Expression of Protective Antigen of *Bacillus anthracis* in Iranian Variety of Lettuce Plastid (*Lactuca sativa L.*)

1Rahimeh Rasouli, 1Hosein Honari, 1Houshang Alizadeh, 2Mehran Gorjian, 3Mokhtar Jalali and 4Mahmoud Aalayi

1College of Agricultural & Natural Resources, University of Tehran, Tehran, Karaj, Iran  
2Department of Biology, School of Sciences, Payamme-Noor University of Mashhad, Mashhad, Iran  
3College of Agricultural, University of Tarbiat Modarres, Tehran, Iran  
4School of Pharmacy, Tabriz University of Medical Science, Tabriz, Iran.

**BV/ HIV Department, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran**

**Abstract:** Anthrax, a fatal disease caused by *Bacillus anthracis*, has introduced new aspects at biological warfare technology. Protective antigen (PA) is the most potent molecule for vaccination against anthrax. Transgenic plastids offer unique advantages in plant biotechnology, including high-level foreign protein expression. In this study, we have constructed a lettuce specific expression vector to transfer PA gene to lettuce chloroplast genome. The vector carries PA gene (domains 2-4) expression cassette using lettuce Prrn promoter and lettuce psbA terminator and a selectable marker cassette constructed. The construct flanked by two adjacent lettuce plastid genome sequences allowing its targeted insertion between the rbcL and accD genes. We showed that lettuce leaf chloroplasts can express PA protein to %7 of the total soluble protein and this confirmed by western blot. Transplastomic T0 plants were fertile. This system will open up of new possibilities for the efficient production of edible pharmaceuticals including vaccines in plants.

**Key words:** *Bacillus anthracis* · Protective antigen · Transplastomic expression vector · *Lactuca sativa*

**INTRODUCTION**

Plants are attractive to produce nontoxic edible vaccines [1]. Chloroplasts are attractive and ideal for scale up of production of recombinant proteins in plant because of presence a large number of transgenic copies per cell. Firstly Transformation to chloroplast was reported in green algae chlamydomonas [2]. Transformation to chloroplast provides advantages including lack of position effect, pleiotropic effects and gene silencing. In addition, it is possible to express multiple proteins via production of polycistronic mRNA. also Chloroplast has maternal inheritance [3, 4, 5]. The biolistic process or PEG method is utilized to delivery plastid vector [6-12]. Lettuce is one of the best candidates among the leafy crops. Use of chloroplast transformation system to leafy crop such as lettuce is ideal for commercial production of edible subunit vaccine and other recombinant proteins. Also it is opening up of facilities for metabolic engineering [13-17]. Scale up of pharmaceutical protein production could be obtained in transgenic lettuce via plastid transformation system. Besides this advantage, it possess extra advantages like being worldwide plant, it is consumable fresh and grows within a few months with high production of biomass, so lettuce has a potential as a bioreactor for producing recombinant proteins and edible vaccines [18, 19, 15].

Anthrax is a disease for animals and humans that caused by *Bacillus Anthracis*. This rod shape, gram positive, non-motile bacteria is listed as a class A agent by the center for disease control (CDC) of USA [20, 12]. Bacterial virulence is related to two plasmids PXO1 and PXO2. PXO1 carries the genes that code for toxin proteins and protective antigen (PA). PA is the most potent molecule that induces protective antibody against Anthrax. This antigen contains several immune dominant epitopes [21-27]. Prevention of disease is possible by vaccination and at present, effective and purified anthrax...
vacines applying PA protein are available at the market. Purity of vaccine, being multiple dose of PA are concerns which necessitate improving edible subunit vaccine [12].

Rakesh Bhatnagar (2002) firstly demonstrated PA expression in nuclear genome of tobacco that has spectinomycin and streptomycin resistance. PsbA (338bp) was expressed the protective antigen (PA) transiently using sets ([28]. Also Yusibov (2005) expressed the protective antigen (PA) transiently using sets ([12]. Gene was used as selection marker gene encoding bioactivity similar to native PA [28]. Also Yusibov (2005) terminator of lettuce plasmid was amplified by PCR primer set and specific primers ([72x598]Pro16s [72x573]was produced that has activity to neutralize toxin in vitro [29]. Koprowski (2005) expressed epitope in hybridoma [29]. Also Yusibov (2005) terminator of lettuce plasmid was amplified by PCR primer set and specific primers ([72x598]Pro16s [72x573]was produced that has activity to neutralize toxin in vitro [29]. Koprowski (2005) expressed epitope in hybridoma [29].

The aim of this study is to produce the Anthrax PA subunit vaccine candidate utilizing the constructed lettuce chloroplast expression vector.

MATERIALS AND METHODS

Seed Sterilization and Explant Preparation: N-96-39 variety of lactuca sativa as used for plasid transformation. Seeds were sterilized in 2.5% hypochlorite containing tween-20 (0.1%) for 25 minutes [31]. Seeds were rinsed three times with sterile distilled water and transferred to petri dishes for 68 hours. After germination they were placed on MS medium containing 0.2 mg/l (BA) and 0.2 mg/l (NAA) with pH 5.7 until they were used for plastid transformation by gene gun.

Construction of Chloroplast Vector: The genome information of lettuce chloroplast was obtained from (DDB)/Gene Bank/EMBL Accession AP007232. rbcL (1100 bp) and accD (1100 bp) genes of lactuca sativa were used as flanking sequences for foreign gene insertion into the chloroplast via homologous recombination. The DNA sequence related to the rbcL gene was amplified by PCR set from lactuca sativa sing PFU (Fermentas) and specific primers (rbcLFor, rbcLRev) (Table 1), containing Apal and Xhol restriction sites, respectively. The produced DNA was cloned into pGEM-T Easy vector. The DNA sequence related to the accD gene was amplified by PCR set and specific primers (accDFor, accDRev), containing NotI and Saci restriction sites, respectively. The PKZs plasmid (This vector provided by University of Munich, Department of Botany) including the prrn-aadA-psbA fragment was digested by PstI and XhoI and inserted into pGEM-T vector.

For construction of selectable marker cassette the 16srRNA promoter region and psbA terminator was obtained from Nicotiana tabaccum genome. The aadA gene was used as selection marker gene encoding aminoglycoside 3’adenyltranserase that confers spectinomycin and streptomycin resistance. PsbA (338bp) terminator of lettuce plasmid was amplified by PCR primer sets (psbATFor, psbATRev), containing Nol, EcoRI, HindIII and Sall restriction sites respectively.

16srRNA (113bp) promoter of lettuce plasmid was amplified by PCR set and with specific primers (Pro16sFor, Pro16sRev), containing psi, EcoRI and SalI restriction sites, respectively. Soeing PCR, one segment (451 bp) was obtain from two fragments (psbA338 bp and 16srRNA113 bp), also one fragment (1551 bp) was obtained from two segments accD (1100bp) and trrn-psbA (451 bp), then produced fragment (1551 bp) was cloned in pGEM-T Easy vector. This vector amplified, it was digested with SacI and NotI enzymes. pGEM-T vector containing rbcL and aadA gene fragments digested with SacI and NotI; then 1551bp fragment insert this vector and after sequencing introduced finally vector as pCL96-39 abbreviated. If pCL96-39 vector digest by Sall and HindIII Enzymes. Target genes can be cloned in the vector. PcL96-39 vector (Figure 1 the map of the constructed plasmid). pCL96-39 vector was sequenced by seq-lab Company.

The PA gene sequence was obtained from NCBI (AF306783) Primers PA gene(PA 2-4For, PA 2-4Rev) were designed by primer III program and all restriction sites were detected in PA segment by Bio labs-NEBcutter. in forward primer ATG nucleotides and spacer sequence related to the pGEM-T Easy vector. The genome information of lettuce chloroplast was obtained from (DDB)/Gene Bank/EMBL Accession AP007232. rbcL (1100 bp) and accD (1100 bp) genes of lactuca sativa were used as flanking sequences for foreign gene insertion into the chloroplast via homologous recombination. The DNA sequence related to the rbcL gene was amplified by PCR set from lactuca sativa sing PFU (Fermentas) and specific primers (rbcLFor, rbcLRev) (Table 1), containing Apal and Xhol restriction sites, respectively. The produced DNA was cloned into pGEM-T Easy vector. The DNA sequence related to the accD gene was amplified by PCR set and specific primers (accDFor, accDRev), containing NotI and Saci restriction sites, respectively. The PKZs plasmid (This vector provided by University of Munich, Department of Botany) including the prrn-aadA-psbA fragment was digested by PstI and XhoI and inserted into pGEM-T vector.

Plastid Transformation of Lettuce: TN96-39 variety of lactuca sativa was grown on MS media without Antibiotic (0.2 mg/l BA 0.05 mg/l NAA pH5.7) under 16h/8h light/dark photo period condition at 25°C. The leaves were cut and left on media for two days to use for biolistic bombardment. Leaves were bombarded using 0.6 nm gold particle coated with pcL96-39 vector using a PDS-1000/He biolistic particle delivery system (Bio rad set). Three series of bombardment were done. Two days after bombardment the leaves were cut and placed on the MS media containing 50 mg/l streptomycine and subcultured to new media every two weeks. Three regeneration preparations were done to confirm the homoplasity of lactuca sativa.
Table 1: list of primers for PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>accDFor</td>
<td>ATGCGGCCGCCCACCCATCCTGTATATTGTCC</td>
</tr>
<tr>
<td>accDRev</td>
<td>GCGCGAGCTTCTCATCCATAGGCTCCCAAAG</td>
</tr>
<tr>
<td>Pro16sFor</td>
<td>CTGCCAGGATATTGTGATTTGTGCTACCC</td>
</tr>
<tr>
<td>Pro16sRev</td>
<td>GAAATTCCGACATTTTCGCCCGAGTTCGCTCC</td>
</tr>
<tr>
<td>pshATFor</td>
<td>GTCGACGAATTCAGCTCTGGAGGAGCAGCAATGAAG</td>
</tr>
<tr>
<td>pshATRev</td>
<td>GCCGCCGGCTGAAACCCTGGCCTTTTGTATTAC</td>
</tr>
<tr>
<td>rbcLFor</td>
<td>ATGGGCCCGATATGAGCTTCATAGGACCAATGAAG</td>
</tr>
<tr>
<td>rbcLRev</td>
<td>CGCTCGAGCGATCCAGGGAAAATACAGG</td>
</tr>
<tr>
<td>pA2-4 For</td>
<td>GTCGACGGAGGTTTATGAAAAATGGAGCACGGCCTT</td>
</tr>
<tr>
<td>pA2-4 Rev</td>
<td>CCAAGCTTCATTAAAATTTTCTGTAGATCCGGT</td>
</tr>
</tbody>
</table>

Fig. 1: Scheme showing pcL96-39 vector, the pcL96-39 vector used for chloroplast transformation. The rbcL and accD genes were used as flanking sequences for homologous recombination.

Fig. 2: Transplastomic plants transformed with pcL96-39 vector in different steps
a: explants in preculture media
b: explants after bombardment
c: explants in 3rd regeneration media
d: transplastomic plants

The regenerated shoots were transferred to new media containing 0.5X MS media with 0.1mg/l NAA and 50 mg/l streptomycin pH 5.7 to induce rooting. For seed production, the transplastomic plants were cultivate for a week in vase containing perlite, in sterile pit soil for 45 days and kept for 2.5 months in greenhouse to produce seeds (Fig. 2).

**Plant PCR Analysis:** The plant leave DNA extraction was done according to Saghai-Maroof method. Briefly Change [32]. PCR with these primers (pA2-4 For, pA2-4 Rev) was done under condition (95°C for 5 min, 95°C 30 cycle 1 min, 61°C 1 min 30-35 cycles, 72°C for 1 cycle 7 min) PCR products were analyzed by agarose gel electrophoresis.

**Plant Protein Extraction:** 0.5 gr of leaves were weighted and then grounded in liquid N2 to a fine powder. Protein was extracted with by using 0.75 ml extraction buffer (Tirs-HCL 50 mM, EDTA 2mM pH:7.5,0.04% v/v (2- mercaptanol). The homogenate was centrifuged (30 min,4000 rpm,4°C) and the supernatant was centrifuged again then supernatant was transferred new 1.5 ml tube and stored -70°C.

**Protein Concentration Determination:** Concentration of protein samples were determined by Bradford method. In this method Bovine serum Albumin was utilized as standard for different concentrations.

**Protein Analysis Sds_Page and Western Blotting:** Total soluble protein of transplastomic plant was used for SDS-PAGE and comassie blue staining.Protein combined with sample loading buffer, boiled and then run on 12% SDS-PAGE gels for 1hr at 100 v.

The Nitrocellulose membrane was placed on the SDS-PAGE gel. The protein bands were transferred to paper for 30 min at 70 volt. The membrane was immersed in Pinesaps. After confirmation of transfer the membrane
was blocked for one hour. Primary antibody IgG (antisera was raised in mouse against purified PA by our team) was used and placed on the shaker for 1 hour. The blot was washed three times with TPBS. Then Secondary antibody (anti-Mouse Secondary Antibody, HRP conjugate and sigma) was added then shake for 1 hour. The DAB substrate solution prepared, then it was added to the membrane finally the reaction was stopped.

RESULTS

Amplification of Genes for Construction of Plasmid Pcl96-39: In order to construct chloroplast vector the \((rbcL1100bp), (accDL1100bp), (prnrn113 \text{ bp}) \) and \((psbA339 \text{ bp}) \) segments were amplified and showed using agarose gel electrophoresis (Fig 3 and Fig 4).

Confirmation of Pa2-4 Domains Sequence: Presence of \(PA\) gene was confirmed by gene specific primers of \(PA2-4\) for and \(PA2-4 \text{ Rev}\) (Fig. 5).

Analysis of Transgenic Lettuce: The \(T_0\) lettuce plant leaves were used for extraction of genomic DNA. The results confirmed the presence of \(PA\) and \(aadA\) genes using agarose gel electrophoresis (Fig 6).

Analysis of Soluble Protein in Transformed Plant Plastid: In order to test the production of \(PA\) protein SDS-PAGE was done. The results showed an extra band in 55 and 72KDa molecular weight area zone (Fig 7).

Western Blot Analysis of Transplastomic Lettuce Plant Extract: Western blot analysis showed that Antibody raised against domain 4 \(PA\) could recognize a 24 kDa band which is comparable to the size of protective antigen PA(63 kD) (Fig 8).
Fig. 8: Immunoblot detection of PA in transplastomic plants. Lane 1-5 transplastomic lettuce lane6 negative control (untransformed plant), M, molecular weight marker.

**Discussion**

It is more than two decade that plants are known as an expression system to produce recombinant proteins. Their simple system is economical and convenient with no human and animal pathogens or bacterial endotoxins. In addition, Chloroplast expression system has advantages of producing high amount of safe and cheap products [7, 33, 34, 35, 1]. There are several publications on expression of recombinant protein using tobacco transplastomic system. Such as: cry2Aa2 46.1%, ctxB-2L21 31.1%, tetC 25%, IFN2b 19%, msi-99 21% and lecA 6.3% of total soluble protein [36, 37, 9, 38, 39, 1, 40, 41]. Our results showed that we have PA as 7% of total soluble protein.

Until now 14 sites are recognized as a flanking regions [42] for construction of chloroplast vectors. We have amplified Tn9639 rbcl-accD sequence from regional lettuce for construction of a pCL96-39 expression vector. Kanamoto (2006) expressed GFP in lettuce chloroplast and they used rbcl-accD as flanking regions and they obtained 36% of total soluble protein expression in lettuce leaf [19]. The results showed that our constructed vector could expresses less amount of the recombinant PA (7%) which could be because of different promoter, terminator as long as inserted gene. Daniell (2005) used the trnI and trnA genes as homologous regions facilitate homologous recombination of the PA gene in chloroplast genome they reached PA expression levels to 14.2% of the TSP in leaves [43]. The reason could be the difference between promoter and terminator as long as tobacco expression system versus lettuce. In this study also rbcl-accD regions were used because of rbcl-accD homologous regions has a high level of gene expression. several elements have affect in chloroplast expression level. Daniell (2009,2010) demonstrated to obtain efficient chloroplast transformation, there is a need of using the endogenous regulatory elements, optimized plant growth regulators and suitable flanking regions [44, 45].

It is known, antibody against PA plays critical role to stimulate an appropriate immunity response in anthrax infection so it can be used as an efficient vaccine [23, 46].

In this study, the amount of external protein produced in lettuce leaves was estimated as 0.4 mg/gr leaves approximately and TSP in transgenic plants was 7%. if the amount as a vaccination dose of PA to vaccination dose is 1.5-7 mg and by assuming there is 10% absorption of that, the vaccination was done by eating lettuce 200gr in weight [19, 43].

Available human vaccine against Anthrax is the culture supernatant of Bacillus anthracis. Therefore, it may has side effects, due to a little amount of lethal and edema factors, so it requires introducing an appropriate expression system for safe and clean vaccine [47].

Our results suggest that plants have potential for a safe, large scale and efficient system for production PA candidate vaccine.

**References**


