Agrobacterium mediated transformation of *cucumis melo* with replicase gene from papaya ringspot virus and regeneration of transformed palnts

N.S. Kottearachchi[1], S.Kertbundit and M.Juricek
Centre for Molecular Genetics and Genetic Engineering, Institute of Science and Technology for Research and Development, Mahidol Univercity, Salaya Campus, Nakornpathom, Thailand.

ABSTRACT

Transgenic muskmelon *(Cucumis melo)* plants were produced efficiently by inoculating cotyledon explants with the *Agrobacterium tumefaciens* strain EHA 101 bearing a Ti binary vector. The T-DNA region of this vector includes chimeric genes for neomycin phosphotransferase II (NPT II) and β-Glucuronidase (GUS). Additionally the plant expressible replicase gene (NIb) of papaya ringspot virus (PRV) was flanked by the NPT II and GUS genes. After co-cultivation for three days, explants were transferred to muskmelon regeneration medium with kanamycin for the selection of transformed tissue. Shoot regeneration occurred within 3-5 weeks after culturing. Detection of GUS activity in 5 plant lines verified the transformation of the T-DNA unit and two of them were further analyzed by PCR amplification for the presence of the replicase gene. These results show that the *Agrobacterium* mediated gene transfer method and regeneration *via* tissue culture are effective methods for the transfer of foreign genes into *Cucumis melo*.

INTRODUCTION

One of the most effective means of gene transfer into dicotyledonous plants has been to utilize the natural transformation mechanism of *Agrobacterium tumefaciens*. It is well known that genes located within the border sequence of the *Agrobacterium* Ti plasmid are inserted into the genome of the host. Utilization of this mechanism for gene transfer requires both susceptibility to infection by *Agrobacterium tumefaciens* and the ability to regenerate plants from individual transformed cells *via* tissue culture.

The cucurbit family includes many high value vegetable and fruit crops (cucumber, melon, squashes). Cucurbits are predominantly infected by three poty viruses, papaya ringspot virus (PRV), watermelon mosaic virus II (WMV II) and Zucchini yellow mosaic virus (ZYMV) (Ling *et al.* 1991). Papaya ringspot virus is classified into two strains which can only be distinguished by host range. Papaya ringspot virus type P infects papaya and has limited host range within the family *Cucurbitaceae*. Papaya ringspot type W infects only cucurbits, but not papaya, causes severe losses of economically important cucurbit crops and is referred to as water melon mosaic virus I (Yeh *et al.* 1992).

Genetically engineered plants resistant to virus diseases have been obtained by transformation with wild type or modified viral genes encoding proteins. This approach was first demonstrated by Powell *et al.* (1986) by expression of a viral coat protein gene. Transgenic resistant plants with viral replicase gene also have been reported for many viruses (Golemboski *et al.* 1990, Brederrde *et al.* 1995, Suzuki *et al.* 1995). Effective protection against these viruses, using one of their coat proteins or nonstructural gene sequences such as a replicase gene would make it much easier to develop cucurbits resistant to these viruses. Therefore, we previously engineered a binary vector bearing a replicase gene of PRV type P, named pSA1039 and transformed it into *Agrobacterium tumefaciens* for future use. As an effort for the production of virus resistant plants we transformed *Cucumis melo* with the replicase gene of papaya ringspot virus using *Agrobacterium* mediated gene transfer technique. This paper describes a successful approach to obtain transformed *Cucumis melo* plants and their regeneration through tissue culture. Moreover this well characterized system can later be used to transfer other useful traits or engineered genes as they become available.

MATERIALS AND METHODS

This study was conducted at the Plant Molecular Laboratory of the Institute of Science and Technology for Research and Development, Mahidol University, Thailand.
Mature seeds of *Cucumis melo* (variety-Thai

[1]Present address: Faculty of Agriculture and Plantation Management, Wayamba University, Makandura, Gonavila, Sri Lanka.

Jumbo) which were extracted under aseptic conditions were soaked in sterile distilled water until the seeds absorbed enough water and settled to the bottom of the bottle. Seeds were then sterilized with 25% Clorox (5.25% sodium hyporchlorite) and a drop of TritonX-100 for 30 min with shaking at 250 rpm. This step was repeated for another 30 min with new Clorox solution. Seeds were then rinsed 4 times with sterile distilled water and placed on a Petri dish with sterile moist tissue paper. The seeds were germinated under 8 hr photoperiod conditions provided by white fluorescent lamps.

The *Agrobacterium* strain carrying PRV-NIb-binary vector (pSA1039) was grown overnight in 5ml of LB (Luria-Bertani medium) broth. 4-5 day old germinated cotyledons were cut on all edges into pieces of approximately 5 mm^2 size and then soaked for 30 min in the fresh overnight culture of *Agrobacterium*. The excised cotyledons were blotted dry to remove excess bacteria and placed on co-cultivation medium supplemented with MS salts and MS vitamins (Murashige and Skoog 1962), 3%(w/v) sucrose, 1mgl^{-1} Benzylaminopurine (BAP) and 0.8% agar. After 3 days of growth in the dark at 27^{0}C, the *Agrobacterium* infected melon cotyledon pieces were transferred to Petri plates containing the selective medium which was made with the same co-cultivation medium in addition to 500 mgl^{-1} cefotoxime (to eliminate bacterial carry-over) and 150 or 75 mgl^{-1} kanamycin (to select transformed tissues). To test the regeneration ability of the medium, untransformed cotyledons were maintained on non-selective medium which was supplemented with the same components as the selective medium except kanamycin. Also, as a negative control, untransformed melon tissues were tested for sensitivity to kanamycin using selective regeneration medium. After 7-8 weeks of growth, developing shoots were transferred to hormone free root inducing medium containing MS salts and vitamins (Murashige and Skoog 1962), 3%(w/v) sucrose, 500mgl^{-1} cefotoxime and 50mgl^{-1} kanamycin.

When the transformed plants grew to about 5cm in height, a fresh leaf lamina was cut into pieces and about 20-30 mg of tissue was incubated with a solution of 5-bromo-4-chloro-3-indol-â -D-glucuronide (X-Gluc) in 200mM Sodium phosphate buffer, pH 7.0 (Jefferson 1987) and incubated at 37^{0}C overnight. Stained tissues were assayed as described by Fitch *et al*. (1992).

Genomic DNA was extracted from young leaf tissue from 4 month old putative transformed plants using the procedure described by Edwards *et.al*. (1991). About 100ng of extracted DNA was subjected to amplification by PCR (polymerase chain reaction) without any further purification in a mixture of 2mM MgCl$_2$, 0.2mM dNTP, 0.6iM primers (designed for replicase gene of PRV) and 5 units of *Amplitaq* polymerase (Perkin Elmer). Amplification was performed in a thermal cycler (Gene amplification system 2400, Perkin Elmer) programmed for 40 cycles of 20 seconds at 95°C, 55°C for 25 sec, 73°C for 2 min.

RESULTS

In the positive control experiment, the explants not inoculated with *Agrobacterium*, were cultured on non selective medium and started shoot regeneration within 2-3 weeks. By the end of fourth week shoot initiation had occurred in 80% of those explants. However none of the explant transformed by *Agrobacterium* grew on selective medium supplemented with 150mgl^{-1} kanamycin. The medium was then modified according to Fung and Grumet (1990) by adding only 75mgl^{-1} kanamycin. Six explants were able to produce shoots (Figure 1). Two weeks after the appearance of shoot buds, shoots were removed and transferred to the root initiation medium. In the root initiation medium the explants were grown until leaves were well formed. Leaves from 6 plants were analyzed for the presence of T-DNA by GUS histochemical staining. Of these plants, only 5 plants produced blue colour. When the GUS positive plants were transferred to rooting medium supplemented with 50mgl^{-1} kanamycin, only two plants produced a good root system.
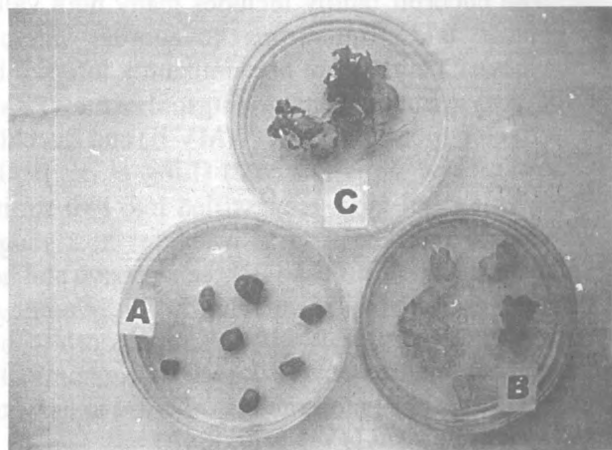

Fig. 1. Cotyledon explants of muskmelon cultured on selective medium with 75 mg l^{-1} kanamycin for four weeks. A: Uninoculated control explants, B: Explants inoculated with *A. Tumefaciens* EIIA 101 harboring pSA1039 plasmid, C: Control explants cultured on medium without kanamycin.

Due to contamination and stunted growth the other 3 plants did not produce any roots. Those well rooted plants were further confirmed for the presence of transgene by PCR amplification. In the transformed

plants (M1 and M5), an amplified band of the same size as Nlb gene was found which was 1.6 kb, while in the untransformed plants this could not be noticed (Figure 2).
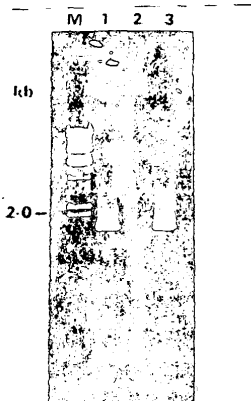

Fig.2. PCR analysis of genomic DNA from transgenic muskmelon plants. Lane M:λ/Hind III digested DNA marker. 1: PCR product obtained from genomic DNA of plant line M1 (transformed). 2:Negative control (untransformed genomic DNA of muskmelon). 3: PCR product obtained from genomic DNA of plant line M5 (transformed).

DISCUSSION

Although Gonsalves et al. (1994) have achieved shoot regeneration on selective medium supplemented with 150mgl^{-1} kanamycin, with 0.9% efficiency, we were not able to do so. According to Fang and Grumet (1990), the kanamycin level at or above 75mgl^{-1} completely inhibited the growth of control explants and therefore this level was chosen for the routine selection of transformed melon. They found that 20%-30% of the explants produced shoots and when they calculated from initial explants to independently derived transgenic plants, the efficiency of transformation and regeneration was 3-7%. Based on these results, the selective regeneration medium was modified by adding 75 mgl^{-1} kanamycin. On this modified medium, 6 explants out of 23 were able to regenerate shoots, thereby confirming the acceptability of this modified medium for transformation via Agrobacterium as observed by Fang and Grumet (1990). In the negative control experiment, even with the reduced level of kanamycin which was 75mgl^{-1} none of the explants were able to produce shoots and explants could not even expand and thicken. These data confirm that the untransformed melon tissues are not able to regenerate under 75mgl^{-1} kanamycin.

The Agrobacterium strain contained the Agrobacterium derived binary vector plasmid pSA1039, its T-DNA region contained a plant expressible bacterial derived neomycin phosphotransferase II (NPT II) gene which upon transfer, genome integration and expression in plant tissues, conferred resistance to the antibiotic

Kanamycin. Therefore the shoots derived from the medium containing 75mgl^{-1} kanamycin indicating that at least the NPT II gene contained within the T-DNA region of the binary vector pSA1039 had been transferred into Cucumis melo tissues by using the disarmed Agrobacterium strain.

β-Glucuronidase catalyses the hydrolysis of substrate 5-bromo-4-chloro-3-indol-β -D- Glucuronide (X - Gluc) and liberates a molecule of 5-bromo-4-chloro-indoxyl (XH) which produces blue colouration in the presence of oxygen. The T-DNA unit of the plasmid pSA1039 contains GUS gene and upon integration into a plant genome, it expresses the β - Glucuronidase enzyme which is easily detectable as reported by Jefferson (1987) in the presence of X-Gluc. β-glucuronidase is absent in most plant species, therefore it is a reliable technique for the confirmation of gene transformation. The intensity of blue colour production was different from plant to plant and it may be due to the factors that affect the integration of the T-DNA unit into the plant genome such as positional effects and the number of insertions.

PCR amplification was accomplished using oligonucleotide primers which were previously designed for the isolation of the Nlb gene of papaya ringspot virus. The expressible form of the Nlb gene was flanked by GUS and NPT II genes in the T-DNA unit of pSA1039 plasmid. The amplification was found to correspond to the exact size of the Nlb gene which was 1.6kb, in plants that were well rooted in 50mgl^{-1} kanamycin medium. All these data confirm the presence of the NPT II gene, GUS gene and Nlb gene of PRV, thereby suggesting the integration of T-DNA unit of the binary vector into the plant genome. Thus, like numerous other dicotyledonous plant species, Cucumis melo can be transformed with Agrobacterium tumefaciens and regenerated into phenotypically normal appearing plants that express foreign genes. Further experiments are now in progress to test whether the transgenic Cucumis melo plants produced from the replicase gene of PRV are resistant for the infections by poty viruses.

REFERENCES

Brederrde FT, Taschner PEM, Posthumus E and Bol JF 1995 Replicase mediated resistance to Alfalfa Mosaic Virus. Virology. 207: 467-474.

Edwards K, Johnstone C, and Thompson C 1991 A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucl. Acids. Res. 19:1349.

Fang G and Grumet R 1990 Agrobacter transformation and regeneration of musk melon

plants. Plant Cell Reports. 9:160-164.

Fitch MMM, Manshard MR, Gonsalves D, Slightom JL and Sanford JC 1992 Virus resistant papaya plants derived from tissues bombarded with coat protein gene of papaya ringspot virus. Biotechnology. 10;1466-1471.

Golemboski DB, Lomonossoff GP and Zaitlin M 1990 Plants transformed with a tobacco mosaic virus non structural gene sequence are resistant to the virus. Proc. Natl. Acad. Sci. USA. 87:6311-6315.

Gonsalves C, Xue B, Yepes M, Fuchs M, Ling K and Namba S 1994 Transferring cucumber mosaic virus-white leaf strain coat protein gene into *Cucumis melo* L. and evaluating transgenic plants for protection against infections. J. Amer. Soc. Hort.Sci. 119(2):345-355.

Jefferson RA 1987 Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Reporter. 5:387-405.

Ling K, Namba S, Gonsalves C, Slightom JL and Gonsalves D 1991 Protection against detrimental effects of poty virus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. Biotechnology. 9:752-758.

Murashige T and Skoog F 1962 A revised medium for rapid growth and Bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-498.

Powel P, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT and Beachy RN 1986 Delay of disease development in transgenic plants that express tobacco mosaic virus coat protein gene. Science. 232:738-743.

Suzuki M, Masutae C, Takanami Y and Kuwata S 1995 Resistance against Cucumber mosaic virus plants expressing the viral replicon. FEBS Letters. 379:26-30.

Yeh SD, Jan FJ, Chiang CH, Doong TJ, Chen MC, Chung PH and Bau HJ 1992 Complete nucleotide sequence and genetic organization of papaya ringspot virus RNA. Journal of General Virology. 73:2531-2541.