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

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

[1]College of Agricultural & Natural Resources, University of Tehran, Tehran, Karaj, Iran
[2]Department of Biology, School of Sciences, Payamme-Noor University of Mashhad, Mashhad, Iran
[3]College of Agricultural, University of Tarbiat Modarres, Tehran, Iran
[4]School 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 T_{03} 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*

Corresponding Author: Houshang Alizadeh, University College of Agricultural & Natural Resources, University of Tehran, Tehran, Karaj, Iran. Tel: +09125601403.

vaccines 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 bioactivity similar to native PA [28]. Also Yusibov (2005) expressed the protective antigen (PA) transiently using by agroinfiltration of *Nicotiana benthamiana*. Antibody was produced that has activity to neutralize toxin in vitro and in vivo similar level to counterpart that was produced in hybridoma [29]. Koprowski (2005) expressed epitope from domain-4 (15 amino acid) of the protective antigen with alfalfa mosaic virus after injections of mice with recombinant plant virus particles,mice elicited an obvious immune response [30].

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 plastid 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 *ApaI* and *XhoI* restriction sites, respectively. The produced DNA was cloned into *pGEM-T Esay vector*. The DNA sequence related to the *accD* gene was amplified by PCR set and specific primers (*accDFor*, *accD*Rev), 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 (*psbAT*For, *psbAT*Rev), containing *NotI, EcoRI, HindIII* and *SalI* restriction sites respectively.

16srRNA (113bp) promoter of lettuce plasmid was amplified by PCRset and with specific primers (*Pro16s*For, *Pro16s*Rev), containing *pstI, EcoRI* and *SalI* restriction sites, respectively. Soeing PCR,one segment (451 bp) was obtain from two fragments (*psbA*338 bp and 16 srRNA113 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 *SalI* 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 segment(GGAGTTT) and restriction sites (gtcgac.ccaagc) in selective vetor was designed stop codon

After PCR by *PFU* enzyme the DNA fragment corresponding to the PA was introduced PcL96-39 via *HindIII* and *SalI* restriction Enzymes.

Plastid Transformation of Lettuce: TN96-39 variety of *lucctuca 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 homoplasty of *lucctuca sativa*.

Table l: list of primers for PCR

	Primer name	Sequence of primers
1	*accDFor*	ATGCGGCCGCCCACCCATCCTGTATATTGTCC
2	*accDRev*	GCGCGAGCTCTTCATCCATAGGCTCCCAAG
3	*Pro16sFor*	CTGCAGGATATTTTGATTTGCTACCC
4	*Pro16sRev*	GAATTCGTCGACATTTCGCCCGGAGTTCGCTCC
5	*psbATFor*	GTCGACGAATTCAAGCTTCTGGAGGAGCAGCAATGAAG
6	*psbATRev*	GCGGCCGCTGCAAACCGCTTTTGATTTAC
7	*rbcLFor*	ATGGGCCCAGTATGGTCGTCCCCTGTTG
8	*rbcLRev*	CGCTCGAGCGATCCAGGGAAAATACAGG
9	*pA2-4 For*	GTCGACGGAGGTTTATGAAAAATGGAGCACGGCTTCT
10	*pA2-4 Rev*	CCAAGCTTCATTAAAATTTTCTTGATCCCGTT

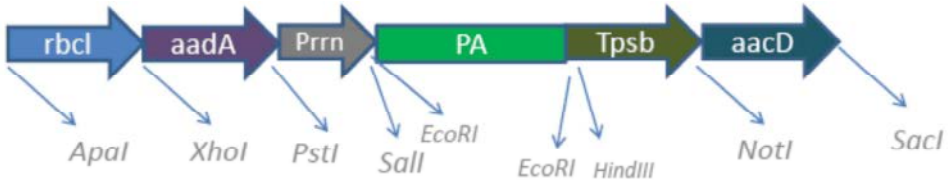

Fig. 1: Scheme showing *pcL96-39* vector, the *pcL96-39* vector used for chloroplast transformation. The *rbcL* and *accD* genes were used as flanking sequences for homologous recombination.

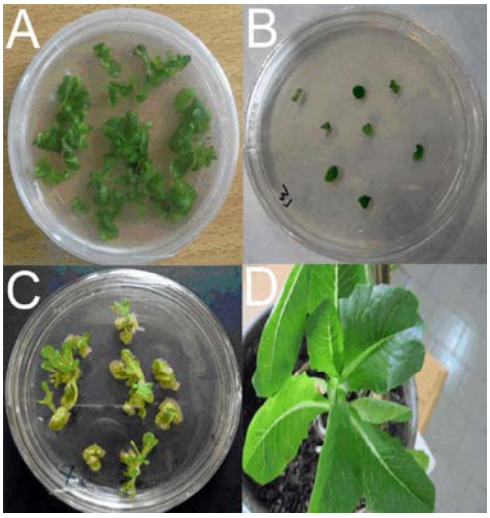

Fig. 2: Transplastomic plants transformed with pcL96-39 vector in different steps
a: explants in preculture media
b: explants after bombardment
c:explants in 3rd regeneration media
d: transplastomic plants

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 N_2 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*), (*accDL*1100bp), (*prrn*113 bp) and (*psbA*339 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* Rev (Fig. 5).

Analysis of Transgenic Lettuce: The T_{03} lettuce plant leaves were used for extraction of genomic DNA. The results confirmed the presence of *PA* and aid 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).

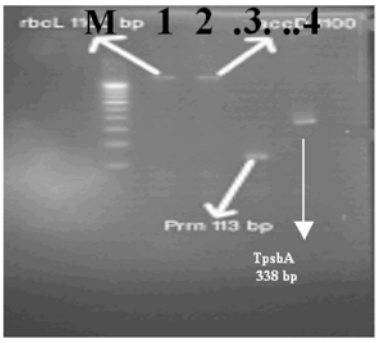

Fig. 3: Lane M is DNA molecular weight. Lanes 1,2,3,4 Amplification of (*rbcL*1100bp), (*accDL1100bp*), (*prrn*113 bp) and (*psb*A339 bp) genes respectively

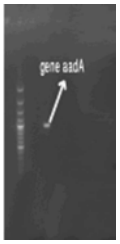

Fig. 4: Lane M: DNA molecular weight, Lane 1: *aadA* gene band.

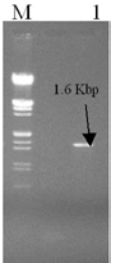

Fig. 5: Confirmation of *PA2-4* domain gene: Lane M is molecular marker Lane 1 is the amplified gene.

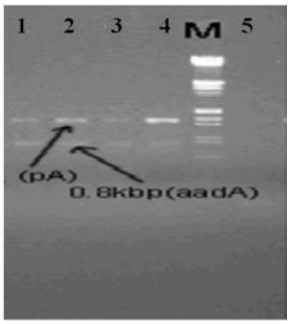

Fig. 6: Confirmation of presence of *PA* and *aadA* gene by PCR in T_{03} plants. Lane 1-4 transplastomic plants, lane M ladder, lane 5 untransformed plant leaf as negative control

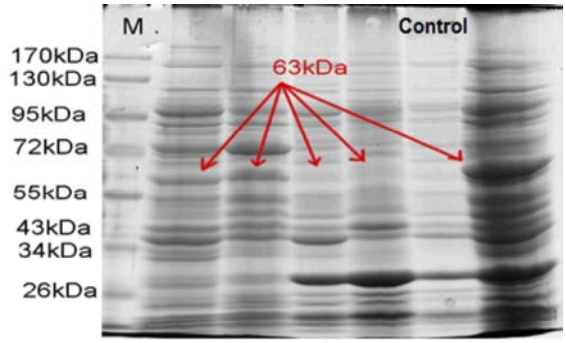

Fig. 7: Expression of (62 KDa) protein in transplastomic plants Lane M: Marker, lane 1-4 and lane 5: Transplastomic plants, lane control: Negative control

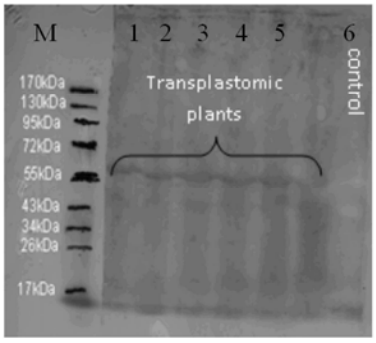

Fig. 8: Immunoblot detection of *PA* in transplastomic plants.Lane 1_5 transplastomic lettuce lane6 negative control (untransformed plant), M, molecular weight marker

DISSCUSION

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 homologouse 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.

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