.6 mg/L BA and 0.24 mg/L ABA (pH 5.8). To obtain the optimum selective concentration of Finale, explants were kept on medium containing 1–10 mg/L Finale during shoot formation. To prevent the growth of Agrobacteria 500 mg/L Claforan was added to medium. In addition, control shoots were exposed to the medium containing Finale. As soon as shoots were formed, explants were transferred to the medium containing to enhance shoots development. Then, shoots were cut from the explants and were transferred to rooting medium (MS medium plus 0.3 mg/L IBA) to get roots. Cotyledon explants without Agrobacterium infection and their shoots were used as control during the experiments in order to confirm the effect of the selective medium and the genetic transformation.

Results

Disinfection methods. It was observed that the seeds surface-disinfected in 15% Chlorox for 15 minutes exhibited 85% normal seedlings without infection. In addition, seedlings grew better, giving rise to robust cotyledons.

Cotyledon age. In preliminary experiments, it was demonstrated that the stage of cotyledon development influenced the shoot formation. When being half-open, cotyledons cut as explants produced a higher percentage of shoots, 80% more than other forms on average *(Table 1)*. Explants from too young or fully opened cotyledons gave very few shoots. The time required to find the stage of half-opening of cotyledons varied with he conditions, temperature and photoperiod. In general, seeds were kept in thermostatic box in dark with a temperature of 32 °C for 1.5 days and then in culture room with a temperature of 25 C at 16h photoperiod for 2 days. In this case, obtained cotyledons were easy to meet the requirements.

Table 1 Cotyledon size effects on shoots formation

	Number of Explants	Number of Shoots	Number of Calli
No-opening	90	3	84
Half-opening	90	17	87
Full-opening	90	1	85

Agrobacterium concentration and soaking time. It was shown that the concentration of *Agrobacterium tumefaciens* was important. Using an overnight bacterial culture, culturing the bacteria in two petri dishes (8 cm in diameter) on solid medium, and harvesting the bacterium in 30ml sterile distilled water, the shoot formation was not effected. Whereas increasing the concentration of Agrobacterium for infection, by culturing in two large petri dishes (12 cm in diameter), shoot formation was strongly inhibited. Soaking time also has an effect on cotyledon explants. It was found that all explants were killed when they were soaked in *Agrobacterium* for one hour. However, 20 minutes soaking were effective for shoot formation, resulting in more shoots *(Table 2)*.

Co-cultivation period and Claforan concentration. Results showed that the control of the growth of Agrobacterium on the induction medium was affected by the previous co-cultivation period. Three day co-cultivation resulted the higher percentage of shoot formation. It was difficult to eliminate the bacteria after five day co-cultivation. As a result, the growing amount of Agrobacterium killed most of the treated explants.

Table 2 Effects of Agrobacterium concentration and inoculation time on callus and shoot formation

	Number of explants	Number of shoots	Number of callus
Two 8 cm petri dishes	280	21	271
Two 12 cm p. dishes	280	2	157
20 minute inoculation	280	24	277
60 minute inoculation	270	0	32

To certain extent, there were little differences with respect to concentrations of Carbenicillin and washing time. To determine the concentration of Claforan needed to eliminate the bacterium, explants were grown on medium containing increasing concentrations of Claforan. It was found that the Claforan at 300mg/L concentration or below could not eliminate the bacterium, for more than 40% of explants were still infected. The concentration as high as 700 mg/L inhibited shoot formation, while the amount of Agrobacterium was not too much (less than 25% of explants were infected) and upward of 1000 mg/L killed 30% of cotyledon explants while only less than 5% of the explants were still infected. Therefore, after several preliminary experiments, 500 mg/L Claforan was considered as the useful concentration for eliminating the bacterium *(Table 3)*.

Table 3 Effects of Claforan on Agrobacterium and explants

Concentration of Claforan	Number of explants	Agrobacterium amount(15 days)	Number of calli	Number of shoots
100 mg/L	280	much	12	0
200 mg/L	280	much	11	0
300 mg/L	280	much	21	0
500 mg/L	280	not too much	267	21
700 mg/L	280	not too much	269	5
1000 mg/L	280	a little	62	0

Finale concentration. It depended on the Finale concentration to select transgenic shoots. It was shown that too high concentration of Finale (more than 3 mg/L) had an effect not only on shoot formation, but also killed cotyledon explants. Too low concentration, like 1 mg/L lacked the capability to select transgenic shoots. Furthermore, experiments indicated that explants grew better and produced more shoots under the condition of 1 mg/L Finale, and yet did not die in 3 mg/L Finale *(Table 4)*.

Table 4 Effect of Finale on explants (31 days after infection)

Concentration of Finale	Number of explants	Number of dead explants	Number of explants with callus	Number of explants with buds
1 mg/L	30	3	25	11
2 mg/L	30	8	13	6
3 mg/L	30	25	0	0
4 mg/L	30	30	0	0
5 mg/L	30	30	0	0
8 mg/L	30	30	0	0
10 mg/L	30	30	0	0
0 mg/L	30	4	24	9

Table 5 Effects of hormone regimes on shoot and callus formation

	Number of explants	Explants with callus	Explants with shoots
0.9 mg/L IAA +0.6 mg/L BA+ 0.24mg/L ABA	280	265	0
1.0 mg/L IAA + 0.6 mg/L BA+ 0.24 mg/L ABA	280	261	15
1.05 mg/L IAA+0.6 mg/L BA+ 0.24 mg/L ABA	280	263	27
1.0 mg/L TDZ + 0.26 mg/L ABA+ 1.5 mg/L IAA	50	46	0
2.4 mg/L IAA + 5.0 mg/L BA	50	48	0

Hormone regime. There were significant differences in regeneration among hormone regimes *(Table 5)*. Only a kind of hormone regime ranked best in inducing shoot formation. The co-cultivation contained 0.9 mg/L IAA, 0.6 mg/L BA, 0.24 mg/L ABA. Then, there was a change in IAA concentration in the regeneration medium, whereas other hormones kept unchanged. In the first medium IAA was 1.0 mg/L, and in second medium IAA was 1.05 mg/L. It was demonstrated that such hormone regime was used to obtain shoots from at best 15% cotyledon explants. The number of shoots obtained per explants ranged from 1 to 10. In general, the explants on which buds appeared could have potential to produce shoots. In rooting medium, which did not contain any other hormone but 0.3 mg/L IBA, callus were at first formed on the basis of shoots, and then roots were obtained from the callus. The forming of roots took about three weeks.

Figure 1 Shoots formed from cotyledonary segments after infection with Agrobacterium tumefaciens on regeneration medium containing Finale (green)

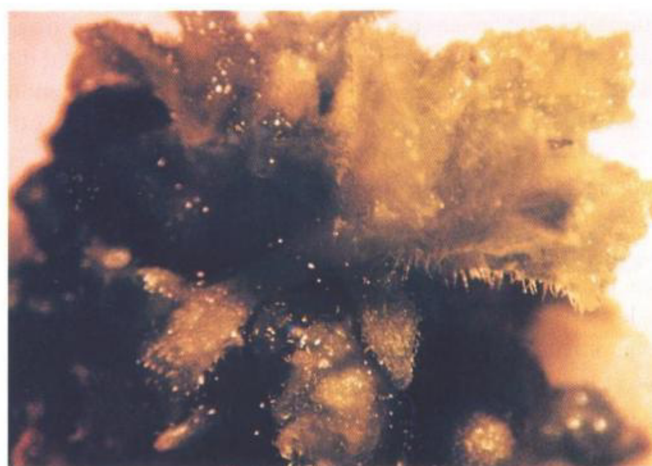

Figure 2 Shoots formed from cotyledonary segments without infection with Agrobacterium tumefaciens on regeneration medium containing Finale (yellow)

Discussion

We have developed a regeneration protocol, which, when combined with the use of Agrobacterium, allows relatively high frequency of transformed plants, by means of experiments with the parameters affecting Agrobacterium-mediated transformation in muskmelon. The protocol was developed using HBS, a generally used muskmelon variety, from which transformed plants have already been obtained, however few succesfull transformation with this or other methods have been reported. The successful transformation, to some extent, is dependent on the fact that during co-cultivation, plasmids are capable of entering the aimed plant cells with ease. One method to improve the transformation efficiency is to change the pH of the medium. The co-cultivation medium was made to pH 5.0, thus resulting in the fact that a large amount of Agrobacterium enter the cotyledon explants as compared to the co-cultivation medium with pH 5.8, because pH 5.0 was not suitable to the growth of Agrobacterium. It was observed that there was but little amount Agrobacterium on the interface between cotyledon explants and medium. It has also been found that after co-cultivation the elimination of the bacterium is a key to successful regeneration. When Agrobacterium grow quickly, there is a competition for nutrients and hormones between bacterium and cotyledon explants, finally Agrobacterium are killing the cotyledon explants. Currently, Claforan or Carbencillian was used to control the growth of Agrobacterium. However, if the antibiotic concentration is too high, it will affect the cotyledon growth, even killing the explants. Because of this, succesfull regeneration procedures of muskmelon *(Bársony* et. al., 1999) might be ineffective when using high concentration of antibiotics in the medium. In principle, the antibiotic concentration as low as possible

is used. To increase the transformation efficiency a low pH medium was used during co-cultivation. We found it is necessary to transfer cotyledon explants to another medium containing Claforan after three-day co-cultivation, because with longer periods it was not possible to eliminate the bacterium completely using a low concentration of Claforan. This result is consistent with that found by *Guowei Fang* et. al., (1990). Even though, we also found that due to effects of light and temperature on Claforan, its function period was on average about two weeks. Therefore, we changed the medium and transferred the explants every two weeks. This is an effective way to control Agrobacterium. On the basis of the experiments, 500 mg/L Claforan was used. This concentration is different from that used by *Guowei Fang* et. al (1990).

Regeneration is a comprehensive result caused by multiple factors, among which hormones played the most important role. As known, growth and reproduction in plants are regulated by hormones inside the plants. In theory, when the ratio IAA/BA is 1, calli are produced, when IAA/BA is more than 1, buds are produced, when IAA/BA is less than 1, roots are produced (Michael et. al., 1993). However, in practice, it was complicated to determine the IAA/BA ratio. Account must be taken into varieties and other conditions, such as growth status and effects of Agrobacterium. It may be due to the fact that changes in concentrations of hormones made it easy for the different positions of cells to hold different concentrations of hormones. As a result, the cell received a stimulation signal, leading to polarity. With the differentiation of the cell, small buds were observed under the microscope. Gradually, some buds developed into shoots. In this study, there was a change in IAA concentration from 0.9 mg/L to 1.05 mg/L. This conclusion is in agreement with the theory of physiology, but further research will be needed.

Successful transformation also required the optimum concentration of selective agents. In our system, Finale was used to select transformed tissue for BAR gene. It was found that cells at different stages possessed different tolerance to Finale. Finale had a detrimental effect on regeneration. Young infected cells were more sensitive than mature ones. On the basis of our experiments, the shoots formed from infected cotyledons with Agrobacterium died under the condition of 3mg/L Finale. Therephore, we used Finale step by step from lower concentration to higher concentration, that is, from 1 mg/L to 3 mg/L. On the other hand, it will be suggested that a better selective agent should be substituted for Finale, which allows us to expose culture to constant selective pressure. GUS gene expression have been confirmed by staining of the transformed tissues. In any case, authentic transgenic plants will have to be identified by Southern blot.

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