African Journal of Biotechnology Vol. 6 (18), pp. 2162-2165, 19 September 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Agrobacterium mediated transformation of Tunisian *Cucumis melo* cv. maazoun

RHIMI Awatef[1], HERNOULD Michel[2] and BOUSSAID Mohamed[1]

[1]Department of Biology, Laboratory of Plant Biotechnology, National Institute of Applied Science and Technology (INSAT), Centre Urbain Nord, B.P. 6761080, Tunis Cedex, Tunisia.
[2]Laboratory of Cellular Biology and Plant Biotechnology, INRA - IBVM - UMR 619 CRA de Bordeaux 71, Avenue Edouard Bourleaux, B.P. 8133883 Villenave d'Ornon Cedex, France.

Accepted 23 February, 2007

Transgenic *Cucumis melo* cv. Maazoun containing the neomycin phosphotransferase II (NPT II) chimeric gene conferring resistance to kanamycin were obtained from cotyledons explants inoculated with *Agrobacterium tumefaciens* (GV3101) that contained the binary vector plasmid pADI. Transformed shoots were obtained on Murashige and Skoog medium supplemented with 1.50 mgl^{-1} IAA, 0.10mgl^{-1} BAP, 0.01 mgl^{-1} NAA and 6 mgl^{-1} kinetin. Transformants were selected by using only 100 mgl^{-1} of kanamycin and 4 days of pre-culture. Putative transformants were confirmed for transgene insertion through polymerase chain reaction (PCR) analysis. From the inoculated explants, 6.66% produced transgenic shoots.

Key words: *Cucumis melo*, genetic transformation, kanamycin, culture medium, PCR analysis.

INTRODUCTION

In Tunisia, *Cucumis melo* is cultured over an area of 9629.39 hectares with a production of 140645 tons per year (DG/PDIA, 2004). However cultures are subject to several viral (e.g. Cucumber Mosaic Virus, CMV) and fungal diseases (e.g. *Sphaerotheca fuliginea*, *Fusarium oxysporum* var. Melonis) that severely limit yield and for which adequate levels of native resistance are not available (Trionfetti et al., 2002; Kuzuya et al., 2003; Vengadesan et al., 2005). Genes resistance against these pathogens exists essentially in wild species (Pitrat and Risser, 1992). However, their introduction via conventional breeding techniques (artificial hybridizations) is very low, expensive and limited because of the interspecific and intergeneric reproductive barriers (Debeaujon and Branchard, 1992; Guis et al., 2000). Plant biotechnology strategies, including genetic transformation could help overcome these problems.

Melon genetic transformation has been achieved for the first time by Fang and Gourmet (1990). A few several procedures for *C. melo* transformation have been established (Vallès and Lasa, 1994; Dias et al., 2002; Akasaka et al., 2004) for genes encoding for kanamycin resistance (Chee, 1990; Sarmento et al., 1992) and coat protein gene conferring virus resistance. Subsequently, transgenic melon plants with useful traits such as long shelf-life of fruit (Chee and Slightom, 1991; Ayub et al., 1996; Vengadesan et al., 2005) and halo-tolerance (Bordas et al., 1997) have been produced. However, melon genetic transformation using *Agrobacterium* strains is at present limited to few varieties. The restriction of the use of this technique was mainly due to the low number of plant regenerated and the high frequency of chimeric plants (Ray et al., 1993; Simandjuntak et al., 1996).

Beji (charentais) and Maazoun (Eastern type) *C. melo* cultivars are widely cultivated in Tunisia and very appreciated by the consumer (Jebberi, 1988). However, the two cultivars are subject to climatic stresses and pathogens. Conventional breeding methods, using wild rustic melon species in order to transfer desirable agronomic genes into the cultivar, are limited because of the intra and interspecific barriers. The present work aims to opti-

*Corresponding author: E-mail: mohamed.boussaid@insat.rnu.tn. Phone: (+216)71703829 / 929. Fax: (+216)71704329.

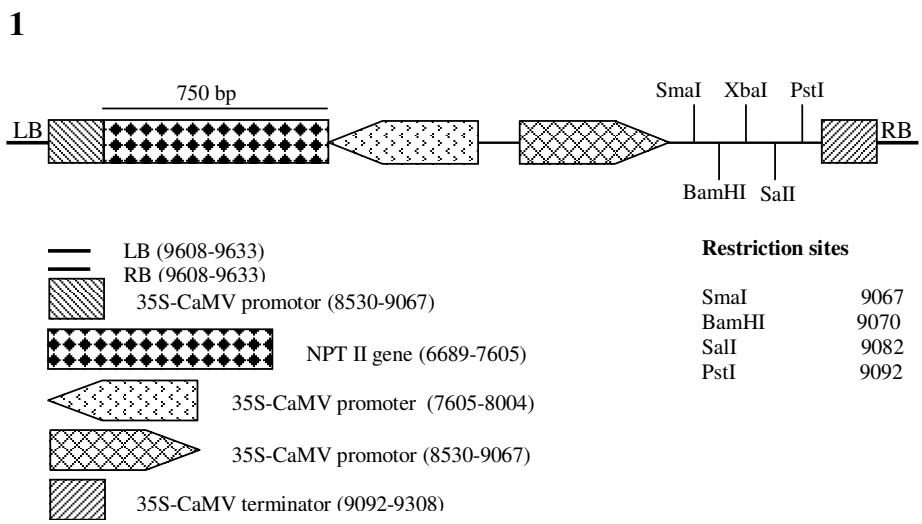

Figure 1. Schematic representation of the T-DNA binary plasmid pADI. This plasmid carries the NPT II gene, conferring resistance to kanamycin, under the control of the 35S-CaMV promoter and 35S-CaMV terminator.

mize a genetic transformation system permitting introduction of desirable genes into *C. melo* cv. Maazoun.

MATERIAL AND METHODS

Plant material

Mature seeds of Maazoun cultivar were provided by INRAT (1998). Seeds were surface sterilized with 5% calcium hypochlorite for 10 min and rinsed three times with sterile distilled water. Seeds were aseptically cultured in Petri dishes (8 x 8 x 2 cm) containing 25 ml of MS medium (Murashige and Skoog, 1962) solidified with 0.8% agar. Cotyledons were taken from ten days old seedlings. They were cut perpendicularly to the longitudinal axis into fragments (7/5 mm). 1 mm wide along the edges was removed.

Media and *in vitro* culture conditions

Explant organogenesis potentialities were tested on the MS basal medium solidified with 0.7% agar and added with NAA (0.01 mgl^{-1}), IAA (1.5 mgl^{-1}), BAP (0.10mgl^{-1}) and kinetin (6 mgl^{-1}). The pH of medium was adjusted to 5.8 before autoclaving at 100 KPa for 20 min. Explants were placed horizontally in Petri dishes containing each 25 ml of medium. Cultures were incubated at 22 ± 1°C under 16 h photoperiod (cool white fluorescent lamps, 5 $Mm^{-2}s^{-1}$). For each experiment, 30 explants were used.

Callus induction medium consisted of MS basal medium supplemented with 1.5 mgl^{-1} IAA and 6 mgl^{-1} kinetin. In the regeneration medium the above growth regulators were replaced by 0.01 mgl^{-1} NAA and 0.1 mgl^{-1} BAP.

Bacteria strain and plasmid vector

Genetic transformation was performed using the *Agrobacterium* strain GV3101 containing the binary plasmid pADI (Figure 1) that carries a neomycin phosphotransferase II (NPT II) gene under the control of CaMV promoter (Hernould et al., Unpublished).

Agrobacterium preparation

A single colony of *Agrobacterium tumefaciens* was inoculated into 40 ml of Lauria-Bertrani medium (bacto-tryptone 10 gl^{-1}, Bacto-yeast extract 5 gl^{-1}, NaCl 10 gl^{-1}) containing 25 mgl^{-1} rifampicine, 10 mgl^{-1} chloramphenicol, 100 mgl^{-1} spectinomycine and 50 mgl^{-1} kanamycin. After 12 h, 40 µl of the above culture were transferred to 40 ml of LB medium, pH 7.2, containing the same antibiotic concentrations. This culture was incubated overnight at 250 rpm on a rotary shaker at 28°C. The 0.8 $OD_{600\ nm}$ culture was pelleted at 3000 rpm at 4°C for 10 min. The pellet was re-suspended in 40 ml MS medium (pH 5.8) supplemented with 200 µM acetosyringone.

Infection, co-cultivation and selection

After four days of pre-culture, explants were inoculated with an overnight culture of *Agrobacterium* diluted to $OD_{600\ nm}$ = 0.8 (10^7 - 10^8 bacteria ml^{-1}) for 20 min. Infected explants were blotted dry using sterile Whatman paper to remove excess bacteria then co-cultivated for three days at 28°C in the dark on callus induction medium. Bacteria were then removed by washing with liquid MS medium containing 300 mgl^{-1} Claventin (Duchhefa, Nederland), explants were transferred onto selective callus induction medium containing 100 mgl^{-1} kanamycin and 300 mgl^{-1} Claventin, and subcultured every week. After four weeks of incubation, green kanamycin resistant calluses were transferred to the regeneration medium containing 100 mgl^{-1} kanamycin and 300 mgl^{-1} Claventin. Resistant organogenic calluses were obtained through several subcultures under the same selective pressure. Putative transgenic plants were then tested using PCR analysis.

DNA preparation and polymerase chain reaction (PCR) analysis

Polymerase chain reaction was used to detect specific DNA sequences of the NPT II gene transferred. Genomic DNA was isolated from putative transformed callus (0.30 g) using a tampon of extraction Tris HCl, pH 8 (0.2 M Tris; 0.025 M Na_2EDTA; 0.5 % (p/v) SDS; 0.25 M NaCl). Polymerase chain reaction of NPT II gene

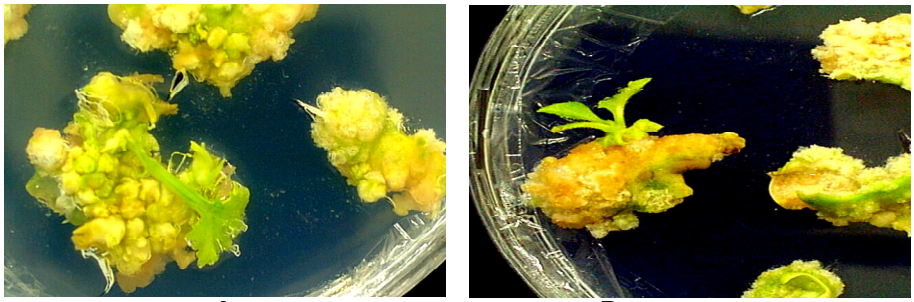

Figure 2. A- Maazoun cotyledon organogenic callus on selection medium. B- Transgenic shoot regenerated on selection medium (cv. Maazoun).

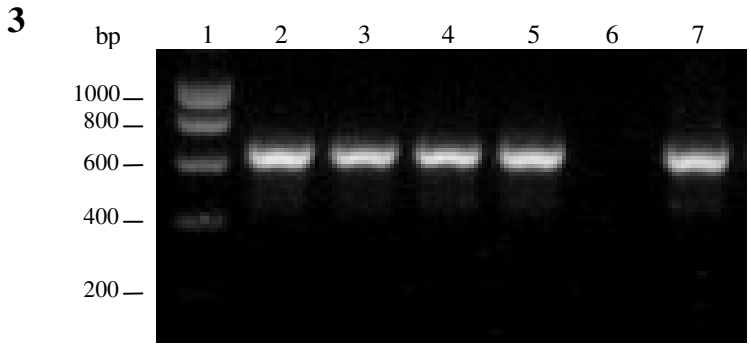

Figure 3. PCR analysis of melon genomic DNA to detect the presence of NPT II gene in transformed (lanes 4, 5 and 7) and non transformed plants (lane 6). Lanes 2 and 3 correspond to PCR analysis of pADI NPT II gene. Lane 1 corresponds to molecular markers.

was performed. The PCR reaction containing 100 ng of total melon DNA was incubated in a final volume of 100 µl with 0.08U μl^{-1} Taq polymerase, 0.50 pmol μl^{-1} for each primer and 200 µM de dNTP. Reaction cycles (35 cycles) consisted of 30 s at 94°C for denaturation, 30 s at 52°C for annealing and 30 s at 72°C for extension. The forward and reverse primers for NPT II gene were 5'-CTTGGGTGGAGAGGCTAT-3' and 5'-AGAACTCGTCAAGAAGGC-3', respectively, amplifying a fragment of 750 bp. PCR products were analysed by 1.5% agarose gel electrophoresis. DNA was visualized under UV and photographed.

RESULTS

Calli formation started after a week for all explants (97%) from the proximal end. Calli were smooth, compact and greenish. Shoots began to differentiate after thirteen days. The induction of adventitious buds reached 79% on MS added with 0.01 mgl^{-1} NAA combined with 0.1 mgl^{-1}BAP.

Selection and regeneration of transgenic plants

Cotyledon explants co-cultivated with bacteria were washed with liquid MS medium containing 300 mgl^{-1} Claventin, blotted on sterile Whatman paper and then placed on the selection medium solidified with 0.7% agar. The agro-infected explants developed green organogenic calluses on selective medium containing 100 mgl^{-1} kanamycin and 300 mgl^{-1} Claventin (23.33%). After four weeks several green calluses regenerated shoots when transferred to MS medium containing 0.01 mgl^{-1} NAA, 0.10 mgl^{-1} BAP, 100 mgl^{-1} kanamycin and 300 mgl^{-1} Claventin. Cotyledon explants formed transgenic shoots, but transformation frequency was low 6.66% (Figures 2A and B).

PCR analysis

Gene transfer mediated by *Agrobacterium* inoculation to cotyledonary explants was tried by transient NPT II expression. The integration of the transgene into the plant genome was confirmed by PCR analysis. 650 bp corresponding to the amplified fragment of NPT II gene was detected (Figure 3). The amplified products were observed in all the organogenic callus tested, confirming the presence of transgenes and non-calli escapes.

DISCUSSION

The genetic transformation via *A. tumefaciens* is commonly used for the genes transfer to the vegetable cells. Many varieties transformed for agronomic genes were obtained. However, the efficiency of this technique can be influenced by many factors including the species or the variety to be transformed, the bacteria used, the explant and the presence of inductive virulence genes substances, particularly the acetosyringone (Wenck et al, 1999; Galperin et al., 2003). The success of the genetic trans-formation requires combination of a transformation vector and a regeneration technique. This combination is coupled with efficient techniques of selection and confirmation of the transformation.

In this paper, we have shown that the potentiality of *C. melo* for genetic transformation depends on regeneration. The melon regeneration using cotyledon as an explant is efficient in terms of regenerated plants and transformation level. To confirm the integration of the foreign DNA into the melon genome, PCR amplification of the NPT II gene was conducted and yielded the correct size of the band (650 bp) corresponding to the NPT II gene in the vectors. Genetic manipulations for disease and pest resistance, as well as abiotic factors require the use of rapid and non-species specific transformation and regeneration system. The use of appropriate constructs, after screening and biosafety concerns have been conducted which allow production constraints of nematodes, fungi, bacteria and virus infection in a sustainable and environmentally friendly manner. It may also be possible to incorporate other characteristics such as drought tolerance, thus increasing the area that can support the growth of melon and contributing significantly to food security and poverty alleviation.

ACKNOWLEDGEMENTS

The authors thank the Tunisian Ministry of Higher Education and Scientific Research and the National Institute of Applied Science and Technology for financial support (Research Grant 99/ UR/09.10).

REFERENCES

Akasaka KY, Tomita KO, Ezura H (2004). Efficient plant regeneration and *Agrobacterium*-mediated transformation via somatic embryogenesis in melon (*Cucumis melo* L.). Plant Sci. 166: 763-769.

Ayub R, Guis M, Ben Amor M, Gillot L, Roustan JP, Latché A, Bouzayen M, Pech JC (1996). Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nat. Biotech. 14: 862-866.

Bordas M, Montesinos C, Dabauza M, Salvador A, Riog A, Serrano R, Moreno V (1997). Transfer of the yeast salt tolerance gene HAL1 to *Cucumis melo* L. cultivars and *in vitro* evaluation of salt tolerance. Transgenic Res. 6: 41-50.

Chee PP (1990). Transformation of *Cucumis sativus* tissue by *Agrobacterium tumefaciens* and the regeneration of transformed plants. Plant Cell Rep. 9: 245-258

Chee PP, Slightom JL (1991). Transfer and expression of cucumber mosaic virus coat protein gene in the genome of *Cucumis sativus*. J. Am. Soc. Hortic. Sci. 116: 1098-1102.

Debeaujon I, Branchard M (1992). Induction of somatic embryogenesis and caulogenesis from cotyledon and leaf protoplast – derived colonies of melon (*Cucumis melo* L.). Plant Cell Rep. 12: 37-40.

DG/PDIA, Ministère de l'Agriculture Tunisienne (2004). Résultats de l'enquête sur le suivi de la campagne agricole 2004/2005 - Direction Générale de la Production Agricole Ministère de l'Agriculture 2004 – 2005.

Dias R de CS, Pico B, Herraiz J, Espinos A, Nuez F (2002). Modifying root structure of cultivated muskmelon to improve vine decline resistance. Hortic. Sci. 37: 1092-1097.

Fang G, Gourmet R (1990). *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. Plant Cell Rep. 9: 160-164.

Galperin M, Patlis L, Ovadia D, Wolf D, Zelcer A, Kenigsbuch D (2003). A melon genotype with superior competence for regeneration and transformation. Plant Breed. 122: 66-69.

Guis M, Ben Amor M, Latché A, Pech JC, Roustan P (2000). A reliable system for the transformation of cantaloupe charentais melon (*Cucumis melo* L. var. cantalupensis) leading to a majority of diploid regenerants. Sci. Hortic. 84: 91-99.

Jebberi H (1988). Le melon. Documents techniques. Ann. Inst. Rech. Agro. Tunisie 104: 36.

Kuzuya M, Hosoya K, Yashiro K, Toita K, Ezura H (2003). Powdery mildew (*Sphaerotheca fuliginea*) resistance in melon is selectable at the haploid level. J. Exp. Bot. 54: 1069-1074.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio-assays with tobacco tissue culture. J. Plant Physiol. 15: 473-497.

Pitrat M, Risser G (1992). Le melon. In: Gallais A, Bannerot H Ed: Amélioration des espèces végétales cultivées (objectif et critères de sélection). INRA. Paris, p. 762.

Ray DG, Mcolley DW, Michael EC (1993). High frequency somatic embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. J. Am. Soc. Hortic. Sci. 118: 425-432.

Sarmento GG, Alpert K, Tang FA, Punja ZK (1992). Factors influencing *Agrobacterium* mediated transformation. Plant Cell Tiss. Org. Cult. 31: 185-193.

Simandjuntak V, Barrett DM, Wrolstad RE (1996). Cultivar and maturity effects on Muskmelon (*Cucumis melo* L.) colour, texture and cell wall polysaccharide composition. J. Sci. Food Agric. 71: 282-290.

Trionfetti NP, Colla G, Granati E, Temperini O, Crino P, Saccardo F (2002). Rootstock resistance to fusarium wilt and effect on fruit yield and quality of two muskmelon cultivars. Sci. Hort. 93: 281-288.

Vallès Mi, Lasa JM (1994). *Agrobacterium* - mediated transformation of commercial melon (*Cucumis melo* L.). Plant Cell Rep. 13: 145-148.

Vengadesan G, Prem Anand R, Selvaraj N, Perl-Treves R, Ganapathi A (2005). Transfer and expression of npt II and bar genes in cucumber (*Cucumis sativus* L.). *In Vitro cell.* Dev. Biol. Plant 41: 17-21.

Wenck RA, Quin M, Whetten WR, Pullman G, Sederoff R (1999). High-effeciency *Agrobacterium* mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). Plant Mol. Biol. 39: 407-416.