Expression of *Shigella flexneri* ipaB Gene in Tobacco

Mandana Ohadi, Rahimeh Rasouli, Elham Darzi-Eslam, Anis Jafari *, and Parastoo Ehsani †

Molecular Biology Unit, Pasteur Institute of Iran, Tehran, Iran
† These authors equally contributed to this work

Abstract

**Background:** Shigellosis is a leading cause of diarrhea in many developing countries and although the disease can be controlled and managed with antibiotics, the constant emergence of resistant species requiring ever newer antibacterial drugs make development of an effective vaccine necessary. The bacteria are highly contagious and since immunity to *Shigella* is serotype-specific a multi-serotype vaccine is required for adequate protection. Proteins encoded by *Shigella* invasion plasmid, which are part of the Type Three Secretion System (TTSS) of this bacteria, are good candidate as vaccine targets since they are both immunogenic and conserved between different *Shigella* species. The advent of molecular farming, which is a low cost system, has opened up new venues for production of recombinant proteins. In view of the difficulties encountered in expressing IpaB in *Escherichia coli* (*E. coli*), the feasibility of the expression of this protein in tobacco has been investigated.

**Methods:** The ipaB gene was cloned in place of the Hygromycin gene in pCambia1304 containing GFP as a reporter gene. The vector was then transferred into competent *Agrobacterium tumefaciens* (A. tumefaciens) strain LBA4404 which was used for agro-infiltration of *Nicotiana tobaccum* (N. tobaccom) leaves. Transformation was confirmed by expression of GFP. The gene was also cloned in pBAD/Genell A and transformed *E. coli* host containing the construct was induced using different amounts of L-arabinose as inducer. Expression of IpaB gene by both hosts was determined by Western blotting using anti-IpaB monoclonal antibody.

**Results:** The data obtained showed that IpaB was expressed in plant leaves but expression in *E. coli* was not detectable.

**Conclusion:** This study showed that *N. tobaccum* is capable of expressing this protein without its specific chaperon and in levels detectable by Western blotting.

**Keywords:** *Escherichia coli*, Recombinant proteins, Shigellosis, Tobacco

Introduction

Diarrhea caused by *Shigella* species is a severe disease with high morbidity and mortality especially in developing countries *. It has been estimated that in Asia 91 million *Shigella* episodes and 414,000 deaths occur annually with *Shigella flexneri* being the most common serotype *. As few as 10 organisms can cause illness and the low infective dose makes these bacteria highly contagious with oral-fecal contact being the predominant route of transmission from person to person. *Shigella dysenteriae* (*S. dysenteriae*), *Shigella*
flexneri, Shigella sonnei, and Shigella boydii are the four species that comprise the Shi-
gellae genus and are divided into over 45 serotypes depending on the structure of the O
antigen component of their outer membrane
lipopolysaccharide 1,3. The Shiga toxin-pro-
ducing S. dysenteriae serotype 1 causes the
most severe infections including hemolytic
uremic syndrome as well as dysentery epi-
demics 4.

Although vaccine development has been a
priority for the World Health Organization for
many years, no licensed vaccine is as yet
available and the emergence of drug resistant
strains makes the development of an effective
candidate vaccine even more urgent 5-7. Approaches to
candidate vaccines have included the use of
ekilled 8, live attenuated 9-12 and recombinant
carrier 13 organisms, polysaccharide conjugates
14,15, and LPS-protein mixtures 16,17. The
shortcomings of these candidate vaccines
have been either poor immunogenicity or high
reactogenicity when tested in humans. Fur-
thermore, it has been shown that immunity to
Shigella is serotype-specific, limiting the
scope of protection offered by these experi-
mentally developed vaccines and necessitat-
ing the development of a multi serotype vac-
cine for adequate protection 3.

Shigellae species, like many gram-negative
bacteria, rely on a TTSS as an essential viru-
lence component which is present at a density
of 50 to 100 per bacterial cell and resembles a
molecular needle and syringe. The needle tip
complex is composed of the invasion plasmid
proteins, IpaB, IpaC and IpaD which are re-
quired for invasion of epithelial cells as well.
These proteins are conserved between differ-
ent Shigella species and serotypes making
them desirable for use as candidate vaccines.
The protective efficacy of antibodies against
IpaB and IpaD (especially IpaB) was demon-
strated recently in a mouse model of intrana-
sal immunization and pulmonary challenge
with homologous and heterologous strains 3.

Hydrophobic IpaB protein is encoded by
1743 bp gene with an apparent molecular
weight of 57 kDa and its production in E. coli
has been difficult. Expression of IpaB in E.
coli has been only achieved fused with thiore-
odoxin or complexed with its chaperon IpgC
18,3 which in both cases require additional
steps to purify the recombinant protein.

Plants have been increasingly used for pro-
duction of genetically engineered biological
products in recent years and the term "Molec-
ular farming" has been coined to describe this
process 19. Several advantages are attributable
to this eukaryotic host such as low cost of
production, ease of scale up, ability to per-
form eukaryotic modification of the product
post-translationally 20. N. tobaccum has been
widely used in molecular farming as a non-
food and non-forage product that is easy to
manipulate and contains high fresh leaf
weight and seed 21. This is the first study on
production of IpaB protein in plants.

Materials and Methods

Constructs for expression in tobacco and E. coli

The IpaB coding region from Shigella flex-
neri for expression in tobacco plants and E.
coli was amplified using the following pri-
mers, which were designed in this study:

ipaB-tob-F: 5’-aata ctc gag gcc gcc acc atg
cat aat gta agc acc aca ac-3’
ipaB-tob-R: 5’-atat ctc gag tca tag ctc atc ttt
ctc aga gtg gtg gtg gtg gtg gtg gtg ace agt
agt ttg ttg cca att g-3’
ipaB-eco-F: 5’-ata gca cca tgg
gac ata atg taa
gca cca ca-3’
ipaB-eco-R: 5’-agc tct aga gta gtt tgt tgc aaa
att g-3’

Forward primer for cloning the PCR prod-
uct in pCambia1304 (a gift from Dr. Rajabi
Memari; Shahid Chamran University of Ah-
vaz), contained restriction site for XhoI (bold)
and Kozak sequence (underlined). In the re-
verse primer a restriction site for X
ho1 (bold),
a KDEL retention signal (underlined), and
His-tag sequence (italic) were included. Re-
striction sites for enzymes NcoI and XbaI
were incorporated in forward and reverse
primers used for amplification of the gene for
E. coli system, respectively.
The chemicals were purchased from Sigma and Merck companies, the enzymes and molecular weight markers were purchased from Fermentas, Lithuania.

The molecular biology techniques were done according to the gene cloning manual. The gene for use in both plant and bacterial system was amplified according to the Enzyme manufacturer protocol (Fermentas, Lithuania), briefly in aliquots of 50 μl containing 500 ng of template, 1.5 U High Fidelity Enzyme Mix, 2 mM MgSO4, 200 μM dNTP mixture 1 μM of each primer (Gene Fanavaran, Tehran). The mixture was subjected to an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension 72°C for 4 min and a final extension of 72°C of 10 min. PCR products were visualized by UV and eluted by gel extraction kit (Fermentas, Lithuania) according to manufacturer’s instructions. The purified fragments were cloned in pTZ57T according to the supplier’s instructions (Fermentas, Lithuania) according to manufacturer’s instructions. The purified fragments were cloned in pTZ57T according to the supplier’s instructions (Fermentas, Lithuania) and sequencing was performed by a commercial facility (Gene Fanavaran, Tehran).

After the sequence verification, the fragment to be cloned in pCambia1304 containing Kanamycin resistance gene was digested with XhoI and ligated with similarly digested vector which had been treated with shrimp alkaline phosphatase in place of hygromycin. Orientation of the cloned gene was determined by digestion with EcoRI.

The vector containing ipaB gene with correct orientation was transformed into competent Agrobacterium tumefaciens strain LB4404 (kindly provided by Dr. Salmanian, National Iranian Genetic Engineering Center) by freeze and thaw method. Transformed bacteria were cultured on LB plates containing kanamycin (50 μg/ml) and incubated at 28°C for 2 to 4 days.

Agro infiltration of leaves of tobacco plant N. tabacum var. samsun (kindly provided by Dr. Rajabi Memari, Shahid Chamran University of Ahvaz) was performed using the method described by D. Aoust et al. Briefly, a single colony of Agrobacterium LBA4404 containing recombinant plasmid was cultured in 5 ml LB medium containing kanamycin (50 mg/ml) at 120 rpm at 28°C for one day. Four hundred μl of the culture (OD=0.8) was centrifuged for 20 min at 4°C (2500 × g). The pellet was dissolved in 1 ml of resuspension solution (10 Mm MgCl2, 10 mM 4-morpholinoethanesulfonic acid (MES), 20 g/l Sucrose, 100 μl Acetosyringone (100 μM) and 1% Tween 20 in MS media) and incubated for 1 hr at 28°C at 120 rpm. Tobacco leaves were immersed in bacterial suspension and transferred to a glass container attached to a vacuum pump (0.5 mbar) for 4 min. Breaking the pressure in container facilitates entry of bacteria into the leaves. The infiltrated leaves were then rinsed by sterile water and put in transparent containers and incubated at 28°C under continuous illumination for 7 days.

Expression of GFP in the leaves was investigated under UV light (350 nm), Image machine 440 CF (Kodak UK). A week after agro infiltration, the leaves were used for total protein extraction. Briefly, tobacco leaves were ground using mortar and pestle and liquid nitrogen and 1 g of leaf-powder was added to 1 ml of protein extraction buffer containing phenylmethylsulfonyl fluoride (2 mM). The plant extract was loaded to a 12% SDS-acrylamide gel and after electrophoresis the proteins were transferred to nitrocellulose membrane (Schleider and Schull, Germany) for Western blotting using semi-dry blotting unit according to manufacturer’s instructions. The membranes were blocked with 0.5% skim milk in Tween-PBS and probed with anti-IpaB MAb (1:1000) a kind gift from Dr. Farida Nato, Pasteur Institute of Paris. Goat anti-mouse antibody (Sigma, USA) was used as the secondary antibody (1:8000) and the membranes were developed using DAB (3-3’ Diaminobenzidine Sigma, USA). The HRP conjugated anti-5xHis antibody was purchased from Qiagen and added in 1/10000 dilution.

The sequence of the gene for IpaB amplified with primers containing NcoI and XbaI.
restriction sites was verified (Gene Fanavaran, Tehran) and after double digestion with these enzymes and gel purification was cloned in similarly digested and purified pBAD/gIIIA (Invitrogen, USA).

Competent *E. coli* Top 10 F’ (Invitrogen, USA) was transformed with the construct and the transformed bacteria were selected on LB plates containing 50 μl ampicillin. The transformed bacteria were induced with different amounts of L-arabinose according to the supplier’s specifications. SDS-PAGE and Western blotting was performed as described above with HRP-conjugated anti-myc (Invitrogen, USA).

**Results**

The two amplified fragments are shown in figure 1 and the slight variation in size between the products is due to the additional sequences required for expression in tobacco.

The sequence of *ipaB* was verified after amplification and also after final cloning in plant expression vector. Prior to sequencing the orientation of the cloned gene in pCambia 1304 was determined by *EcoR* I (Figure 2) which has one site in the vector and another in the *ipaB* gene located at position 353 bp. Therefore, gene cloned with correct orientation produced a 1413 bp fragment after digestion with *EcoR* I.

The transformed *A. tumefaciens* were then selected on plates containing kanamycin and transformation was further verified by colony PCR (Figure 3).

Expression of GFP was detected by UV showing that *Agrobacterium* expression cassette containing *IpaB* and GFP genes had been successfully transferred (Figure 4).
Western blot analysis showed 57 kDa protein corresponding to IpaB protein that is absent in extracts of non-transformed leaves using anti His antibody (Figure 5).

After subcloning ipaB in pBAD/gIII expression vector and its transformation into E. coli (Top10), IpaB failed to be synthesized under any of the conditions used. Co-expression of the gene in the presence of plasmids containing non-specific chaperones such as DnaK, groE was also attempted, but did not result in production of the protein (data not shown).

Discussion

Recent studies have highlighted the importance of the Shigellae invasion proteins especially IpaB in inducing immunity against these bacteria which are a frequent cause of dysentery in areas of poor hygiene. These proteins are not produced by these bacteria in high abundance and are not readily purified from bacterial cultures. Therefore, to produce and purify the IpaB protein, recombinant technology was used. However, our attempt to express this protein in E. coli (Top10 expression host recommended by Invitrogen) using pBAD/gIII did not result in production of IpaB protein.

It had been shown that this protein could be expressed in E. coli fused to thioredoxin (Trx1) a small cytoplasmic protein which has been successfully employed as a fusion partner to increase the expression level and solubility of heterologous proteins in E. coli and seems to act mainly as a chaperone. Therefore, it seems that expression of IpaB in E. coli requires the presence of a chaperone either as a fusion partner, as was demonstrated by Picking et al who used Trx1, or IpgC which is specific for this and IpaC protein. Both of these strategies have disadvantages of requiring additional steps and time for cloning, separation and purification of IpaB, which lengthens the purification process and increases cost. IpaC also has been expressed in transgenic Arabidopsis. This protein is essential for bacterial entry into epithelial cells, capable of eliciting protective antibody responses in animal models. The expression level was 0.2% total plant protein.

Plants can produce proteins that are difficult or impossible to produce in bacteria, because unlike bacteria, inclusion bodies are not formed in plants eliminating the need for renaturation of product, thus avoiding any loss of activity. In addition, it is possible to lead the toxic proteins to intracellular compartments to protect cells from the toxic effects of the products. Transient expression also provides this advantage in addition to fast and higher expression level of recombinant proteins. A variety of genes have been expressed using agroinfiltration including interferons, growth hormone, antibodies and vaccine candidates. In addition, because of using non transgenic plants and short production period in controlled area, transient expression of the recombinant proteins does not produce any genetically modified foods and preventing social concerns.

The objective of this study was transient expression of ipaB using agro-infiltration technique. As efficiency of agro-infiltration depends on the ability of bacterial penetration inside the leaf tissue, the infiltration time and vacuum force is important. We found that 4 min infiltration at 0.5 mbar is sufficient for the transfer of Agrobacteria into tobacco leaves. Our findings also showed that in younger leaves the amount of recombinant

Figure 5. Western blot of leaf extract containing IpaB. Using anti His antibody, 1) infiltrated leaves, 2) MW, 3) plant negative control
proteins synthesized is higher indicating that the age of leaves affects the gene expression level (data not shown).

We used Kozak and KDEL sequences for enhancing the production of recombinant proteins. Kozak sequence lies within the 5' untranslated region of eukaryotic genes and directs translation of mRNA by causing the ribosome to pause and recognize the ATG codon. KDEL is a tetra amino acid sequence which keeps the protein from secreting out of endoplasmic reticulum, thus protecting the recombinant proteins from cytoplasmic proteases as well as preventing the addition of complex type glycosides.

pCambia1304 has been used to produce transgenic lines and contains GFP/GUS as reporter genes and hygromycin as antibiotic marker. Since in transient expression there is no need for plant selective marker we were able to use the selective marker site for expression of ipaB and GFP as a marker to determine the area of infiltration of the vector and the possibility of expression. To the best of our knowledge the gene replacement in this vector and the use of non-fused GFP as a reporter gene for successful transfer of Agrobacteria into tobacco leaves has not been previously documented.

Transient expression of recombinant proteins using agro infiltration is also more convenient for developing countries that do not have biosafety regulations for releasing the transgenic plants.

Acknowledgement

We gratefully acknowledge the technical help on detection of GFP given by Ms. Torkashvand from the Biotechnology Department of the Pasteur Institute of Iran.

References

1. Niyogi SK. Shigellosis. J Microbiol 2005;43(2): 133-143.
2. WHO. Shigellosis: disease burden, epidemiology and case management. Wkly Epidemiol Rec 2005; 80:93-100.
3. Martinez-Becerra FJ, Kissmann JM, Diaz-McNair J, Choudhari SP, Quick AM, Mellado-Sanchez G, et al. Broadly protective Shigella vaccine based on type III secretion apparatus protein. Infect Immun 2012;80(3):1222-1231.
4. Fontaine A, Arondel J, Sansonetti PJ. Role of Shiga toxin in the pathogenesis of bacillary dysentery, studied by using a Tox' mutant of Shigella dysenteriae 1. Infect Immun 1988;56(12):3099-3109.
5. WHO Initiative for Vaccine Research (IVR). Diarrheal diseases. 2009. Available from: http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index.html.
6. Wong MR, Reddy V, Hanson H, Johnson KM, Tsoi B, Cokes C, et al. Antimicrobial resistance trends of Shigella serotypes in New York City, 2006-2009. Microb Drug Resist 2010;16(2):155-161.
7. Ye C, Lan R, Xia S, Zhang J, Sun Q, Zhang S, et al. Emergence of a new multidrug-resistant serotype X variant in an epidemic clone of Shigella flexneri. J Clin Microbiol 2010;48(2):419-426.
8. McKenzie R, Walker RI, Nabors GS, Van De Verg LL, Carpenter C, Gomes G, et al. Safety and immunogenicity of an oral, inactivated, whole-cell vaccine for Shigella sonnei: preclinical studies and a Phase I trial. Vaccine 2006;24(18):3735-3745.
9. Barnoy S, Jeong KI, Helm RF, Suvarnapunya AE, Ranallo RT, Tzipori S, et al. Characterization of WRSs2 and WRSs3, new second-generation virG (icsA)-based Shigella sonnei vaccine candidates with the potential for reduced reactogenicity. Vaccine 2010;28(6):1642-1654.
10. Katz DE, Coster TS, Wolf MK, Trespalacios FC, Cohen D, Robins G, et al. Two studies evaluating the safety and immunogenicity of a live, attenuated Shigella flexneri 2a vaccine (SC602) and excretion of vaccine organisms in North American volunteers. Infect Immun 2004;72(2):923-930.
11. Kotloff KL, Pasetti MF, Barry EM, Nataro JP, Wasserman SS, Sztein MB, et al. Deletion in the Shigella enterotoxin genes further attenuates Shigella flexneri 2a bearing guanine auxotrophy in a phase 1 trial of CVD 1204 and CVD 1208. J Infect Dis 2004; 190:1745-1754.
12. Rahman KM, Arifeen SE, Zaman K, Rahman M, Raqib R, Yunus M et al. Safety, dose, immunogenicity, and transmissibility of an oral live attenuated Shigella flexneri 2a vaccine candidate (SC602) among healthy adults and school children in Matlab, Bangladesh. Vaccine 2011;29(6):1347-1354.
13. Kotloff KL, Losonsky AG, Nataro JP, Wasserman SS, Hale TL, Taylor ND, et al. Evaluation of the safety, immunogenicity, and efficacy in healthy vaccination of Shigella flexneri 2a bearing guanine auxotrophy in a phase 1 trial of CVD 1204 and CVD 1208. J Infect Dis 2004; 190:1745-1754.
adults of four doses of live oral hybrid Escherichia coli-Shigella flexneri 2a vaccine strain EcSf2a-2. Vaccine 1995;13(5):495-502.

14. Cohen D, Ashkenazi S, Green M, Lerman Y, Slep- pon R, Robin G, et al. Safety and immunogenicity of investigational Shigella conjugate vaccines in Israeli volunteers. Infect Immun 1996;64(10):4074-4077.

15. Passwell JH, Harlev E, Ashkenazi S, Chu C, Miron D, Ramon R, et al. Safety and immunogenicity of improved Shigella O-specific polysaccharide-protein conjugate vaccines in adults in Israel. Infect Immun 2001;69(3):1351-1357.

16. Fries LF, Montemarano AD, Mallett CP, Taylor DN, Hale TL, Lowell GH. Safety and immunogenicity of a proteosome-Shigella flexneri 2a lipopolysaccharide vaccine administered intranasally to healthy adults. Infect Immun 2001;69(7):4545-4553.

17. Tribble D, Kaminski R, Cantrell J, Nelson M, Porter C, Baqar S, et al. Safety and immunogenicity of a Shigella flexneri 2a Invaplex 50 intranasal vaccine in adult volunteers. Vaccine 2010;28(37):6076-6085.

18. Picking WL, Mertz JA, Marquart ME, Picking WD. Cloning, expression, and affinity purification of recombinant shigella flexneri invasion plasmid antigens IpaB and IpaC. Protein Expr Purif 1996;8(4):401-408.

19. Daniell H, Khan MS, Allison L. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends Plant Sci 2002;7(2):84-91.

20. Mett V, Farrance CE, Green BJ, Yusibov V. Plants as biofactories. Biotechnol. 2008;36(6):354-358.

21. Daniell H. Medical molecular farming: expression of antibodies, biopharmaceuticals and edible vaccines in plants. London: Kluwer Academic Publishers; 2003.

22. Sambrook J, Fritsch ER, Maniatis T. Molecular cloning, a laboratory manual. 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2003.

23. Shereman S, Bevan MW. A rapid transformation method for Solanum tuberosum using binary Agrobacterium tumefaciens vectors. Plant Cell Rep 1987;7(1):13-16.

24. D’Aoust MA, Lavoie PO, Belles-Isles J, Bechtold N, Martel M, Vézina LP. Transient expression of antibodies in plants using syringe agroinfiltration. Methods Mol Biol 2009;483:43-50.

25. Jurado P, de Lorenzo V, Fernandez LA. Thioredoxin fusions increase folding of a single chain Fv antibodies in the cytoplasm of Escherichia coli: evidence that chaperone activity is the prime effect of thioredoxin. J Mol Biol 2006;357(1):49-61.

26. Lammeli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680-685.

27. MacRaea AF, Preiszner J, Ng S, Bolla RI. Expression of His-tagged Shigella IpaC in Arabidopsis. J Biotechnol 2004;112(3):247-253.

28. Maghari BM, Ardekani AM. Genetically modified foods and social concerns. Avicenna J Med Biotech 2011;3(3):109-117.

29. Ma JK, Drake WMP, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 2003;4(10):794-805.

30. Fischer R, Schillberg S. Molecular farming, plant made pharmaceuticals and technical proteins. Weinheim: Wiley-VCH Verlag GmbH & Co; 2004.

31. Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 1987;15(20):8125-8148.