

# A reliable system for the transformation of cantaloupe charentais melon (*Cucumis melo* L. var. *cantalupensis*) leading to a majority of diploid regenerants

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## Abstract

An efficient system of transformation leading to a majority of transformed diploid plants from leaf explants of *Cucumis melo* L. var. *Cantalupensis* (cv. Védrantais) was developed. Several regeneration protocols using cotyledon or leaf explants were analysed with particular emphasis on the regeneration efficiency and the ploidy level of the regenerated melon plants. The use of leaf explants excised from 10 day-old seedlings, cultured in Murashige and Skoog's medium supplemented with 1  $\mu\text{M}$  6-benzylaminopurine (BAP) and 1  $\mu\text{M}$  6-( $\gamma,\gamma$ -dimethylallylamino)-purine (2iP), resulted in a high regeneration frequency (73%). In these conditions, more than 84% of the regenerated plants were found to be diploid. Addition of an *Agrobacterium*-mediated transformation step did not significantly change the percentage (81.8%) of diploid plants regenerated. This protocol was successfully used to produce diploid transgenic melon plants expressing the antisense ACC oxidase gene, encoding ACC oxidase which catalyses the last step of ethylene biosynthesis. Ethylene production and ACC oxidase activity of the leaf explants from transgenic plants was reduced by more than 80% as compared to the control untransformed tissues. This transformation/regeneration method could be routinely used for the introduction of other genes of interest in melon.

*Keywords:* *Cucumis melo* L.; *Agrobacterium tumefaciens*; Genetic transformation; Ploidy level

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## 1. Introduction

The development of efficient transformation methods is important for the successful application of biotechnological techniques for crop improvement. Over the last few years, a variety of *Agrobacterium*-mediated and particle bombardment transformation procedures have been described, leading to regeneration of transgenic melon plants (see Guis et al., 1998 for review). However, a high proportion of transgenic melon plants were found to be tetraploid (Fang and Grumet, 1990; Dong et al., 1991; Gonsalves et al., 1994; Valles and Lasa, 1994; Ayub et al., 1996). Phenotype alterations with respect to diploid plants were important. They included short internodes and reduced productivity due to low fertility (Nugent and Ray, 1992). Moreover, tetraploid fruits are not marketable due to their smaller size, flatness and propensity to crack (Nugent and Ray, 1992). The spontaneous generation of tetraploid plants from diploid genotypes was found to occur universally in melon tissue culture and can be considered as a factor limiting the further development of the genetic manipulation of this species (Ezura et al., 1992). This phenomenon is not restricted to melon and has also been observed among other cucurbitaceae species such as cucumber (Colijn-Hooymans et al., 1994).

The purpose of the present paper was firstly to develop a simple and efficient regeneration system, allowing the production at high frequency of diploid plants of the cantaloupe charentais melon (*Cucumis melo* L., var. *catalupensis*, cv. Védraçais), a genotype frequently used in breeding programs. Then, we demonstrated that the method was amenable to *Agrobacterium*-mediated transformation using an ACC oxidase gene, encoding the last enzyme of the ethylene biosynthesis pathway, in the antisense orientation.

## 2. Material and methods

### 2.1. Plant culture

Mature seeds of *Cucumis melo* L., cv. Védraçais, were obtained from Tezier (France), sterilized as described by Guis et al. (1997) and germinated in culture tubes on hormone-free half-strength Murashige and Skoog's medium (Murashige and Skoog, 1962). In the experiments reported here, all the constituents were added to the medium and the pH adjusted to 5.7 before autoclaving at 120°C for 20 min. Cultures were maintained at  $25 \pm 1^\circ\text{C}$ , under a 16 h light/8 h dark cycle with a light flux of  $100 \text{ mol m}^{-2} \text{ s}^{-1}$  (OSRAM L36W/36 Nature tubes).

### 2.2. Plant regeneration

Somatic embryos were obtained from cotyledon explants cultured using the induction medium and growth conditions described by Guis et al. (1997). Shoot

formation was induced from cotyledon explants excised from 2 day-old germinated seeds following the method of Dirks and van Buggenum (1989). Regeneration of plants from leaf explants was obtained using the conditions described by Kathal et al. (1988) with slight modifications. Briefly, leaves (lamina with small petiole) of 0.3–0.5 cm were excised from 10 day-old aseptic seedlings. The leaves were transversally cut into two parts after having removed the petiole and some of the basal part. Leaf explants were then incubated on Murashige and Skoog's medium (Murashige and Skoog, 1962) supplemented with 1  $\mu\text{M}$  6-benzylaminopurine (BAP) and 1  $\mu\text{M}$  6-( $\gamma,\gamma$ -dimethylallylamino)-purine (2iP) and solidified by 0.7 g l<sup>-1</sup> agar. After 3 weeks of culture, shoots formed from the basal part of the leaf explants were excised and incubated on development medium (DM) consisting of MS medium supplemented with 1  $\mu\text{M}$  BAP and 0.2  $\mu\text{M}$  gibberelic acid (GA3) and 0.8 g l<sup>-1</sup> agar. They were then placed in the rooting medium consisting of MS medium without growth regulator. As soon as roots appeared, the plantlets were acclimatized according to Guis et al. (1997), and transferred to the greenhouse following the cultural practice described by Ayub et al. (1996).

### 2.3. *Agrobacterium*-mediated transformation

The disarmed strain of *Agrobacterium tumefaciens*, LBA4404, was used for the genetic transformation of the melon leaf explants. The binary vector, pGA643 (Fig. 1A), contained a NOS/NPTII chimeric gene, a selectable marker for kanamycin resistance and a CaMV35S/antisense cDNA encoding melon ACC oxidase (*MEL1*) (Ayub et al., 1996). Young, non-expanded leaf explants were incubated in a gently shaken *A. tumefaciens* suspension (10<sup>6</sup>–10<sup>8</sup> cells/ml) for 30 min. The explants were then blotted on Whatman n<sup>o</sup>1 paper for 10 min, placed abaxially on the culture medium and incubated at 27°C for 3 days. Then, the explants were transferred to the regeneration medium, supplemented with 100 mg l<sup>-1</sup> kanamycin and 500 mg l<sup>-1</sup> carbenicillin.

### 2.4. Southern blot analysis

Genomic DNA from young leaves of in vitro regenerated plants was extracted following the method of Doyle and Doyle (1990). For Southern blot analysis, 20  $\mu\text{g}$  of genomic DNA, digested with HindIII, was separated by electrophoresis in a 0.8% agarose gel and transferred to Gene screen membranes (Dupont). The subsequent hybridizations were based on the protocol of Sambrook et al. (1989) using a <sup>32</sup>P-labeled NPTII coding sequence (Ready to Go, Pharmacia) as a probe.

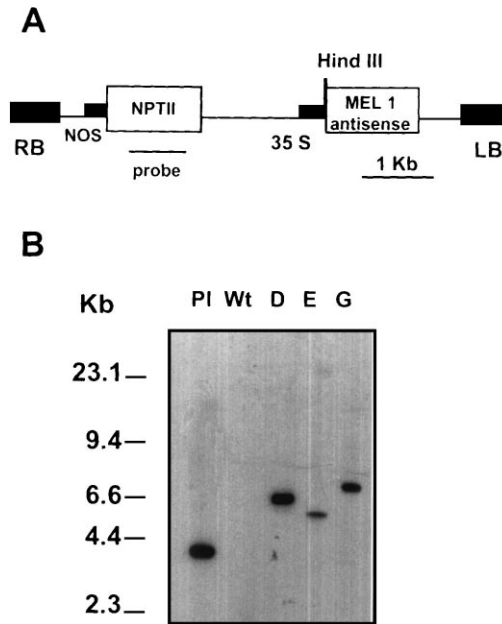


Fig. 1. Molecular characterization of transformed melon plants. (A) Partial restriction map of the T-DNA from pGA643-MEL1 antisense. The pMEL1 cDNA was inserted in reverse orientation between the CAMV 35S promoter and the T7-5 terminator. The T-DNA contains the NPTII gene under the control of the nopaline synthase (NOS) promoter and terminator. (B) Southern blot analysis of transgenic melon plants. Total genomic DNA (20  $\mu$ g) digested with HindIII and hybridized with a  $^{32}$ P-labeled probe specific for the coding region of the NPTII gene. *Lane 1*: digested pGA643-MEL1 antisense; *Lane 2*: digested DNA from a wild-type melon; *Lanes 3–5*: digested DNA from antisense lines D, E, and G, respectively.

### 2.5. Ploidy estimation

The ploidy was determined by counting the number of chloroplasts in ten stomatal guard cells of two leaves, excised from each acclimatized plant, according to the method described by Fassiolutis and Nelson (1992) for muskmelon genotypes. The ploidy level was confirmed by flow cytometry. Crude samples of nuclei were prepared from leaves of acclimatized plants according to the method of Galbraith et al. (1991). The DNA content of the isolated nuclei was analysed with a flow cytometer (Cell Analyser CAII, Partec), calibrated using the 2C peak from nuclei of young leaves of diploid plants derived from seed. The ploidy levels of acclimatized plants, obtained using each of the regeneration systems, were estimated by the two methods. For statistical analysis, data were transformed using an arcsine transformation and compared using Newman–Keuls test at  $P = 0.05$ . A high correlation ( $r = 0.98$ ,  $P < 0.01$ ) between the two methods was observed in our experimental conditions.

## 2.6. Ethylene measurements

Ethylene measurements were performed on five leaf disks (1 cm<sup>2</sup>) transferred to a 25 ml flask, capped with a rubber septum, containing 5 ml of regeneration medium. After a 4 h incubation period at 25°C, a 1 ml gas sample of head space was withdrawn with a hypodermic syringe and the ethylene content determined by gas chromatography as described by Ayub et al. (1996).

## 2.7. ACC oxidase activity *in vivo*

The assay of ACC oxidase in the leaf tissues was based on the conversion of exogenous ACC to ethylene. Leaf disks (1 cm<sup>2</sup>) were excised from 10 day-old WT and AS plants and incubated in the presence of 250 µM ACC as previously described (Ben Amor et al., 1998). Ethylene produced was then measured by gas chromatography.

# 3. Results and discussion

## 3.1. Regeneration of melon plants

A number of explants and regeneration media were tested with special attention being paid to the regeneration rate and to the ploidy of the regenerated melon plants (Table 1). The percentages of regeneration obtained via shoot organogenesis from cotyledon (84.6%) or leaf (72%) explants were found greater than somatic embryogenesis frequency from cotyledon explants (42%). However, the production of acclimatized melon plants seems to be independent of the

Table 1

Ploidy levels of *in vitro* regenerated melon plants produced via shoot organogenesis or somatic embryogenesis from cotyledon or leaf explants<sup>a</sup>

Explants	System of regeneration	Regeneration (%)	Acclimatized plants per explant	Diploids (%)	Tetraploids (%)
Cotyledon <sup>b</sup>	Organogenesis	84.6a	2.0 ± 0.9a	18.6c	81.4a
Cotyledon <sup>c</sup>	Embryogenesis	42.0c	1.6 ± 0.5a	71.3b	28.7b
Leaf <sup>d</sup>	Organogenesis	72.0b	2.1 ± 0.7a	84.6a	15.4c

<sup>a</sup> Values within columns followed by different letters are significantly different by Newman-Keuls test at  $P = 0.05$ .

<sup>b</sup> Cotyledons excised from 2 day-old seedlings grown *in vitro*.

<sup>c</sup> Cotyledons excised from quiescent seeds.

<sup>d</sup> Leaves excised from 10 day-old seedlings grown *in vitro*.

regeneration methods, since the number of acclimatized plants per explant was not significantly different for the three regeneration systems. In contrast, the ploidy of the regenerated plants was greatly affected by the regeneration method (Table 1). Indeed, more than 80% of the regenerated plants were found to be diploid when shoot formation was induced from leaf explants, whereas only 15% were diploid when the regeneration occurred from 2 day-old cotyledon explants. Our data are consistent with those obtained by Kathal et al. (1992) which show that the majority of leaf-derived regenerants were diploid. More recently, Yadav et al. (1996) described a highly efficient system for shoot regeneration of melon plants from well developed leaves (3–4 cm) excised from greenhouse-grown plants, but more than 40% of the regenerated plants were found to be tetraploid.

These results show that the nature of the explant and the stage of development of the tissues greatly affect the ploidy of the regenerated melon plants. Flow cytometry performed on the explant tissues suggest that 90% of cells from mature cotyledons and nearly 100% of young leaf cells were  $2n$  while more than 80% of cells from 2 day-old germinated cotyledons were  $4n$ . These data agree with the observations of Colijn-Hooymans et al. (1994) and Gilissen et al. (1993) who found that, in the cucumber, the percentage of tetraploid cells increased during cotyledon development. This phenomenon has also been described in *Arabidopsis*, where endoduplication of the cotyledon cells is frequently observed, leading to polyploidy, while the endoduplication of leaf cells occurred at low frequency (Galbraith et al., 1991). These data indicate that the ploidy level of regenerated melon plants can be correlated to the ploidy level of the explant source.

In our conditions, the regeneration system, based on shoot formation from very young leaf explants, excised from in vitro-grown plants, allowed the production of a majority of diploid melon plants. This system was selected for further genetic transformation studies.

### 3.2. Melon transformation and analysis of the transgenic plants

Leaf explants were cocultured with *A. tumefaciens* strain LBA4404 containing the vector pGA643 harboring the antisense ACC oxidase gene placed under the control of the constitutive 35S promoter. Addition of the *Agrobacterium*-mediated transformation step did not significantly affect the level of regeneration or the percentage of diploid regenerated plants (Table 2). In the presence of  $100 \text{ mg l}^{-1}$  kanamycin, more than 80% of the putatively transformed regenerated plants, were found to be diploid. Furthermore, the frequency of transgenic plants generated per explant (2.4%) was consistent with previously reported melon transformation frequencies ranging from 1% (Gaba et al., 1992) to 7% (Fang and Grumet, 1990).

Table 2

Influence of an *Agrobacterium*-mediated transformation step on the regeneration frequency and ploidy level of melon plants recovered from leaf explants

Treatment	Regeneration (%)	Acclimatized plants per explant	Diploids (%)
Control	65.3	2.3 ± 0.9	83.4
<i>Agrobacterium</i> transformation			
Kanamycin 0 mg/l	64.8	2.1 ± 0.2	84.3
Kanamycin 100 mg/l	61.9	0.2 ± 0.01	81.8

Three putative primary transformants, selected on the basis of the presence of the NPTII gene assessed by a polymerase chain reaction (PCR)-based method were analysed by Southern blot, using a probe generated from the NPTII coding sequence (Fig. 1B). Genomic DNA digested with HindIII, which cuts only once within the T-DNA but not in the transgene, released only one fragment from transformed plants but not from untransformed plants. This data indicates that only one copy of the T-DNA has been incorporated into the genome of the regenerated melon plants.

Expression of the transgene was assessed by measuring ethylene production and ACC oxidase activity by leaf explants of wild-type (WT) and antisense ACC oxidase (AS) melon plants. Ethylene production by leaf explants from AS plants was always lower than in WT tissues (Table 3). The capacity of the explants from AS melon line G to produce ethylene was reduced by more than 70%. The low level of ethylene production by tissues of AS plants can be attributed to a substantial reduction in the ACC oxidase activity (Table 3). This enzyme controls one of the rate-limiting reactions in the biosynthesis of ethylene, as previously described in transgenic tomato plants (Hamilton et al., 1990).

In conclusion, the method developed in this paper for the transformation/regeneration of melon plants allows the production of a high frequency of diploid transgenic melon plants. Since biotechnological strategies are now used in melon

Table 3

Ethylene production and ACC oxidase activity of leaves from wild type (WT) and antisense ACC oxidase (AS) melons<sup>a</sup>

Genotypes	Ethylene production (nl g <sup>-1</sup> h <sup>-1</sup> )	ACC oxidase activity (nl g <sup>-1</sup> h <sup>-1</sup> )
WT	25.7 ± 2.1	77.1 ± 5.2
AS line D	15.1 ± 1.9	45.3 ± 3.7
AS line E	16.2 ± 1.7	50.7 ± 5.6
AS line G	7.3 ± 0.9	22.2 ± 3.9

<sup>a</sup> Values represent the mean ± SE of three replicates.

breeding programs and since transformation is regarded as a difficult problem, the *Agrobacterium*-mediated transformation procedure described here could be used for the production of transgenic melon plants from *C. melo* genotypes.

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