Research in Plant Sciences, 2013, Vol. 1, No. 1, 1-3
Available online at http://pubs.sciepub.com/plant/1/1/1

DOI:10.12691/plant-1-1-1

Transformation of Iranian Melon for Increasing Resistance to Fungal Diseases

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Received December 28, 2012; Revised January 19, 2013; Accepted February 28, 2013

Abstract Melon (*Cucumis melo* L.) is one of the most important horticultural crops that fungal diseases are one of factors limiting its production. So, resistant cultivars have particular importance. *Chitinase* and *β-Glucanase* are well-known enzymes for increasing plant resistance to fungal pathogens. Thereby, transformation of Khatooni cultivar as the most important melon in Iran was carried out. Gene constructs consisting of the *chitinase*, *β-1, 3 Glucanase*, a selector gene (ntpII) and cauliflower mosaic virus (CaMV) 35S promoter were transferred by *Agrobacterium*. Transgenic plants were regenerated from medium containing kanamycin (25mg/l) cefotaxime (300mg/l) and BAP (1mg/l). The presences of gene constructs were proved by PCR using specific primers. In this study, the efficacy of transgenic shoots in Khatooni cultivar was 2.7 percent.

Keywords: *gene transfer, pathogenic fungus, melon, chitinase, glucanase*

1. Introduction

Genetic transformation begins with the activation of the vir gene region, whereby *A. tumefaciens* is able to recognize the host plant. vir gene expression is triggered by phenols [14,15] and monosaccharides [16,17,18]. Melon is one of the major horticultural crops in tropical and subtropical regions. Iran with more than one million tons productions and about 85 thousand hectares of under cultivation area is the third largest producer country of melon in the world [1]. Yield and quality of the products affected by biological stress, including fungal diseases greatly decreased. Plant breeding for biological stress has its complexity; beside today transformation of resistance genes with known mechanism in many species has been done with success. However, genetic resources in melon cultivars for resistance to fungi in its gene pool are limited and in the other hand, traditional breeding methods and crosses efficiency between melon species is low; hence the use of gene transfer technology can effectively increase melon yield and quality. Although melon is susceptible to inoculate with *Agrobacterium* and have a good response for complete plant regeneration from leaf cotyledonary explants in tissue culture conditions, but the efficiency of transgenic plant production in most of the cases are still low (2-7%); so, development of a transgenic system in melons, for better production of transgenic crops is necessary [2,3,4]. In recent years, specific genes controlling resistance against the fungus has attracted the researcher interest. In this regard, until now some proteins and poly antifungal peptides have been isolated from different groups of organisms. Hydrolytic enzymes have effect on degradation of fungal cell wall and have directly antifungal activity, which increases plant resistance to fungal agents [5]

Among them *Chitinase* and *β-Glucanase* are very effective enzymes that distinctly are used in a wide range of fungal resistance in plants [6]. The genes producing these enzymes can be induced in plants and induce specific biochemical responses in the host plant, so the stability of plant against fungal agents attack will increase [7]. In this study, transformation of Persian melon cultivar Khatooni (*Cucumis melo* CV. Khatooni) as the most important commercial cultivars of Iran, by the gene construct pBI121-chi glu containing the coding genes of Chitinase and β-1, 3 glucanase, was investigate.

2. Materials and Methods

2.1. Plant material

Seeds of mature khatooni melon after being uncoated were disinfected with 70% ethanol for 30 seconds and then 5% sodium hypochlorite (NaOCl) for 20min. afterwards were washed three times with sterile distilled water and were placed on ½ MS (MS; Murushige and Skoog, 1962) medium containing 0.5mg/l benzyl amino purine (BAP) at 24℃ and 16 hours photoperiod for sprouting. Seven day cotyledonary leaves explants along with some sub-cotyledon parts within 0.5cm^2 were selected as explants.

2.2. Bacterial Strains and Growth Conditions

A single bacterial colony of *Agrobacterium tumefaciens* strain LBA4404 containing binary vector pBI121 containing *Chitinase* and β-*Glucanase* genes with NPTII (kanamycin resistance factor as selectors) in LB medium

containing 50mg/l kanamycin and 75mg/l reef ampicillin in dark conditions at 28 ℃ with 180rpm in a shaker incubator to achieve OD_{600} nm = 0.8 concentration was cultured. The bacterial suspensions after grown overnight, centrifuged, and were ready for inoculation.

2.3. Plant Transformation and Selection

Prepared explant were inoculate with bacteria suspensions for 30 second and placed on co-cultivate MS medium without antibiotics, containing one mg/l BAP for 48h in the dark with temperatures of 25 ℃ for effective transformation of T-DNA to leaf cotyledon explants. Then, explants were washed with distilled water and placed on MS selector medium containing one mg/l BAP, 25mg/l kanamycin and 300mg/l cefotaxime; for direct regeneration of shoots in a period of three to four weeks (kanamycin were used as operating selector and cefotaxime were used to control bacterial growth). Resistant explant to kanamycin that produced shoot were transferred to elongation medium containing one mg/l BAP, one mg/l GA_3, 50mg/l kanamycin and 300mg/l cefotaxime and were allowed to grow for two weeks. Separated selective shoots were transferred to rooting MS medium without antibiotics with one mg/l BAP. After two weeks, rooted plantlets were obtained.

2.4. Molecular Analysis of Transgenic Plants

For molecular analysis, samples of young leaves of transgenic and non-transgenic melon plants were powdered with liquid nitrogen and DNA contact was extracted using CTAB [8]. DNA fragments containing *Chitinase* and *β-Glucanase* genes were amplified by PCR (Techne, Burlington, NJ, USA) with specific primers (Figure 2). The PCR process included a 95 ℃ for five min followed by 30 cycles for 30s at 94 ℃, one min at 60 ℃ for annealing, one min at 72 ℃ for elongation and final extension at 72 ℃ for 5min. Anticipated PCR products size were about 680bp and 870bp for β-glucanase and chitinase genes respectively. The PCR products were electrophoresis with a one kilo base DNA ladder on 1% agarose gel.

3. Results

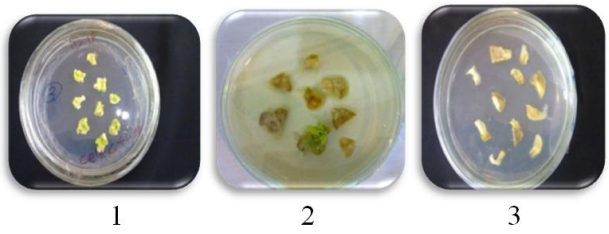

Figure 1. Transfer explants on selector medium: 1) inoculated explants with Agrobacterium 2) The transgenic explants left green and non-transgenic explants have been yellow in kanamycin medium 3) control explants after a while without regeneration became necrosis

In this study seven-day leaf cotyledonary explants were inoculate for 30 seconds with overnight suspensions of *Agrobacterium* containing pBI121 plasmid and *Chitinase and β-Glucanase* genes. Afterwards have been co-cultivated on MS medium for 48 h in dark conditions, then explants were transferred to selective medium (containing 25mg/l kanamycin and 300mg/l cefotaxime) (Figure 1).

12 regenerated shoots that showed resistance were transferred to elongation medium. Appropriate kanamycin was used as a selector plants that is in the range of 25-50mg/l for melon. At higher levels, even transgenic plants would die. In this medium, some undesirable transgenic explants that had remained alive got necrosis and died (Figure 2).

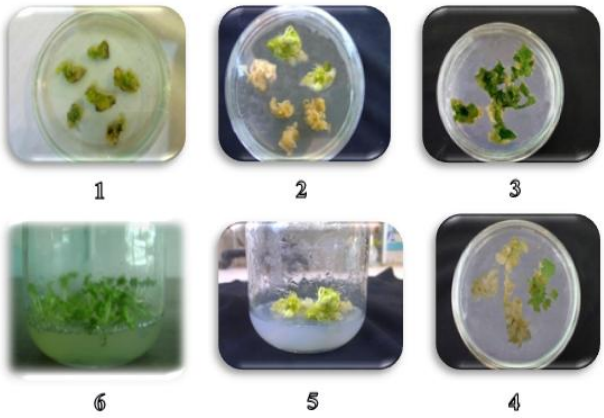

Figure 2. Direct regeneration of shoots from leaf cotyledonary explants on selective medium: 1) generate a new shoot buds of the inoculated explants 2) desirable transgenic seedlings remained green and Some unfavorable transgenic got yellow and lost 3) growth of resistant shoot in selective medium with more kanamycin (50mg/l) 4) remove of non-transgenic escaped shoot 5) growth of resistant shoots in elongation medium 6) kanamycin-resistant shoots elongation

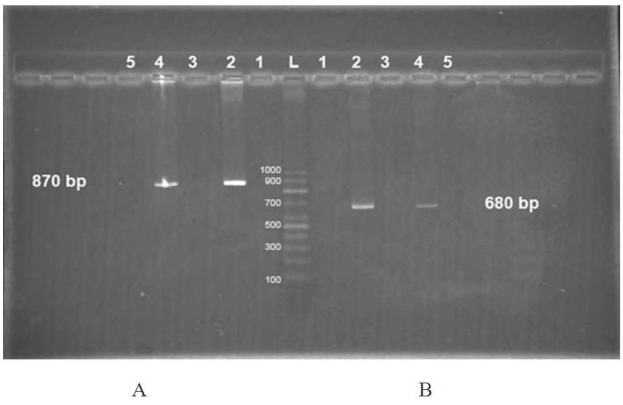

Figure 3. Electrophoresis product of PCR reaction for gene of chitinase (A) and β-1, 3 glucanase (B), agarose gel 1%: L) DNA ladder (1kb) 2) DNA plasmid pBI121 3) DNA genome of non-transgenic plant (control) 4) DNA genome of transgenic plants 5) negative control (water).

Finally, transgenic shoots that remained green were transferred to rooting medium containing one mg/l IBA and 200mg/l cefotaxime (eliminating the kanamycin and BAP) (Figure 2). In this study, regeneration of the explants on MS selective medium was low and had less than three percent performance. Genomic PCR analysis was performed to identify transgenic plants. Pieces of genomic DNA extracted from leaves of kanamycin-resistant plants for genes A-*chitinase* (870bp) and B- *β-glucanase* (680bp) and genomic DNA of control plants were amplified with specific primers. PCR analysis have confirmed the presence of binary plasmids containing fragments of *chitinase and β-glucanase* genes in the genome of transgenic plants while in non-transgenic

control plants no band due to the presence of these two genes were observed (Figure 3).

4. Discussion

Transformation of *chitinase* gene into plants against fungal diseases has been highly done [9,10,11]. Although the efficiency of it's enzyme is usually limited for resistant to fungal agents in most products [12], but it has well synergistic effects with *β-glucanase* in *in vitro* and *in vivo* conditions. They are the most important hydrolytic enzymes using together in plant for increasing resistance against fungal agents [6,7]. Leaf cotyledonary explants has a better performance because of more viability in selective medium than other explants. Transformation via leaf disk method and the use of LBA 4404 could increase gene transfer efficiencies in melon varieties [4].There are so many reports about results of the transgenic melon [4], but generally regeneration efficiency of transgenic varieties of melon are low and dependent on the genotype and factors such as strains of bacteria and the physiological and cultivation conditions [4,13]; In this study, the efficacy of production of transgenic shoots in Khatooni cultivar was 2.7 percent.

5. Conclusion

Totally, melon breeding through biotechnology is much more economical than traditional breeding methods and gene transfer has high efficacy to increase the yield and quality of melon cultivars. Production of resistant melon to fungi disease causes a reliable source of resistance genes in melons gene pool that we have done this in present study. As far as we know, this is the first gene transfer attempt in khatooni cultivar melon that could be useful for future studies.

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