Transformation Fava Beans by Agrobacterium using Chitinase, Glucanase and CryIA (b) genes
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Abstract— In this study two plasmid vectors that are appropriate for plant transformation were made by preparation of gene cassettes for β-1, 3-glucanase from barley, chitinase from bean and cryIA (b) from Bacillus thuringiensis (BT). Each of these genes were cloned under the control of the CaMV35S promoter and the Nos terminator in pBI121 binary vector. pBI-Chi and pBI-Glu and recombinant plasmid vectors were constructed via cloning of chitinase, β-1,3-glucanase and cryIA (b) genes, respectively, instead of the gus gene in T-DNA region of pBI121 vector. Construction of pBI-ChiGlu recombinant plasmid vector was performed by means of cloning both of the complete chitinase and glucanase gene cassettes in pBI121 vector, with the intention of production synergistic effects against fungal infection. pBI-ChiBt recombinant plasmid vector containing both of the complete chitinase and Bt gene cassettes was also constructed in order to contemporaneous plants resistance to pest pathogens and fungal in a single transformation event. pBI-ChiGlu and pBI-ChiBt that have been introduced into the A. tumefaciens strain LBA4404 that was subsequently used for transformation. Results indicate that embryogenic calli are well appropriate as objective material for Agrobacterium tumefaciens-mediated transformation in Faba bean. Seventeen well established shoots were transferred to new MLS medium including suitable antibiotics. Finally six independent transgenic plants were successfully rooted on kanamycin-containing selection media and then transferred to soil after 20 days. Four plants out of six putative transgenic plants displayed to contain the end part of the chit transgene and nos terminator. The corresponding piece, 700 bp of the chit gene, was amplified using specific primer. These putative transgenic plants were also be measured for the presence of the bgn13.1 and cryIA (b) genes by PCR using specific primers. Two pieces with expected sizes (1221 bp and 640 bp) for bgn13.1 and cryIA (b) genes subsequently was amplified from three the transgenic plants and other three plants had not these fragments. A group of virG primers was used to discover Agrobacterium pollution that might have threw off the selection. Expression of chit, and β-1,3-glucanase genes at the transcriptional level in the transformed plants were proven by means of RT-PCR but expression of cryIA(b) was not proven. The expected dimension of the amplified DNA pieces were identified in the transformed plants, that confirming the permanent annexation and expression of T-DNA in the genome of kanamycin-screened plants.

Keywords— Agrobacterium, β-1,3-glucanase; BT; Chitinase; Cloning; Recombinant Plasmid

I. INTRODUCTION

Faba bean ( Vicia faba L.) is one of the most important food legumes in some countries such as Iran, Egypt, Greece, Ethiopia and Nepal. Like many other crops, the production of this crop is challenged by a number of plant pathogens and parasites. One of the most important fungal disease for this crop is Faba bean Root Rot, and causes considerable losses in the yield of fava bean in Iran (fig 1). The mycoparasitic process involves varied fungal cell wall degrading enzymes, including hydrolytic enzymes (Chet et al., 1998). Among these enzymes, β-1,3-glucanases and chitinases are appealing molecules because they have activity against a vasty range of fungi (Walsh et al., 2000). β-1,3-glucanases and Chitinases have been shown to have strong antifungal activities when used in combination (Lorito et al., 1998). Chitinases also confer broad resistance to other biotic and abiotic stresses, such as salinity, heavy metals and bacterial pathogens (Dana et al., 2006).

In this study the chitinase gene from bean, the β-1,3-glucanase gene from barley and cryIA (b) from Bacillus thuringiensis (BT) are used and have been replaced instead T-DNA of pBI121 plasmid (Fig 2). Faba bean cultivar is transformed by Agrobacterium-mediated transformation method. The plants that is being transgenic are analyzed by PCR and RT-PCR, and tested for their antifungal activity.

Fig 1. Aphanomyces root rot or common root rot of legumes
II. MATERIALS AND METHODS

An economically important faba bean of the Dehano cultivar, was used as the receptor. Fusarium solani, the causal important agent of Faba bean root rot was propagated in whole molecular experiments.E. coli O157H7 was used in whole molecular experiments. E. coli O157H7 was transformed using the CaCl2 method. Cloneis of the A.tumefaciens LBA4404, placing recombinant plasmids (pBI-ChiGlu and pBI-ChiBt that have been made by method of Mohsenpour et al,2008) containing β-1,3-glucanase , cryIA (b) and chitinase genes, were grown in EMB medium supplemented with 50 mg/l of kanamycin and allowed to grow overnight at 27°C with constant shaking (200 rpm) to mid-log phase(Fig 3 and Fig 4).

The bacterial cells were provided by centrifugation and resuspended in infection medium Murashige and Skoog (MS) medium, 70 mg/l of sucrose, (pH 5.7) and were then shaken for 2.5 h at 27°C. Explants of axis below the cotyledons were infected with A. tumefaciens LBA4404. Infected explants with chitinase, cryIA (b) and glucanase genes, were grown in EMB medium supplemented with 50 mg/l of kanamycin and allowed to grow overnight at 27°C with constant shaking (200 rpm) to mid-log phase(Fig 3 and Fig 4).

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An agar disc (5 mm in diameter) containing Fusarium solani, which was taken from the fungus in an actively growing state, was placed at the center of a Petri dish. In continuance the plates were incubated at 30°C for 3 days. Wells were subsequently pierced into the agar at a interval of 25 mm from the center of the plates. Then for the samples to be examined those were mixed with 10 µl of 15 mM acetate sodium buffer, pH 4 and placed in the wells.

| Primer number and application | Sequence |
|------------------------------|----------|
| 1-for Chit forward | 5-GAGTGTGGTGAGGTCTGTGG-3 |
| 2-for Chit reverse | 5-GCCATAACCGAATCCAAAGCA-3 |
| 3-for β-1,3-glucanase forward | 5-GAACATCAAGTACATCGCCG-3 |
| 4-for β-1,3-glucanase reverse | 5-GAGTGGTGTGGATGTGTAG-3 |
| 5-for cryIA (b) forward | 5-CTCTAGGTTGGAAGGATTGAG-3 |
| 6-for cryIA (b) reverse | 5-GTGAGCATCGGTGATAG-3 |
III. RESULTS

pBI-ChiGlu and pBI-ChiBt that have been introduced into the A. tumefaciens strain LBA4404 was subsequently used for transformation. Shoots were cocultivated and after 4 days, the slices were rinsed and transferred to selection medium containing cefotaxime and kanamycin. These results indicate that embryogenic calli are well appropriate as objective material for Agrobacterium tumefaciens-mediated transformation in Faba bean. Most shoots that was growing on selective media were shown to be non-transgenic. Seventeen well established shoots (3-4.5 cm long) were transferred to new MLS medium including suitable antibiotics. Finally six independent transgenic plants were successfully rooted on kanamycin-containing selection medium and then transferred to soil after 20 days. Only one well-grown shoot from each infected explant was transferred to rooting medium.

Four plants out of six putative transgenic plants displayed to contain the end part of the chit transgene and nos terminator. The corresponding piece, 700 bp of the chit gene, was amplified using specific primer. These putative transgenic plants were also be measured for the presence of the bgn13.1 and cryIA (b) genes by PCR using specific primers. Two pieces with expected sizes (1221 bp and 640 bp) for bgn13.1 and cryIA (b) genes subsequently was amplified from three the transgenic plants and other three plants had not these fragments. The results this study indicated that the transgenic plants were transformed by these three genes. The chit42, bgn13.1 and cryIA (b) specific primers did not amplify the corresponding pieces in the untransformed samples. A group of virG primers was used to discover Agrobacterium pollution that might have threw off the selection. PCR under various qualifications showed no scramble bands using transgenic plant DNA as template. A 738 bp band was identification using Agrobacterium DNA as control. Expression of chit, and β-1,3-glucanases genes at the transcriptional level in the transformed plants were proven by means of RT-PCR and expression of cryIA(b) was not proven.

RNA was isolated from leaf tissues for production of cDNA. The expected dimension of the amplified cDNA pieces were identified in the transformed plants that confirming the permanent annexation and expression of T-DNA in the genome of kanamycin-screened plants. Non-transformed plants were applied as negative controls and no PCR products were recognized. For test the antifungal activity of expressed Chit42 and Bgn13.1 from transgenic plants on the actively growing phytopathogenic fungus fusarium solani, total protein extracts from leaves of transgenic and non-transgenic plants were examined with using the radial diffusion assay. Antifungal activity was detected in extracts from two RT-PCR positive plants. Antifungal activity was not looked in other leaf juices.

IV. DISCUSSION AND CONCLUSIONS

Crops often suffer from diverse diseases caused by microbes covering phytopathogenic fungi, resulting in reduced yield and quality of products. Faba bean can be attacked by fungal diseases, such as rust (Uromyces viciae-faba) and chocolate spot (Botrytis fabae). It is also attacked by the black bean aphid (Aphis fabae) and Faba bean Root Rot. This fungus causes serious losses in yield. Cell wall degrading enzymes such as glucanases and chitinases from mycoparasitic fungi have shown high antifungal activity versus a wide range of economically important fungi (Harigui et al., 2006). The last two decades attempts have been made on the transgenic expression of plant fungal chitinases in crops, and significant developments in the resistance to fungal diseases have been registered (Nandakumar et al., 2005; Rajasekaran et al., 2005; Pappinen et al., 2002). Plant chitinases have been indicated that enchain fungal growth in vitro by degrading chitin polymers in fungal cell walls (Noel et al., 2005). Copy number of the transgene is one of the most important agents implicated in the durability of gene expression in transgenic plants (Katleen et al., 2005). Meaningful differences in the level of expression of the chitinase gene among the transgenic for cotton have been reported (Tohidfar et al., 2005). A positive relation was appointed between the level of chitinase expression and prevention of fungal growth and damage. Many reports have demonstrated that β-1,3-glucanases are distinct in their gene regulation (Romero et al., 1998). Before, activity of β-1,3-glucanases in pea seedlings has been proved. All β-1,3-glucanases are expressed in leaves in response to pathogens. The function of β-1,3-glucanases in floral tissues is well understood in anthers. Some researches have been performed to expand transgenic crop plants that have raised expression levels of the cell wall degrading enzymes in hopes of producing fungal disease-resistant varieties (Datta et al., 2001). Prior studies have also shown that elevated chitinase activity in transgenic strawberry (ASAO et al. 1997), rice (NISHIZAWA et al. 1999), tobacco (EMANI et al. 2003). There are many reports about the benefits of using chitinases for plant preservation (Jayaraj and Punja, 2007; Xiao et al., 2007). However, there are almost no reports demonstrating that chitinase genes derived from plants or bacteria allow resistance to several fungi in transgenic plants, showing that these enzymes have a slender range of antifungal activity (Dana et al., 2006). The failed use of plant chitinases for plant protection maybe comes from the fact that the gene codes for enzymes that usually affect only the hyphal tip and lack the capability to efficiently degrade spores or hard chitin constructions (Joosten et al., 1995).

Whereas fungal chitinases are able to lyse not only the hyphal tip, but also the strict chit wall of the complete hyphae and other fungal maintenance structures (Lorito et al., 1998). Also, there are some researches on the expansion of transgenic plants with glucanase genes for producing fungal disease resistant varieties (Datta et al., 2001). Multiple reports explain the synergic interplay effects of β-1,3-glucanase and chitinase in antifungal protection and these enzymes are non-toxic (Moravcikova et al., 2007; Dana et al., 2006). Usage of multifold resistant transgenes with distinct mechanisms to crop genetic manipulations could widen the range and increase the level of resistance in transgenic plants (Zhu et al., 2007). Some PCR-positive plants failed to express the corresponding mRNA when further analyzed by the RT-PCR method, thus demonstrating the silencing of these genes. Silencing of transgenes have also been reported in some other transgenic...
plants (Khan et al., 2008; Chang et al., 2002). Crude protein juices from the transgenic plants were able to prolong the hyphal growth of the fungal pathogen F. solani in the in vitro experiment.

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