Journal of Plant Molecular Breeding (JPMB)
Vol. 2/No. 1/June 2014/ 1-11

Genetic transformation of Tomato with three pathogenesis-related protein genes for increased resistance to *Fusarium oxysporum* f.sp. lycopersici

B. Dolatabadi[1,2*], Gh. Ranjbar[1], M. Tohidfar[3], A. Dehestani[2]

1. Department of Agronomy and Plant Breeding, Sari University of Agricultural Sciences and Natural Resources, Sari, Iran.
2. Genetics and Agricultural Biotechnology Institute of Tabarestan, Sari University of Agricultural Sciences and Natural Resources, Sari, Iran.
3. Agricultural Biotechnology Research Institute of Iran, Karaj, Iran.

[*]Corresponding Author, Email: bandd_7159@yahoo.com

Received: May 2014 Accepted: June 2014

Abstract

Fusarium wilt caused by *Fusarium oxysporum* f.sp. Lycopersici is one of the major obstacles to the production of tomato which causes huge losses in tomato products worldwide. In order to increase the tolerance to this disease, a triple structure containing PR1, chitinase and glucanase genes controlled by 35S promoter was transferred to tomato. Eight days after planting on pre-culture medium, explants were inoculated by *Agrobacterium tumefaciens* strain LBA4404 containing the aforementioned plasmid. When the regenerated shoots grew to 2-3 cm, they were cut and transferred to rooting medium.The plantlets were then transferred to pots filled with a soil mixture of peat moss and perlite for further acclimatization. The putative transgenic plant lines were analyzed by multiplex PCR and the transcription of the transgenes was confirmed by RT-PCR method using the specific primers. The estimated value for the frequency of the simultaneous transfer of chitinase, glucanase and PR1 genes to tomato was 2.7%. Protein extracts of transgenic plants expressing chitinase, glucanase and PR1 genes inhibited *in vitro* hyphal growth of *F. oxysporum* f.sp. lycopersici. Compared with non-transgenic control plants, despite some alterations in chlorophyll content no other morphological changes were observed in transgenic plants. The total content of chlorophyll "a" and "b" in transgenic plants were 31.8 and 36.2 % higher than that of control plants, respectively, which may be attributed to metabolic changes due to simultaneous expression of three transgenes.

Key words: Chitinase, *Fusarium oxysporum*, Glucanase, *PR1*, Transgenic Tomato.

Introduction

Today increasing food production commensurate with population growth is one of the main objectives all over the world. Reducing the yield loss caused by plant diseases have been focused in recent years and various technologies were implemented to achieve this goal (Giovanni *et al*, 2004). Tomato (*Lycopersicon esculentum*) is one of the world's most important crops due to the high value of its fruits both for fresh market consumption and numerous types of processed products (Giovanni *et al*,

2004). Pathogenic microorganisms such as viruses, bacteria, and specially fungi cause severe losses and drastic decrease in the annual fruit production (Barone & Frusciante, 2007). The *Fusarium* wilt disease causes loss of tomato crops worldwide and first described in 1895 by G.E. Massi (Jones & Woltz, 1981). This fungal disease has been reported from all geographical areas (Tanyolac & Akkale, 2010).

There are 3 common procedures for controlling fungal diseases: 1) agricultural operations, 2) utilization of chemical compounds and 3) using resistant varieties (Barone & Frusciante, 2007). *Fusarium oxysporum* f.sp. Lycopersici is a soil-borne pathogen, it remains in contaminated soils for several years. Therefore, controlling *Fusarium* wilt in the first two months of planting is very difficult and expensive. Mean while, generating resistant cultivars can play a significant role in increasing tomato production (Jones & Woltz, 1981).

At the present time, one of the most prevalent strategies is producing transgenic plants which are resistant to fungous diseases. A group of plant-coded proteins induced by different stress stimuli, named "pathogenesis-related proteins" (PRs) are believed to have an important role in plant defense against pathogenes (Edreva, 2005). These proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origins (Van Loon, 1985). Toxicity of PRs can be generally accounted for their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability. Thus, hydrolytic enzymes(β-1,3-glucanases, chitinases and proteinases) can be valuable tools in weakening and decomposing of fungal cell walls (van Loon *et al*, 2006). Chitinases are found in a wide range of organisms including bacteria, fungi and organic plants which play various physiological roles (Felse & Panda, 1999). Chitinase produced in microorganisms is reported as the main bio-control agent for different kinds of fungal diseases in plants (Freeman *et al*, 2004). Furthermore, β-1,3-glucanase which was well examined at physiological and molecular levels, plays an extensive role in defense reactions (Simmons *et al*, 1992). β-1,3-gluconase together with chitinase are expressed in response to pathogenic pollutions, wounds, ethylene treatment and chemical tensions (Li *et al*, 2001). The enzyme β-1,3-glucanase is able to decompose the available glucan on cellular wall of fungus and decrease the damages. On the other hand, this enzyme is able to exacerbate the activity of chitinase enzyme. Therefore, when these two enzymes are available in a transgenic plant, the plant will be able to represent a better and permeated resistance in a wide spectrum of pathogenic fungi. In cereals, for example, it will display high resistance to diseases, including yellow rust, brown rust and powdery mildew (Selitrennikoff, 2001). Several studies on transgenic tobacco which contains chitinase and glucanase genes controlled by CaMV35S revealed that the growth of *Rhizoctonia solani* was reduced which demonstrated the fact that the genes were applied separately (Jach *et al*, 1995). The genes encoding bean chitinase and tobacco β-1, 3 glucanase were introduced into the tomato line A53 (*Lycopersicon esculentum* cv.A53) via an Agrobacterium mediated transformation system. Transformants were obtained and confirmed by PCR and Southern blot analysis. The transgene copy numbers

ranged between 1 and 8 copies. The foreign genes expression in the obtained transgenic plants showed resistance to *Fusarium* wilt disease (Bo *et al*, 2003). Regarding the importance of the proposed issue, this research was considered to produce transgenic tomato resistant against *Fusarium* disease with simultaneous transfer of three resistant genes including PR1, chitinase and β-1,3- glucanase.

Materials and Methods

Plant materials and growth conditions

Seeds of commercial tomato cultivars (Sheffellat) were provided by Agricultural and Natural Resources Research Center of Mazandaran. For sterilization, seeds were first immersed in 70% ethanol for 30 seconds and then rinsed by distilled water and incubated in 1% solution of sodium hypochlorite for 15 min. Finally, it was rinsed three times (each time for 3-5 min) by sterile distilled water and disinfected seeds were cultured on Ms-medium (Murashige & Skoog, 1962) including 30 gl^{-1} sucrose and 8 gl^{-1}agar for germination. Afterwards, they were incubated at 25°C and 16/8 light/dark photoperiod. The cotyledons were separated as explants from 8-day plantlets and were cultured on pre-treatment medium containing MS basal medium, 0.1 mgl^{-1} naphthalene acetic acid (NAA) and 0.1 mgl^{-1} 6-benzylaminopurine (BAP) and were incubated at 25°C under dark conditions for 72 hours.

Gene Construct

Strain LBA4404 of *Agrobacterium tumefaciens* containing plasmid PBI121 ChiGluPRl (+) (Raufi *et al*, 2012) was used in this study. This plasmid contains three genes of chitinase, glucanase and PR1 with separate promoter (*CaMV*35s) and terminator (Nos). The selectable marker gene was neomycin phosphotransferase with Nos promoter and terminator (Figure1).

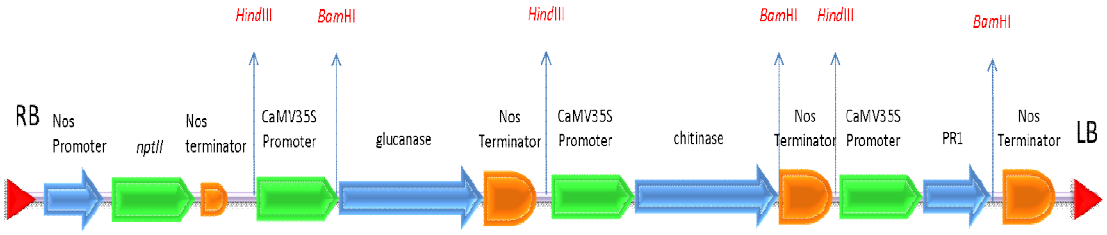

Figure 1. Schematic profile of plasmidic vector PBI121 Chi Glu PR1(+)

Preparation of bacterial suspension and inoculation of explants

Agrobacterium containing neo-compound plasmid was grown in Luria-Bertani (LB) medium containing 50mgl^{-1}kanamycin, 50 mgl^{-1} rifampicin and 200 µM acetosyringone at 28°C with shaking (220 rpm). The explants which were previously incubated on pretreatment medium, were transferred to bacterial suspension and were shaken for 30 min at 28 °C. The explants were then blotted on sterile filter papers and were placed on a medium containing basal MS salts, 0.1 mgl^{-1} NAA, 0.1 mgl^{-1} BAP and 200 µM acetosyringone at 25°C and darkness for 48 hours.

Regeneration and selection of transgenic plants

After co-cultivation, infected slices were rinsed by MS medium and distilled water, which had an appropriate concentration of cefotaxime (500 mg/l), for Agrobacterium removal. They were then transferred to basal MS with 0.5 mgl^{-1}Indoleacetic acid (IAA), 0.5 mgl^{-1}zeatinriboside, 300 mgl^{-1} cefotaxime and 25 mgl^{-1} kanamycin. When the adventitious shoots grew to about 2-3 cm, they were transferred to rooting medium (basal MS with 200 mgl^{-1} cefotaxime and 25 mgl^{-1} kanamycin).

Molecular confirmation of probable transgenic plantlets with multiplex PCR

Genomic DNA was extracted from leaves using Dellaporta method (Dellaporta *et al,* 1983). To confirm the simultaneous integration of genes including chitinase, glucanase and PR1 in genomes of putative transgenic plants, multiplex polymerase chain reaction (multiplex PCR) was performed in 25 µl containing 1x PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.32 µM specific forward and reverse primers of both chitinase and glucanase genes, 0.4 µM specific forward and reverse primers of PR1 gene, 40ng of genomic DNA and 1 unit of Taq DNA polymerase (Sina Gene). Amplification consisted of 3 min at 94°C (initial denaturation), 35 cycles of 1 min at 94ºC (denaturation), 1 min at 58ºC (annealing), and 3 min at 72ºC (extension) followed by 5 min at 72ºC (Final extension). PCR products were separated in 1% agarose gel. For reliable screening, bacterial contamination was also checked by amplification of virG gene in PCR using virG gene specific primers. The virG containing plantlets were considered as false positive transgenic selection. The sequence of primers used in this reaction is as follows (Raufi*et al*, 2012):

R (chi) 5' GCCATAACCGACTCC AAGCA3'
F (chi) 5' GAGTGGTGTGGATGCTGTTG 3'
R (Glu) 5' TCTCCGACACCACCACCTTC 3'
F (Glu) 5' CA GGTCCAAGGGCATCAA CG 3'
R (PR1) 5' TTAGTATGGACTTTCGCC 3'
F (PR1) 5' GTCATGGGATTTGTTCTC 3'

Analysis of gene expression through RT-PCR

Total RNA was isolated from leaves of transgenic and non- transgenic tomato plants using Trizolr agent. Then the first strand cDNA was generated using the oligo (dT) by the "first strand cDNA synthesis kit" (Fermentas). PCR amplification was achieved using the first strand cDNA as template. This reaction was performed in a 25 μl containing 1x PCR buffer, 2 μl cDNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 unit Taq DNA polymerase and 0.4 μM each of primers. PCR was carried out as follows: an initial denaturation at 94ºC for 3 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 60°C for chitinase and glucanase genes and at 57°C for PR1 gene for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 5 min. The PCR products were separated by electrophoresis on 1% (w/v) agarose gel.

Disk diffusion Bioassay

The effect of protein extracts of transgenic plants on the growth of *Fusarium oxysporum* f. sp. Lycopersici was studied on PDA media. The leaf material (700 mg) was grounded to a fine powder in liquid nitrogen using a mortar and pestle. About 750µl of extraction buffer (acetate sodium

100 mM, 2-mercaptoethanol 8 mM and phenylmethylsulfonyl fluoride 1mM, PH 6.5) was added to the leave powder. The extracts were then shaken for 1 h at 4ºC and sub-sequently centrifuged at 13000 g for 15 min at 4ºC. Protein concentrations were estimated using Bradford method (Bradford, 1976). A piece of agar including the fungal isolates was placed at the center of each of the PDA petri dishes. Petri dishes were then kept at 24°C and the six paper discs were placed in such a way that they surrounded agar segment symmetrically and the samples (containing proteins) were added to discs. They were again kept in incubator at 24°C. In order to compensate the reduction in enzymatic activity of samples during maintenance, a protein sample was again added to discs 18 hours later.

Studying morphological changes in transgenic plants

Transgenic plants were evaluated for probable physiological alterations compared with control plants. For this purpose, the chlorophyll levels of transgenic and control plants were measured as follows: About 0.5g of plant leaf was well crushed with 10 ml acetone. The solution was then filtered using filter paper and the volume reached to 25 ml. The absorption level was then measured at wavelengths of 662 and 645 respectively by spectrophotometer and the values of chlorophylls “a” and “b” were calculated using the following formula:

Chlorophyll a= 11.75× A662 - 2.35× A645

Chlorophyll b= 18.61× A645 - 3.96× A662

Results and Discussion

In current research, 82 among the 960 explants were regenerated into rootless green stems. When the plantlets grew to 2-3 cm, they were transferred to rooting medium (basic MS) and 40 of the 82 plantlets generated roots. they were transferred to the pots filled with a soil mixture of peat moss and perlite (Figure 2). Figure 3 shows the PCR analysis of the putative transgenic plants in the presence of chitinase, glucanase and PR1 genes. Two of the 25 plants were positive for all of the three genes (lanes 2 and 3 in Figure 3), however one plant was positive for chitinase and glucanase gene and negative for PR1 gene (lane 4 in Figure 3). Plasmid PBI 121 ChiGluPRl (+) was used as positive control and water was used as negative control (lane 6 and 1, Figure 3, respectively). A total of 25 putative transgenic plants were generated, out of which 24 plants contained chi, glu and pr1 genes and only one of the plants contained chi and glu genes (as determined by PCR).

Chang *et al* (2002) reported the achievement of double transfer of chitinase and glucanase genes into pea genome at the level of 1.6%. In this study the rate of simultaneous transfer of chitinase, glucanase and PR1 genes was estimated as 2.7%. Nookarajuand Agrawal (2012) transferred chitinase and β-1, 3- glucanase genes from wheat to grape genome using agrobacterium to increase resistance to *Plasmoparaviticola* and observed that the transgenic plants demonstrated high levels of resistance to the pathogen.

When the resistance to a specific disease is conditioned by a single gene, resistance breaking events would frequently occur. Using a plasmid construct containing multiple resistant genes encoding for antifungal proteins under the control of individual and strong promoters would be an appropriate approach for producing fungal resistant transgenic plants (Mohsenpour *et al*, 2008).

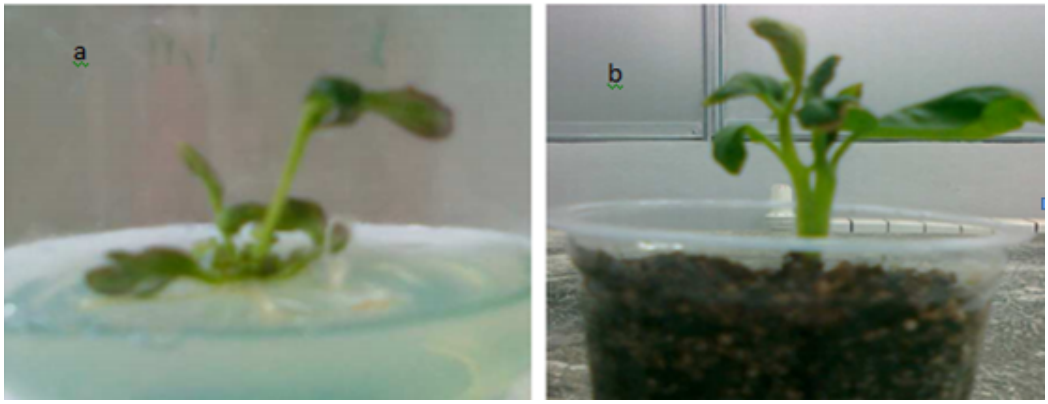

Figure 2. a) Potential transgenic plantlet which produced root in regeneration medium. b) Potential transgenic plantlet after transferring on soil.

The results indicated that one of the obtained transgenic plants contained chitinase and glucanase genes while PR1 gene was not present in this line. T-DNA transformation into plant cell is initiated at the right border and terminated at the left border (Mohseni Azar *et al*, 2012). The right border sequence promotes T-DNA transfer and integration (Gheysan *et al*, 1998). Therefore, gene sequences adjacent to the right border are more likely to be integrated into the host genome. The breakage probably happens more frequently in DNA regions away from the sequences of right border. Chen *et al* (1997) showed that 44 % of transgenic wheat lines carried incomplete T-DNA segments. Most of these breakages occur at the left border (Wu *et al*, 2006). Among 260 transgenic barley plants, only 3 percent had complete T-DNA (Bartlett *et al*, 2008). The deletion of some parts of T-DNA can interfere with the performance of the gene. In a study on transgenic plants, it was determined that 37.5 percent of transgenic plants have broken T-DNA segments (Hensel *et al*, 2012).

To verify the absence of agrobacterium in putative transgenic plants, polymerase chain reaction was performed using specific primers for vir G gene. Among the tested plants, three plants exhibited the 390 bp band corresponding to virG gene, demonstrating that the agrobacterium cells are present on plant tissues. Further analysis making were necessary to confirm whether they are real transgenics or false-positive results due to agrobacterium contamination. The absence of this band in other transgenic plants indicates that they are real transgenics with transgene integration. The results indicated that cefotaxime application did not remove the agrobacterium cells and the resulted adventitious shoots were somehow infected with these cells.

Several authors reported similar results when regenerating transgenic shoots after agrobacterium infection (Pena *et al*, 2010).

It was previously reported that the presence of bacterial colonies resistant to kanamycin in some tissues, especially at the cut zone of explants, reduces the antibiotic toxicity and allows the

reproduction of non-transgenic cells in the selection medium (Dominguez *et al*, 2004).

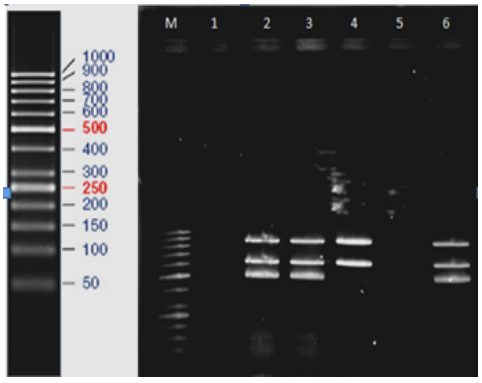

Figure 3. Polymerase chain reaction using the specific primers of genes including PR1, chitinase and glucanase. M: 50 bp DNA ladder, 1: negative control (water), 2 & 3: Transgenic plant containing all three genes, 4: transgenic plant containing two genes of glucanase and chitinase, 5: control plant , 6: positive control (Plasmid PBI121 ChiGluPRl (+)).

Gene transcription analysis through RT-PCR

The presence of 872, 629 and 510 bp fragments indicates transcription of chitinase, glucanase and PR1 genes, respectively. The lanes 5, 9 and 15 are positive controls corresponding to chitinase, glucanase and PR1 genes, respectively. The lanes 1, 10 and 11 are negative controls. The lanes 4, 8 and 14 correspond to non-transgenic plants. The lanes 1 and 3 correspond to chitinase gene, lanes 6 and 7 are related to glucanase gene. The lanes 12 and 13 are related to PR1 gene (Figure 4).

Evaluation of antifungal activity

Inhibitory activity of recombinant chitinase, glucanase and PR1 proteins in the protein extracts of transgenic plants was evaluated on *Fusarium oxysporum* f. sp. lycopersici.

For this purpose, inhibitory effect of the protein extracts on the growth of fungal hyphae was assessed using PDA plates. Results showed that protein extracts containing recombinant proteins of the transferred genes, inhibited the growth of fungal hyphae (5 and 6, Figure 5).

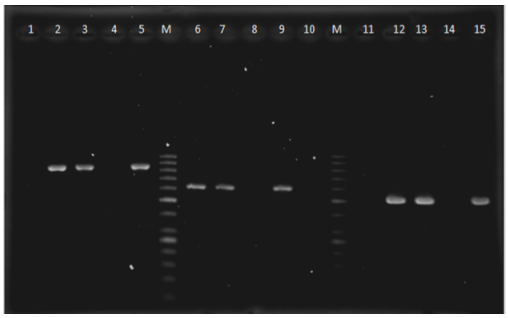

Figure 4: RT-PCR test for transgenic tomato lines with pBI 121 PR1 chi Glu. M: 50 bp DNA ladder , 5, 9 , 15 positive control related to genes including chitinase , glucanase and PR1 , 1 , 10 , and 11 negative control , 4 , 8 , 14 control plant , 2 and 3 related to chitinase gene, 6 and 7 related to glucanase gene, 12 and 13 related to PR1 gene.

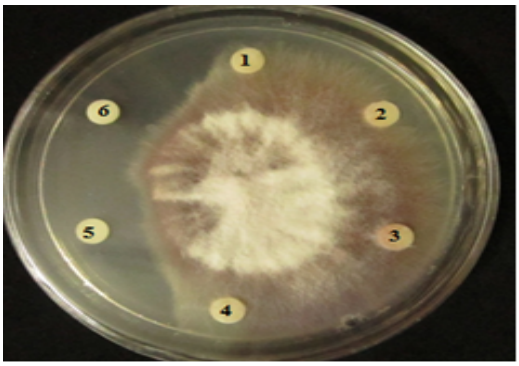

Figure 5: studying anti-fungous activity of protein extract of transgenic plant on the fungus *fusarium oxysporum* f . sp. Lycopersici, 1: 50 µg protein extract of non- transgenic plant, 2: 100 µg protein extract of non- transgenic plant , 3: 50 µl extraction buffer, 4: 100 µl extraction buffer, 5: 50 µg protein extract of transgenic plant, 6: 100 µg protein extract of transgenic plant.

Study of morphological changes in transgenic plants

Phenotypically, transgenic plants were similar to non- transgenic plants. The only difference between these two plant types was the color of leaves. Obviously, the leaves of transgenic plants were darker (dark green) than those of non- transgenic plants (Figure 6).

Figure 6: Difference in chlorophyll values between transgenic plant and non- transgenic plant (control).

To assess this alteration, the available chlorophyll in the leaves of transgenic and non- transgenic plants was measured.

In the leaves of non- transgenic and transgenic plants, the values of chlorophyll "a" were 15.41 and 20.31, respectively. On the other hand, the values of chlorophyll "b" were 5.66 and 7.71 for the leaves of non- transgenic and transgenic plants, respectively. The accumulation of PRs in the plants induces SAR (System of Acquired Resistance) genes (Ward *et al*, 1991). SAR phenomenon creates several morphological changes in the stressed plant (Ross, 1961). Some morphological and biochemical changes are created in relation to SAR phenomenon such as cellular death (Low & Merdia, 1996), increased synthesis of phytoalexins (Neuenschwader *et al*, 1996), accumulation of pathogenesis-related proteins (Jeun, 2000) and changes in chlorophyll value (Milavec *et al*, 2001). SAR phenomenon in broad bean plants increased chlorophyll value (Maggie *et al*, 2006).

Acknowledgements

The authors are grateful to Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) for providing the instruments to perform this research.

References

Barone, A. and Frusciante, L. 2007. Molecular marker-assisted selection for resistance to pathogens in tomato, Marker-Assisted Selection, Current status and future perspectives in crops, livestock, forestryand fish. pp 153-164.

Bo, o., Han-Xia, L. and Zhi-Biao. Y. 2003. Increased resistance to *fusarium* wilt in transgenic tomato expressing bivalent hydrolytic Enzymes. Plant Physio & Mol Biol, 29: 179-184.

Bartlett, J. G., Alves, S. C., Smedley, M., Snape, J.W. and Harwood, W.A. 2008. High-throughput Agrobacterium - mediated barley transformation. Plant Meth, 4: 22.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of micro gramquantities of protein utilizing the principle of protein dye binding. Anal Biochem, 72:248-254.

Chang, M. M., Culley, D., Choi, J. J. and Hadwiger, L .A. 2002. Agrobacterium-mediated co-transformation of a pea β-1, 3-glucanase and chitinase genes in

potato (*Solanumtuberosum* L. c.v. Russet Burbank) using a single selectable marker. Plant Sci., 163: 83-89.

Cheng, M., Fry, J., Pang, E. S., Zhou, H., Hironaka, C. M., Duncan, D. R., Conner, T.W. and wan, Y. 1997. Genetic transformation of wheat mediated by Agrobacterium tumefaciens. Plant Physio, 115: 971-980.

Dellaporta, S. L., Wood, J. and Hicks, J. B. 1983. A plant DNA mini preparation: version II. Plant Mol Bio Rep, 1:19-21.

Dom´ınguez, A., Cervera, M., P´erez, R., Romero, J., Fagoaga, C., Cubero, J., L´opez, M.M., Ju´arez, J., Navarro, L. and Pe˜na, L. 2004. Characterisation of regenerants obtained under selective conditions after Agrobacterium-mediated transformation of citrus explants reveals production of silenced and chimeric plants at unexpected high frequencies. Mol Breeding, 14: 171–183.

Edreva, A. 2005. Pathogensis-Related Proteins: research. Gen Appl Plant Physio, 31: 105-124.

Felse, P. A. and Panda, T. 1999. Regulation and cloning of microbial chitinase genes. Appl Microbiol Biotechnol, 51: 141-151.

Freeman, S., Minzm, O., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., Nitzani, Y., Kirshner, B., Rav-David, D., Bilu, A., Dag, A., Shafir, S. and Elad. Y. 2004. Trichodermabiocontrol of Colletotrichumacutatum and Botrytis cinerea and survival in strawberry. E J P P, 110: 361-370.

Gheysen, g., Angenon, g. and Van Montagu, M. 1998. Agrobacterium mediated plant transformation: A scientifically intriguing story with significant application. In Transgenic Plant Research, Eds. Lindsey, K., Harwood Academic, A. M. Sterdam, pp:1-33.

Giovanni, C. D., Orco, P. D., Bruno, A., Ciccarese, F., Lotti, C. and Ricciardi, L. 2004. Identification of PCR-based markers (RAPD, AFLP) linked to a novel powdery mildew resistance gene (ol-2) in tomato. Plant Sci, 166: 41-48.

Hensel, G., Oleszczuk, S. S., Daghma, D. E., Zimny, J., Melzer, M. and Kumlehn, J. 2012. Analysis of T-DNA integration and generative segregation in transgenic winter triticale (x TriticosecaleWittmack). BMC Plant Bio, 12:171.

Jach, G., Gornhardt, B., J. Mundy, J., Logemann, J., Pinsdorf, E., R leah, Schell, J. and Maas. C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J, 8: 97-109.

Jeun, Y. C. 2000. Immunolocazation of PR-protein P14 in leaves of tomato plants exhibiting systemic acquired resistance against Phytophthoriainfestanse induced by pre-treatment with 3-aminobutyric acid and preinoculation with Tobacco necrosis virus. J Plant Dis, 107: 352-367.

Jones, J. P. and Woltz, S. S. 1981. Fusarium-incited disease of tomato and potato and their control. Pennsylvania State University Press, pp 157-168.

Li, W. L., Faris, J. D., Muthukrishnan, S., Liu, D. J., Chen, P. D. and Gill, B.S. 2001. Isolationand characterization of novel cDNA clones of acidic chitinasesand β-1,3-glucanases from wheat spikes infected by *Fusariumgraminearum*. Theor Appl Genet, 102: 353–362.

Low, P. S. and Merdia, J. R. 1996. The oxidative burst in plant defense function and signal transduction. Plant Physiol, 96: 533-542.

Maggie, H., Saidea, A., EI-Abbasi, I. H. and Mikhail, M.S. 2006. Inducing Resistance against Faba Bean Chocolate Spot Disease. Egypt J Phytopathol, 34: 69-79.

Milavec, M., Ravnikar, M. and Kovac, M. 2001. Peroxidases and photosyntthetic pigments in susceptible potato infected with potato virus YNTN.Plant Physio Biochem, 39: 891-898.

MohseniAzar, M., Nazeri, S., Malboobi, M. A., Ghadimzadeh, M., barzegari, M. and farokhzad. A. 2012. Optimizing Factors Affecting Agrobacterium Tumefaciens Mediated Transformation Of Dwarf Rootstock Of Apple (Malus Domestica Borkh Cv Gami Almasi). Agri biotech, 10: 7-15.

Mohsenpour, M., Babaeian, N. A., Tohidfar, M. and Habashi, A. A. 2008. Design and construction of four recombinant plasmid vectors containing chitinase, glucanase and BT genes, suitable for planttransformation. J Agric Sci Natural Resour, 15:41-47.

Murashige, T. and Skoog, F. 1962. A revised mediumfor rapid growth and bioassays with tobacco tissue cultures. Plant Physio, 15:473-497.

Neuenschwader, U., Lawton, K. and Ryals, J. 1996. Systemic acquired resistance in plant-microbe interact. Chapman and Hall New York, 1: 81-108.

Nookaraju, A. and Agrawal, D. C. 2012. Enhanced tolerance of transgenic grapevines expressingchitinase and b-1,3-glucanase genes to downy mildew. Plant Cell Tiss Organ Cult, 111:15–28.

Pena, L., Cervera, M., Fagoaga, C., Romero, J., Ballester, A., Soler, N., Pons, E., Rodr´ıguez, A., Peris, J., Ju´arez, J. and Navarro, L. 2010. Compendium of Transgenic Crop Plants, Volum 5: Transgenic Tropical and Subtropical Fruits and Nuts: Citrus fruit.

Raufi A., Tohidfar, M., Soluki, M. and Mohsenpour M. 2012. Isolation and Cloning of Two Genes from PR1 Family and Construction of Treble Plasmids Containing 3 Groups of Genes for Producing Transformed Plants Resistant to Fungal Diseases. J Agric Biotechnol, 2:27-46.

Selitrennikoff, C. P. 2001. Antifungal proteins. ApplEnvMicrobiol, 67: 2883-2894.

Ross, A. F. 1961. Systemic acquired resistance induced by localized virus in fection in plants. Virology, 14: 340-358.

Simmons, C. A., Litts, J. C., Huang, N. and Rodriguez, R. L. 1992. Structure of arice β-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid, and fungal elicitors. Plant Mol Bio, 18: 33–45

Tanyolac, B. and Akkale, C. 2010. Screening of resistance genes to fusarium root rot and fusarium wilt diseases in F3 family lines of tomato (*Lycopersicon esculentum*)using RAPD and CAPs markers. AJB, 9: 2727-2730.

Van Loon, L.C. 1985. Pathogenesis-related proteins. Plant Mol Biol, 4: 111-116.

Van Loon, L. C., Rep, M. and Pieterse, C. M. J. 2006. Significance of inducible defense-related proteins in infected plants. Phytopathol, 9: 342-431.

Ward, E.R., Uknes, S.J. Williams, S.C. Dincher,S.S. Widerhold, D.L. Alexander,D.C. Ahl-Goy, P. Métraux, J.P. and Ryals,J.A. 1991. Coordinate gene activity in response to agentsthat induce systemic acquired resistance. Plant Cell, 3: 1085-1094.

Wu, H., Sparks, C. A. and Jones, H. D. 2006. Characterisation of T-DNA loci and vector backbone sequences in transgenic wheat produced by Agrobacterium-mediated transformation. Mol Breeding, 18:195–208.

انتقال سه ژن رمزگردان پروتئین‌های مرتبط با بیماریزایی در گیاه گوجه‌فرنگی برای افزایش مقاومت به بیماری قارچی *Fusarium oxysporum* f. sp. Lycopersici

بهناز دولت‌آبادی[۲و۱*]، غلامعلی رنجبر[۱]، مسعود توحیدفر[۳]، علی دهستانی[۲]

۱- گروه کشاورزی و اصلاح نباتات، دانشگاه علوم کشاورزی و منابع طبیعی ساری

۲- پژوهشکده ژنتیک و زیست‌فناوری کشاورزی طبرستان، دانشگاه علوم کشاورزی و منابع طبیعی ساری

۳- پژوهشکده بیوتکنولوژی کشاورزی کرج

[*]نویسنده مسئول: bandd_7159@yahoo.com

چکیده

گوجه‌فرنگی یک محصول تجاری مهم در سراسر دنیاست. یکی از محدودیت‌های اصلی در کاشت گوجه‌فرنگی بیماری پژمردگی فوزاریومی است که باعث از دست رفتن تولیدات گوجه فرنگی در سراسر جهان می‌شود. به منظور افزایش مقاومت در گوجه‌فرنگی سازه سه‌گانه حاوی ژن‌های PR۱، کیتیناز و گلوکاناز تحت پیشبرهای مستقل 35s به گوجه‌فرنگی منتقل گردید. ریزنمونه‌های ۸ روزه پس از قرارگیری بر روی محیط پیش کشت، به وسیله‌ی اگروباکتریوم سویه LBA4404 حاوی ناقل مورد نظر تلقیح شدند.گیاهچه‌های باززایی شده زمانی که به طول ۲-۳ سانتیمتر رسیدند به محیط کشت ریشه‌زایی منتقل شدند. پس از ریشه‌زایی، گیاهچه‌های حاصل به گلدان‌هایی با ترکیب خاکی مناسب انتقال یافتند. صحت انتقال و ادغام ژن‌ها توسط واکنش multiplex PCR تایید و نسخه‌برداری از ژن‌های انتقالی نیز با روش RT-PCR با استفاده از آغازگرهای اختصاصی ژن‌های مذکور اثبات شد. نسبت انتقال همزمان سه ژن کیتیناز، گلوکاناز و PR1 برابر ۲/۷ درصد بود. عصاره پروتئینی گیاهان دارای ژن‌های کیتیناز، گلوکاناز و PR1 توانست رشد قارچ *Fusariumo xysporum* f. sp. Lycopersici را درشرایط درون شیشه‌ای کنترل نماید. از نظر مورفولوژیکی گیاهان تراریخته مشابه گیاهان غیرتراریخته بودند تنها تفاوت مشاهده شده در میزان کلروفیل بود. میزان کلروفیل a و b در گیاهان تراریخته به غیرتراریخته به ترتیب ۳۱.۸ و ۳۶.۲ درصد افزایش داشت.

کلمات کلیدی:گوجه‌فرنگی، کیتیناز، گلوکاناز، PR1، *Fusarium oxysporum*.