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Full Length Research Paper

Isolation of OmpA gene from *Salmonella typhimurium* and transformation into alfalfa in order to develop an edible plant based vaccine

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The outer membrane protein A (OmpA) of *Salmonella typhimurium* may contribute to immunity and virulence in livestock animals. Introduction of this gene in forage crops like alfalfa may be an alternative and effective way to produce animal edible vaccine. In the present study, the OmpA gene was obtained after polymerase chain reaction (PCR) amplification and sequencing. We successfully identified the complete ORF encoding this protein. In order to express OmpA protein in alfalfa, the gene was inserted into a plant expression vector PBI121.The recombinant OmpA was expressed in *Escherichia coli* TG1. The new construct was used to transform the *Agrobacterium tumefaciens* Strain LBA4404 before plant transformation. Transgenic alfalfa plants were then developed by introducing OmpA gene in the plant genome under the control of Camv35s promoter and for the first time we expressed this protein in alfalfa. Releasing this one new transgenic variety may be a considerable progress towards release varieties which enables the production of edible vaccine.

Key words: Outer membrane protein A (OmpA), sub cloning, plantibodies, transgenic alfalfa, bioreactor, edible vaccine.

INTRODUCTION

Outer membrane proteins comprise almost 50% of the bacterial membranes of Gram-negative bacteria (Koebnik et al., 2000). The outer membrane protein A (OmpA) super family is one of the most abundant outer membrane proteins in prokaryotes. OmpA is the most widely studied (Thanassi and Hultgren, 2000). Its main role is to provide integrity to the membrane by ensuring physical linkage between the outer membrane and the underlying peptidoglycan layer as well as having importance in bacterial conjugation (Skurray and Reeves, 1974; Ried and Henning, 1987). It also serves as a receptor to some bacteriophages (Morona et al., 1985) and colicins (Foulds and Barrett, 1973). Another research reported the uses of this protein in vaccine production (Schorr et al., 1991; Haddad et al., 1995). In this study the OmpA gene was amplified from *Salmonella typhimurium* strain (UUCC101) which is a cause of common typhoid disease or salmonellosis in livestock. The disease is characterized by fever, anorexia, septic shock with or without dysentery. Protein of this gene in lower doses can be used as a vaccine. In an alternative way rather than the over expression of this gene in hosts like microorganisms, uses of plants as a bioreactor are another profitable ways.

Plant-based systems are increasingly used for the production of recombinant proteins including antibodies. Plant-based systems have several advantages over the other production systems, such as the ability to carry out necessary post-translational modifications not available in bacterial systems, as well as greater safety and lower production costs compare to animal- based systems. Plant-based technology has been recently reviewed, with full description of commonly used plants (Stoger et al.,

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Abbreviations: OmpA, Outer membrane protein A; **LB,** Luria-Bertani; **PCR,** polymerase chain reaction; **MS,** Murashige and Skoog; **YEP,** yeast extract peptone; **2,4-D,** 2, 4-dichlorophenoxyacetic acid; **BAP,** benzylaminopurine; **EtBr,** ethidium bromide; **MCS,** multi cloning site.

2002). Processing of transgenic crops would require relatively little capital investment, making the commercial production of biopharmaceuticals an exciting prospect. It has been estimated that the cost of producing recombinant proteins in plants could be 10 to 50 fold lower than producing the same protein in *Escherichia coli* or mammalian cells. Several proteins, enzymes and antibodies have been produced in plants and used in clinical trials, with a prospect of commercial exploitation (Ma et al., 2005). In this study we express *S. typhimurium* OmpA protein into plant host which is alfalfa. The introduction of exogenous genes into the adequately modified T-DNA of *Agrobacterium* cells and following infection of a vegetable tissue led to gene's stable integration in the plant genome.

MATERIALS AND METHODS

Bacterial strains and culture media

The *S. typhimurium* strain UUCC101 was obtained from the bacterial strain collection in the Department of Medicine biotechnology at the Tarbiat Modares University. *E. coli* Strain TG1 and *Agrobacterium* LBA4404 were obtained from Cinnagen. *E. coli* strains were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone (Bacto; Cat. No. 211701), 0.5% (w/v) yeast extract (Bacto; Cat. No. 212730), 1% (w/v) NaCl (Fisher; Cat. No. 642-500). *E. coli* TG1 cells containing pTZ57R plasmid were grown in LB broth containing 100 µg/ml ampicillin (Sigma; Cat. No. A9393). *E. coli* and *Agrobacterium* cultures were incubated at 37 and 28°C, respectively, in a shaking incubator.

Plasmid construction

The OmpA gene sequence was taken from *S. typhimurium* (NCBI GenBank GI: 47798). The OmpA encoding region was amplified using the designed primers Back: 5´-GGAAATTCGAGCTCTTA GTGAGATGGTGAC-3´ and Forward: 5´TCTAGAGGATCCTAAA CAATGGTCCTGCTACAGTCA-3´. Each polymerase chain reaction (PCR) mixture was prepared in a final volume of 50 µl containing 50 ng template DNA, 50 pM forward primer, 50 pM of the corresponding backward primer, 40pM dNTPs, 25 mM MgCl2, 1.25 U Taq DNA polymerase (Cinnagen) and 5 µl 10 × PCR buffer II (Cinnagen). Hot start PCR was performed at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s and elongation at 72°C for 1 min. The reaction was completed by a final extension time at 72°C for 1 min. The amplified variable regions were purified by electrophoresis on a 1.5% agarose gel and subsequently extracted with an AccuePrep Gel Extraction Kit (Bioneer) according to the manufacturer's instructions. Amplified DNA was cloned into T/A vector (pTZ57R) using the InsT/A clone PCR product Cloning kit from Fermentas (Cat. No.k1214) which is a convenient system for direct one-step cloning of PCR-amplified DNA fragments. pTZ57R plasmid was cut with BamH I and Sac I (Roche Applied Science). After another electrophoresis on a 1.5% agarose gel, the variable regions were excised and extracted with the AccuPrep Gel Extraction Kit (Bioneer). The fragment of interest was ligated with the correspondingly cut vector pBI121 in a 10 µl volume containing 50 ng vector DNA, threefold molar excess of the PCR product and 5 U of T4 DNA ligase (Fermentas) for 16 h at 14°C. *E. coli* TG1 cells (50 µl) were transformed with 2 µl of the ligation product via $CaCl_2$ method. One milliliter of prewarmed SOC medium (Sambrook et al., 2001) was immediately added and the cells were grown at 37°C for 1 h shaking with 250 rpm. Cells were plated on LB agar medium containing kanamycin (25 µg/ml) and incubated overnight at 30°C. Colonies were picked for plasmid extraction using the Accuprep Plasmid Extraction Kit (Bioneer). The engineered pBI 121 plasmids were used to transform the *Agrobacterium* strain LBA 4404 using a freeze and thawing standard protocol (Sambrook et al., 2001).

Transformation

Sterilized alfalfa shoots were propagated in vitro on Murashige and Skoog (1962) medium supplemented with 3% sucrose and solidified with 0.8% agar. These plants were subcultured every 2 weeks and the basal nodes of *in vitro* shoots were transferred to MS medium supplemented with 9% sucrose for microtuberization. Axenic plants of alfalfa were maintained under *in vitro* growth conditions at 26°C and 16 h photoperiod in the laboratory. For alfalfa transformation, *Agrobacterium* cells were used for transformation. Leaf explants of *in vitro*-grown were inoculated with *Agrobacterium* in a yeast extract peptone (YEP) liquid medium for 10 min. For effective transformation, the explants were placed in a co-culture medium containing 2.0 mg/l of 2, 4-dichlorophenoxyacetic acid (2,4-D). Two days later, the explants were transferred to the regeneration medium (MS medium, supplemented with 2.0 mg/l 6-benzylaminopurine (BAP), 0.01 mg/l α-naphthalene acetic acid (NAA), 200 mg/l cefatoxim and 100 mg/l kanamycin). The explants were transferred to the fresh medium at 2 weeks intervals. As a control, non-inoculated explants were cultured in the same medium without hormones and antibiotics. The induced shoots were then dissected from the explants and transferred to MS medium containing cefatoxim (200 mg/l) for *Agrobacterium* elimination and kanamycin (100 mg) as a selective marker.

PCR and southern blotting analysis

After rooting of the regenerated alfalfa plants on free antibiotic medium, they were transferred into the greenhouse and maintained to maturity. Genomic DNA from 200 mg each of non-transgenic plants as negative control and all putative kanamycin resistant plants was extracted from transgenic plants according to Albani et al. (1992). Transformed and control plant genomic DNA was used as a template to detect the OmpA gene by PCR under the conditions that were described before and with specific primers. The 1200 bp amplified DNA fragments were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide (EtBr). Transgenic plant Genomic DNA (10 mg) was digested with HindIII, SalI, XhoI and PstI and then was electrophoresed on a 0.8% agrose gel. After electrophoresing, the DNA was transferred onto a Hybond N+ nylon membrane (Amersham) using capillary transfer method and then hybridized according to Sambrook et al. (2001). The probe was prepared by means of PCR and labeled by [a-32P] dCTP. The primers and the composition of PCR system was the same to that in plasmid construction. PCR was performed also in the same way as described in plasmid construction.

RESULTS

Construction and transformation of vectors

The isolation and PCR amplification of the gene encoding the OmpA was performed. OmpA gene of the *S. typhimurium* was sub cloned into the expression vector pTZ57R. Targeting the restriction sites BamH1 and Sac1

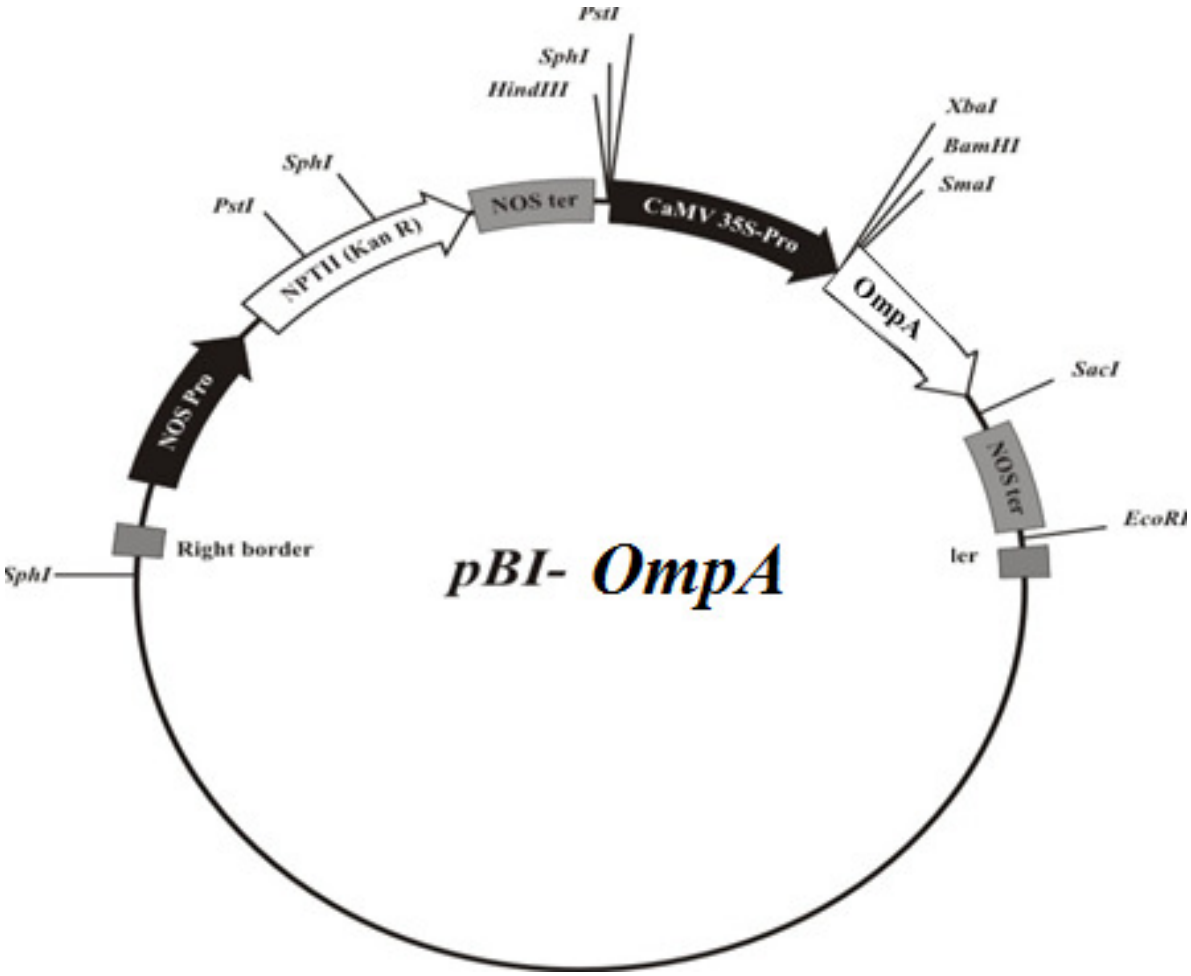

Figure 1. Schematic representation of pBI-OmpA construction. OmpA gene inserted between CaMV 35S promoter and NOS terminator.

in pTZ57R plasmid facilitated subcloning. New construct (plasmid) was transformed into TG1 *E.coli* and the colonies appeared on the kanamycin containing plate. After the extraction, pTZ57R and pBI121 plasmids were cut by BamH1 and Sac1 restriction enzymes. Electrophoresis showed 1200 bp bands from pTZ57R and 12000 and 1900 bp GUS removed gene from pBI121. The amplified 1200 and 12000 bp bands were gel-purified from agarose. During the ligation, OmpA gene inserted into pBI121 plasmid and new recombinant plasmid was transferred to *Agrobacterium* strain C58GV 3101 (Figure 1). When the leaf explants were inoculated with *Agrobacterium* immediately after excision, shoots on the cut edges of the explants were observed in the presence of 100 mg/l kanamycin and 200 mg/l cefatoxim after two weeks (Figure 2). The putatively transformed shoots were excised when they were about 1 cm tall and transferred to a shoot elongation medium containing cefatoxim and kanamycin. Consequently shots were transferred to rooting media and after hardenization to the greenhouse. The genes that had been stably integrated into the plant genome were translated under the control of CaMV35S, resulting in the expression of the OmpA gene and the growth of transformants on medium supplemented with kanamycin.

Expression of OmpA fragment in plants

We obtained more than 50 kanamycin-resistant putative transformants on selective media and carried out further analysis of OmpA gene expressions on selected transgenic plants. Untransformed alfalfa plants that had been regenerated from leaf discs without kanamycin selection, were used as negative controls. PCR analysis was carried out as the first method to confirm the transgenic nature of the regenerated plants. The presence of OmpA DNA in the genomic DNA isolated from regenerated alfalfa was confirmed. Transformants were detected by PCR amplification of inserted OmpA with specific primers.

Figure 2. Comparison of Transformed and Non-transformed leaves on selective media containing 100 mg /l kanamycin and 250 mg/l cefatoxim.

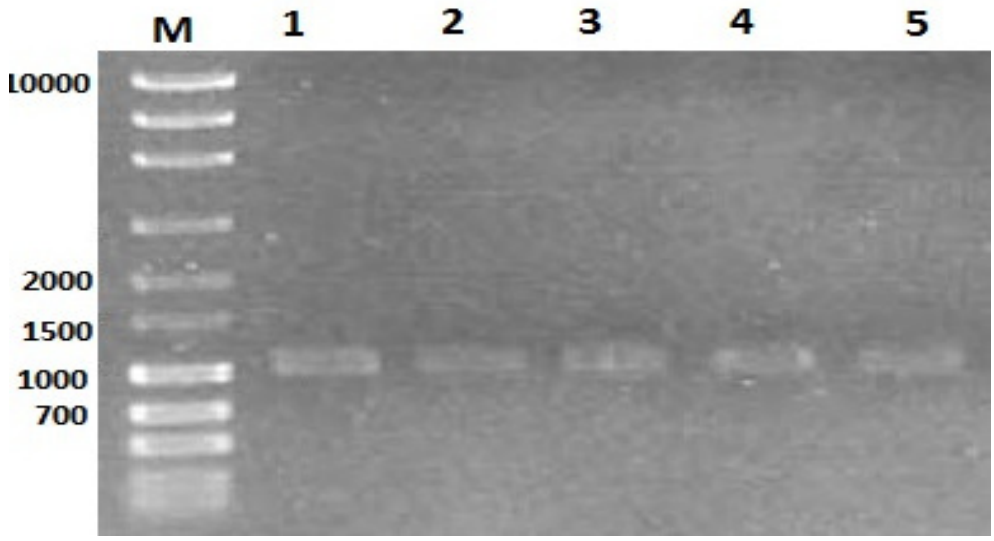

Figure 3. Agarose gel electrophoresis of OmpA amplification products from transgenic plants. Lane 1 MassRulerTM Express Forward DNA Ladder (Fermentas; Cat. No. SM1283); lanes 1 to 5 transgenic alfalfa plants.

The expected 1200 bp OmpA bands were found in the transformants (Figure 3). No DNA product was detected in untransformed control plant DNA. The same fragment was amplified using plasmid pBI 121 as our positive control.

Southern blotting analysis

The copy number of OmpA in an alfalfa genome was determined by DNA-gel blot analysis. Alfalfa chromosomal DNA was digested with HindIII, SalI, XhoI and PstI

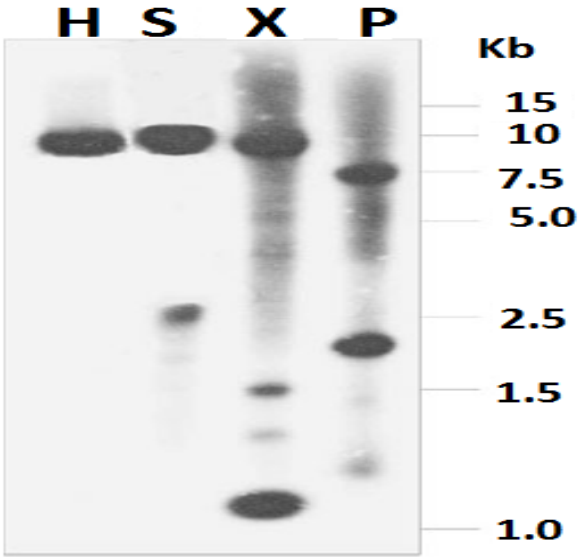

Figure 4. DNA-southern blot analysis of OmpA cDNA. The full-length OmpA cDNA was used as a probe. Numbers on the right are molecular weight markers in kilo bases.

and transferred to the membrane. The hybridization was carried out under high-stringency conditions using the full-length OmpA cDNA as a probe. Digestion with HindIII, which did not cut this gene showed a single hybridization band of a high molecular mass. For SalI (one site at 227) or PstI (one site at 453) treatment, two hybridization bands were detected. When the genomic DNA was digested by XhoI (two sites at 18 and 563), a specific hybridization band about 500 bp and upper bands were generated (Figure 4). These results indicate that this gene has only one copy in the alfalfa genome.

DISCUSSION

Expression of the first recombinant antibody in tobacco plant opens the field of the expression of recombinant antibodies as a real alternative to eliminate many constraints of monoclonal antibody production in bioreactors (Hiatt et al., 1980s). Expression of antigens in plants is being studied for their potential uses in biotechnology. We have described here for the first time, the expression of an OmpA gene in plants. To facilitate immunohistochemical, biochemical and bioassay investigation, large amount of OmpA is required; therefore we use the CaMV 35S promoter which is very strong constitutive promoter, causing high levels of gene expression in dicot plants. Before that, we use the T/A cloning vector (PTZ57R) because we want to take advantages of inserted multi cloning site (MCS) which is beneficial for cloning and also M13 primers around the MCS that facilitate the gene sequencing using this plasmid resulting PCR product ligated into a linear vector with a 3´ terminal 'T' or 'U' at both ends. Infectious *Agrobacterium* strain was LBA4404. The chromosome in *Agrobacterium* and activating potency of genes in virulence region are important internal factors influencing the infecting ability of *Agrobacterium* LBA4404. This bacterium not only has different chromosome background but also different Vir-helper plasmid with different levels of activating potency. Among leaves, stem fragments and root fragments, the optimum explants used in transformation of alfalfa are leaves. The reason is that the frequency of shoot induction of leaves is the highest and moreover, *Agrobacterium* has the strongest ability to infect leaves. Transformation efficiency can be improved by adding hormone to co-cultivation medium, because it can accelerate cell division of explants and maintain cell activity, consequently in favor of cell growth after transformation. The addition of hormone to co-cultivation medium resulted to increase in the frequency of Kanamycin resistant shoots for uninfected leaves. The possible reason is that shoot induction of alfalfa happened at early stage and could be started in short period. Therefore, the addition of hormone to co-cultivation medium likely started shoot induction and shoots continued developing even on selection medium. Based on the above reason, the solid MS medium without any hormone was used as co-cultivation medium in our case. Southern blotting and a hybridizing band were obtained from the transgenic plants (Figure 4). It indicated that the OmpA gene had been integrated into the genome of alfalfa.

REFERENCES

Albani D, Sardana R, Altosaar I, Arnison PG, Fabijanski SF (1992). A Brassica napus gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic plants. Plant J. 2: 332–342.

Foulds J, Barrett C (1973). Characterization of *Escherichia coli* mutants tolerant to bacteriocin JF246-2: New classes of tolerant mutants. J. Bacteriol. 116: 885–892

Haddad D, Liljeqvist S, Kumar S, Hansson M, Stahl S, Perlmann H, Perlmann P, Berzins K (1995). Surface display compared with periplasmic expression of a malarial antigen in *Salmonella typhimurium* and its implications for immunogenicity. FEMS Immunol. Med. Microbiol. 12: 175–186.

Koebnik R, Locher KP, Van Gelder P (2000). Structure and function of bacterial outer membrane proteins:barrels in a nutshell. Molec. Microbiol. 37(2): 239–253.

Ma JK, Barros E, Bock R, Christou P, Dale PJ, Dix PJ, Fischer R, Irwin J, Mahoney R, Pezzotti M, Schillberg S, Sparrow P, Stoger E, Twyman RM (2005). Molecular farming for new drugs and vaccines current perspectives on the production of pharmaceuticals in transgenic plants, EMBO Rep. 6: 593-599.

Morona R, Kramer C, Henning U (1985). Bacteriophage receptor area of outer-membrane protein OmpA of *Escherichia-coli* K-12. J. Bacteriol. 164 (2): 539–543.

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

Ried G, Henning U (1987). A unique amino-acid substitution in the outer-membrane protein OmpA causes conjugation deficiency in *Escherichia-coli* K-12.FEBS Lett. 223 (2): 387-390.

Schorr J, Knapp B, Hundt E, Kupper HA, Amann E (1991). Surface expression of malarial antigens in *Salmonella typhimurium*: induction of serum antibody response upon oral vaccination of mice. Vaccine 9: 675–681

Skurray RA, Reeves P (1974). F factor-mediated immunity to lethal zygosis in *Escherichia-coli* K-12. J. Bacteriol. 117: 100-106.

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM (2002). Practical considerations for pharmaceutical antibody production in different crop systems. Mol. Breed. 9: 149-158.

Thanassi DG, Hultgren SJ (2000). Multiple pathways allow protein secretion across the bacterial outer membrane. Curr. Opin. Cell Biol. 12: 420-430.