MELON REGENERATION AND TRANSFORMATION USING AN APPLE ACC OXIDASE ANTISENSE GENE

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ABSTRACT

The efficiency of melon regeneration protocols using cotyledon and leaf melon explants was evaluated in terms of number of regenerated plants, ploidy and transformation level. Young leaf explants were found to regenerate at a higher rate of diploidy, and they were selected as a recipient tissue for transformation. ***Agrobacterium tumefaciens*** *gene transfer methods showed a good efficiency of transformation around 3%. A construction containing an antisense ACC oxidase gene, named pAP4, the last enzyme of the ethylene biosynthetic pathway, was used for transforming melon leaves. In the transgenic plants, the pAP4 gene was detected and the fruits showed a low ethylene production.*

Key words: ***Cucumis melo*** *L., genetic transformation, climacteric fruits.*

RESUMO

REGENERAÇÃO E TRANSFORMAÇÃO DE MELOEIRO, COM UM CLONE DA ACC OXIDASE DE MAÇÃ EM ORIENTAÇÃO "ANTISENSE". Estudou-se a eficiência de protocolos de regeneração a partir de cotilédones e de folhas de meloeiro, avaliando-se a taxa de regeneração, poliploidia e transformação. A regeneração a partir de folhas jovens permitiu a obtenção de altas taxas de plantas diplóides, constituindo-se no explante selecionado para a transformação genética. O método de transfomação por ***Agrobacterium tumefaciens*** *proporcionou boa eficiência de transformação, em torno de 3%. Uma construção contendo o gene da ACC oxidase "antisense", denominado pAP4, última enzima da via de biossíntese do etileno, foi usada para a transformação dos meloeiros. Nas plantas tranformadas, detectou-se a presença do gene "antisense" e os frutos apresentaram baixos níveis de etileno.*

Palavras-chave: ***Cucumis melo*** *L., transformação genética, frutos climatéricos.*

INTRODUCTION

Melons (*Cucumis melo* Naud), cv. Gaúcho, exhibit good sensory attributes but have a poor keeping quality post-harvest, limiting their commercial acceptance.

The plant hormone ethylene regulates the ripening of climacteric fruit and therefore represents an obvious target for the controlling of climacteric fruit ripening by genetic manipulation. Strong inhibition of the ripening process has been achieved in transgenic tomato by incorporation of silencing genes coding for two enzymes of the ethylene biosynthetic pathway, ACC oxidase and ACC synthase (HAMILTON *et al.*, 1990; OELLER *et al.*, 1991; AYUB *et al.*, 1996; PETERS *et al.*, 1999).

Genetic plant manipulation needs, as a preliminary step, the calibration of regeneration and transformation protocols (GABA *et al.*, 1992; CLOUGH & HAAM, 1995; AYUB *et al.*, 1996; PETERS *et al.*, 1999).

The transformation of the melon was achieved by FANG & GOURMET (1990) who reported the first transgenic plants regenerated from cotyledons explants by *Agrobacterium* inoculation. Subsequently, several transgenic melon plants have been obtained from cotyledon explants following an *Agrobacterium* method or a particle bombardment transformation procedure (GABA *et al.*, 1992; CLOUGH & HAAM, 1995; AYUB *et al.*, 1996; PETERS *et al.*, 1999). All these authors have reported a high frequency of tetraploids among regenerated plants. However, tetraploid fruits are not marketable due to their characteristics of a smaller size, flat shape and easy cracking (NUGENT & RAY, 1992). Generation of spontaneous tetraploids from a diploid genotype was found to be a universal phenomenon in melon tissue culture (EZURA *et al.*, 1992) and they can be considered a limiting factor for further development of genetic manipulation for the melon species. Several protocols have been published on melon regeneration either through somatic embryogenesis (BRANCHARD E CHATEAU, 1988) or organogenisis (DIRKS & VAN BUGGENNUM., 1989; FASSIOLOTIS & NELSON, 1992; FICADENTI & ROTINO, 1995), but the number of tetraploid plants recovered was not always documented. The first objective of this study is to select a simple and efficient regeneration system leading to the lowest number of tetraploid plants. This protocol would then be combined with an *Agrobacterium* gene transfer of an antisense ACC oxidase gene (pAP4), the last enzyme of the ethylene biosynthetic pathway. Diploid regenerated plants exhibiting fruits expressing the transgene were recovered after *Agrobacterium* infection.

MATERIAL AND METHODS

Plant Material

Seeds of *Cucumis melo* Naud., cv Gaúcho, were used. Before disinfecting, seed coats were removed and soaked in sterile water for 2 hours. Seeds were than washed in a solution of 70% ethanol for 2 minutes and rinsed in sterile distilled water for 5 min. After that, seeds were disinfected in a solution of 2,5% calcium hipocloride containing 0,2% Tween-20 for 20 min and subsequently washed three times in sterile distilled water.

Regeneration

Cotyledon organogenesis was induced following the DIRKS and VAN BUGGENUM (1989) method on explants from two days old germinated seeds. The protocol described by KATHAL *et al.* (1988) was used with minor modifications for leaf organogenesis. To obtain leaf explants, 10 seeds were germinated per Magenta box containing 10 mL of MS medium (MURASHIG & SKOOG, 1962) supplied with 1% sucrose and solidified with 0,7% agar. The pH of all media was adjusted to

5.7 before autoclaving at 120°C for 25 min. The first leaf with an average diameter of 0,6 cm was excised from eight day-old germinated seedlings. Leaf explants, each one corresponding to a half of transversally cut leaf, were placed on organogenic induction medium composed of MS medium supplemented with Benzyl- Amino-Purine (BAP) and phosphate inosine (2iP) at 1 μM, 3% sucrose and solidified with 0,7% agar. Shoots were excised from the explants and transferred to a development medium containing MS, 1 μM BAP, 3% sucrose and solidified with 0,7% agar. Giberelic Acid (GA_3), sterilized by filtration, was added at the concentration of 0,19 μM to the autoclaved medium. After 3 weeks, elongated shoots were separated and transferred to the rooting medium (same medium as above without growth regulators). Rooted plantlets were rinsed free of medium and transplanted to pots containing a mix of vermiculita and sterile soil, and incubated for a month at 25°C under a light flux of 100 $\mu moL.m^{-2}.s^{-1}$ and 16 h of photoperiod. Plants were transferred to the greenhouse and trained in trellis with standard cultural practices for fertilization and pesticide treatment. Flowers were self-pollinated and 4 fruits were kept on each plant.

Ploidy estimation

The ploidy level was determined following exactly the methodology described by AYUB *et al.* (1996).

Melon transformation

For the transformation disarmed *Agrobacterium tumefaciens* LBA4404 containing the binary vector pGA643 (AN *et al.,* 1988) with a NPTII chimeric gene, a selectable marker for kanamycin resistance, and a CaMV35S promoter were used. The antisense cDNA pAP4 was inserted into t-DNA region encoding for an apple fruit ACC oxidase (PETERS *et al.*, 1999).

Young non-expanded leaves were prepared as described above and immersed in an *Agrobacterium* suspension culture (OD_{600}=0,4 to 0,6) for 20 min under gentle agitation. Explant leaves were then blotted on Whatman filter paper and allowed to dry out for 10 min and placed for three days on cocultivation in induction medium. After this period, explants were transferred to selective medium. Consisting of induction medium supplemented with 100 $mg.L^{-1}$ kanamycin 500 $mg.L^{-1}$ carbenicilin for explants transformed with *Agrobacterium*. Selection was also performed in development medium using 150 $mg.L^{-1}$ kanamycin and in rooting medium where the concentration of kanamycin was lowered to 100 $mg.L^{-1}$.

Polymerase chain reaction (PCR) analysis

Genomic DNA from young leaves of regenerated plants was extracted following the method of Doyle & Doyle (1990).

For PCR analysis, two primers derived from *pAP4as* were used:

5' – GCT TGA AAG TGA CTC GTA GCA – 3'

5' – TAG CTA GAT TAG ATT CGC ATT A – 3'

The endogenous sequence corresponding to melon ACC oxidase was used as a positive control (AYUB *et al.*, 1996).

5' – AAG GAT CCG CAC AAA CCA AAT CTT GTA C - 3'

5' – AAG GAT CCT AAG CTG AAA GTG AAT TTA AAT TA - 3'

PCR amplifications were performed on 100 ng of total melon DNA with a robocycler Progene R-150 using the following conditions: 95°C for 30 sec, annealing at 70°C for 1 min and DNA synthesis at 72°C for 30 sec, 36 cycles and a final step of 72°C for 5 min. Results were viewed by agarose gel electrophoresis containing 0,7% agarose.

Ethylene measurements

Internal ethylene concentration of fruits was monitored using an external gas collection apparatus (SALTVEIT, 1993). Gas samples were taken every early morning through a serum stopper and analyzed by chromatography as described by SILVA (2000).

RESULTS AND DISCUSSION

Effects of explant on regeneration and poliploidy

Explants were tested with special attention to the regeneration rate and to the number of tetraploid plants recovered (Table 1). A high regeneration frequency (80%) was obtained with cotyledons from two days old seedlings on a MS medium supplemented with 5 μM BAP. Adventious shoots preferentially located along the cut basal edge of the explant were clearly visible after one week of culture. Non expanded leaves, incubated on organogenic induction medium regenerated 50%. Under these conditions leaf explants showed an overall expansion, about a two-fold increase, within the first days of culture. After four weeks, shoots appeared directly at the junction of the lamina and petiole. In two explants, plantlets could be obtained after three months. Cotyledon organogenesis reached the highest regeneration rate producing two to three plantlets per explant, while from leaf organogenesis, the average was one to two plants per explant. However, the most important difference was observed in the number of undesirable tetraploids regenerated from the two explants. Organogenesis from two day old cotyledons have recovered 75% of tetraploid plantlets, whereas only 15% was recovered from leaf explants. For this reason the leaves were selected for further genetic transformation experiments. These results were in accordance with those of COLIJN-HOOYMANS *et al.* (1994), in cucumber, where the percentage of tetraploid cells increased during the development of the cotyledon. Also, in *Arabidopsis*, multiploidy in cotyledon explants have been observed while leaf endoduplication occurred at very low frequency (AKAMA *et al.,* 1992; GALBRAITH *et al.*, 1991). Using older leaves as an explant, YADAV *et al.* (1996) reported 40% of tetraploid regenerants.

TABLE 1 - Ploidy levels of *in vitro* regenerated melon plants by organogenesis from cotyledon and leaf explants[1]

Explants	Regeneration (%)[2]	Diploids (%)[2]	Tetraploids (%)[2]
Cotyledon	82 a	25 b	75 a
Leaf	50 b	85 a	15 b

[1]Average from 150 explants.

[2]Data with commom letter are not statistically different using Duncan test at P=0,05.

Evaluation of transgenic plants

The use of a selection medium containing 150 $mg.L^{-1}$ kanamycin lead to very few plants able to root on kanamycin containing medium (1 rooted plant to 100 explants) in the control treatment. In contrast, twelve rooted plantlets were recovered after *Agrobacterium* transformation. Nine of these plantlets were diploid (Table 2).

TABLE 2 - Leaf transformation by *Agrobacterium tumefaciens* containing the pAP4 antisense gene

Total explants treated	Regenerants	Plants rooted on kanamycin Containing medium	Diploids	Plants PCR positives	Transformation frequence (%)
100	52	12	9	3	3

Genomic DNA of the putative transformed plants were subjected to an analysis by PCR using two pairs of primers, one to identify the pAP4 antisense gene and the second one to detect the endogenous ACC oxidase gene. Only three plants presented a PCR amplification band corresponding to pAP4 antisense. No pAP4 antisense amplification was observed in control plants (Figure 1). However, the endogenous sequence was amplified in all treatments. The amplification from pAP4 plasmid show that the endogenous ACC oxidase gene is present only in melon plants and the size of pAP4 melon plants corresponds exactly to pAP4 plasmid.

The 3% frequency of transgenic plants per initial number of explants placed in culture is consistent with the usual melon transformation frequency that ranged from 1% (GABA *et al.*, 1992) to 7% (FANG & GOURMET, 1990).

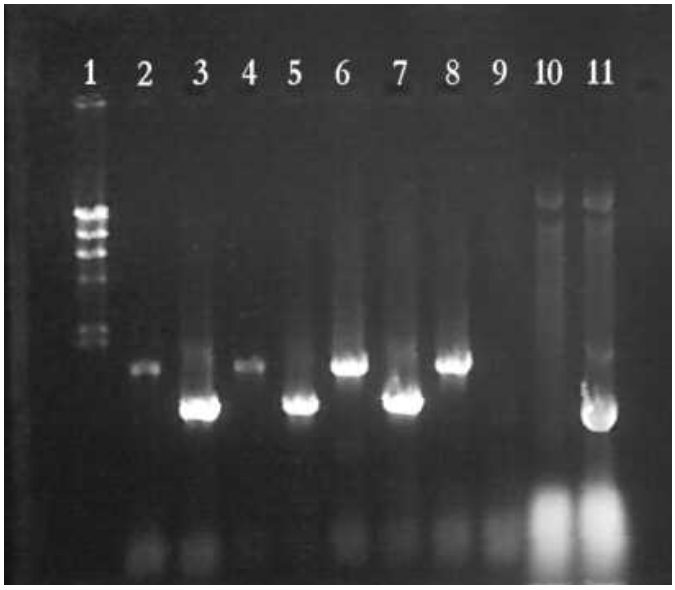

Figure 1 - PCR analysis of melon genomic DNA (lines 2 to 9) to detect the presence of pAP4 antisense gene (lines 3,5, 7 and 9) and endogenous ACC oxidase gene (lines 2, 4, 6 and 8) in transformed (lines 2 to 7) and non transformed plants (lines 8 and 9). Lines 10 and 11 correspond to PCR analysis of pAP4 antisense gene using endogenous ACC oxidase primers (line 10) and pAP4 antisense primers (line 11). Line 1 corresponds to molecular markers.

Transformed plants had normal growth and phenotypic appearance. They presented fertile flowers and set fruits that developed normally. In the control plants the melon ripening is characterized by an increase in ethylene concentration that can reach 6,25 ppm at its maximum. However, in transgenic plants there was a strong reduction of ethylene production, reaching maximum of 0,54 ppm (Table 3). In addition, the seeds obtained from these fruits kept the kanamycin resistance when germinated on MS medium containing 150 $mg.L^{-1}$ kanamycin (data not showed). Melons which are expressing a high ethylene inhibition will be further evaluated in terms of quality attributes and shelf life improvements.

TABLE 3 - Ethylene concentration on transformed and non transformed melon fruits[1]

Line	Ethylene concentration (ppm)
Control	6,25a[2]
AS1[3]	0,34b
AS2	0,31b
AS3	0,08c

[1]Average from 40 fruits in climacteric stage.
[2]Data with common letter are not statistically different using Duncan test at P=0.05.
AS1, AS2,AS3: Lines of antisense melons.

CONCLUSION

The melon regeneration using young leaf melons as an explant is efficient in terms of regenerated plants, ploidy and transformation level. Transformed plants with pAP4 clone have normal growth and phenotypic appearance. The ethylene production is strongly reduced in transgenic fruits.

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