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A plant based protective antigen [PA(dIV)] vaccine expressed in chloroplasts demonstrates protective immunity in mice against anthrax☆

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ABSTRACT

The currently available anthrax vaccines are limited by being incompletely characterized, potentially reactogenic and have an expanded dosage schedule. Plant based vaccines offer safe alternative for vaccine production. In the present study, we expressed domain IV of *Bacillus anthracis* protective antigen gene [PA(dIV)] in *planta* (by nuclear agrobacterium and chloroplast transformation) and *E. coli* [rPA(dIV)]. The presence of transgene and the expression of PA(dIV) in *planta* was confirmed by molecular analysis. Expression levels up to 5.3% of total soluble protein (TSP) were obtained with AT rich (71.8% AT content) PA(dIV) gene in transplastomic plants while 0.8% of TSP was obtained in nuclear transformants. Further, we investigated the protective response of plant and *E. coli* derived PA(dIV) in mice by intraperitoneal (i.p.) and oral immunizations with or without adjuvant. Antibody titers of $>10^4$ were induced upon i.p. and oral immunizations with plant derived PA(dIV) and oral immunization with *E. coli* derived PA(dIV). Intraperitoneal injections with adjuvanted *E. coli* derived PA(dIV), generated highest antibody titers of $>10^5$. All the immunized groups demonstrated predominant IgG1 titers over IgG2a indicating a polarized Th2 type response. We also evaluated the mucosal antibody response in orally immunized groups. When fecal extracts were analyzed, low sIgA titer was demonstrated in adjuvanted plant and *E. coli* derived PA(dIV) groups. Further, PA(dIV) antisera enhanced *B. anthracis* spore uptake by macrophages *in vitro* and also demonstrated an anti-germinating effect suggesting a potent role at mucosal surfaces. The antibodies from various groups were efficient in neutralizing the lethal toxin *in vitro*. When mice were challenged with *B. anthracis*, mice immunized with adjuvanted plant PA(dIV) imparted 60% and 40% protection while *E. coli* derived PA(dIV) conferred 100% and 80% protection upon i.p. and oral immunizations. Thus, our study is the first attempt in highlighting the efficacy of plant expressed PA(dIV) by oral immunization in murine model.

Abbreviations: AP, alkaline phosphatase; AVA, anthrax vaccine adsorbed; BAP, benzyl amino purine; BC, before challenge titers; cfu, colony forming units; CTAB, cetyltrimethyl ammonium bromide; CT, cholera toxin; DMEM, Dulbecco's modified eagle medium; EF, edema factor; ELISA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(2 ethanesulfonic acid); i.p., intraperitoneal; IPTG, isopropyl β-D-1-thiogalactopyranoside; LeTx, lethal toxin; LF, lethal factor; MAP kinase, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-5 diphenyltetrazolium bromide; MTD, mean time death; MWCO, molecular weight cut-off; NAA, α-naphthalene acetic acid; NBT, nitroblue tetrazolium; (NTdIV Nu), domain 4 from nuclear transformed plants; PA(dIV), domain IV of protective antigen *B. anthracis*; PAGE, poly acrylamide gel electrophoresis; PBS, phosphate buffered saline; PGA, poly-D-glutamic acid; PMSF, phenyl methyl sulfonyl fluoride; PA, protective antigen; SDS, sodium dodecyl sulphate; SM, selection medium; TSP, total soluble protein; TMB, tetramethylbenzidine; RM, regeneration medium; UTR, untranslated region; WT, wild type.

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1. Introduction

Anthrax, a zoonotic disease is caused by the gram-positive spore-forming bacterium '*Bacillus anthracis*'. Recently, anthrax has attracted considerable attention due to malevolent use of its spores as a biological weapon. The anthrax bioterror attacks in late 2001 in US *via* postal mail affected 22 people of which 5 succumbed to fatal death [1]. Anthrax is considered as one of the most resilient agents; the spores survive for decades and easily disseminate in the environment [2,3]. Anthrax spores can enter into the host through skin, inhalation or ingestion. The disease can take its worse shape leading to toxemia and death within few days of defined clinical symptoms [4].

The pathogenesis of anthrax is attributed to its 'tripartite exotoxin' comprising of protective antigen (PA), lethal factor (LF) and edema factor (EF) that exert their toxic effects in binary combinations and also the 'poly-D-glutamic acid (PGA)' component of the capsule that inhibit phagocytosis [5]. PA is the central component of anthrax toxin and as the name suggests provides protection against the disease. LF and EF are enzymatic components and form lethal toxin and edema toxin respectively, when combined with PA. The intoxication process is mediated by the binding of PA83 to the mammalian host cell receptors [6]. Upon proteolytic cleavage of 20 kDa protein at the furin protease site, the PA63 protein gets activated and oligomerises to form a heptameric prepore complex which competitively binds EF and LF. The heptameric complex is internalized by receptor mediated endocytosis. Under the influence of acidic pH in the late endosome, EF and LF are released and translocated into cytosol where they exert their toxic effects. LF, a Zn metalloprotease disrupts mitogen activated protein kinase (MAPK) signal transduction pathway leading to shock like symptoms and death [7]. EF, a calmodulin dependent adenylate cyclase augments cellular cAMP levels leading to imbalance in water homeostasis and edema [8]. In concern with the biological threat and potential lethal effects of *B. anthracis*, it becomes imperative to prevent the disease.

Vaccination is the most reliable means of prophylaxis against anthrax. All the current human anthrax vaccines have PA as the predominant or only component. The current licensed vaccines, the US based anthrax vaccine adsorbed (AVA) and the British vaccines are derived from the culture filtrates of acapsular, toxigenic strains of *B. anthracis* adsorbed to aluminum adjuvant [9,10]. Although, these vaccines proved to be protective in various animal models and humans [11–13] questions related to the characterization of vaccine components for each batch, reactogenicity, length of immunization schedule, route of administration remain a matter of concern [10]. Thus, the next generation vaccines focused on the development of subunit vaccines. The subunit vaccines since, free of pathogens are considered to be more safe and least reactogenic [14]. Since, PA is the most immunogenic component of anthrax toxin, the primary subunit vaccines focused on development of PA based vaccines.

Immunization with PA provides complete protection against anthrax spore challenge [11–13,15,16]. However, recent reports indicate that not all the antibodies generated against PA are protective. In fact, a subset of antibodies promote lethal toxin mediated killing [17]. The degree of immune response and the level of protection with recombinant PA vaccine was also not consistent in various animal models [18]. Moreover, these vaccines were prone to rapid degradation of protein thus, questioning the stability of the molecule for long term storage [19].

Studies have shown that 'Domain IV' (596–735 aa of PA) [PA(dIV)], the receptor binding element of PA, is sufficient to generate complete protection against toxin and spore challenge suggesting that dominant protective epitopes are present in this region [20,21]. Epitopes mapped to this region have shown to be protective in mice model [22,23]. Genetic deletion of PA(dIV) from *B. anthracis* genome showed a drastic decline in the virulence [24]. Also, monoclonal antibodies against this molecule are efficient in blocking PA from binding to the receptor [20,25]. Therefore, PA(dIV) can be used in effective vaccination against anthrax.

In order to overcome the problems associated with the stability and safety, vaccine antigens can be successfully expressed in plant system. Plant based vaccines are advantageous in being cost effective, provide easy scale up and are devoid of bacterial contaminants. Several studies have demonstrated the feasibility of expression of vaccine candidates in plant system. A plant produced poultry vaccine against Newcastle disease virus has already been commercialized (www.dowagrosciences.com) and many antigens expressed in plants, e.g.: HBSAg, Norwalk virus capsid protein, *E. coli* heat labile toxin, rabies glycoprotein have shown to be protective in clinical trials [26,27].

Most of the current approaches used in generating plant based vaccines relied on agrobacterium mediated transformation, that target the genes to nucleus. The impinging problems of nuclear transformation associated with low expression levels, position effects due to random gene integration, and safety due to environmental dissemination of genes by pollen has hampered its expediency for commercialization [26,28]. Chloroplast transformation technology has offered immense relief in this regard by generating enormous expression levels [29]. Chloroplast transcription machinery is akin to the prokaryotic system. Therefore, multiple genes can be processed thus, facilitating the expression of polyvalent vaccines. Other significant attributes include targeted gene integration that precludes position effects and the containment of transgenes due to maternal inheritance [30]. More importantly, the proteins expressed in plastids were found to be stable in the gut and were efficiently transported to the circulatory system [31]. Previous studies have demonstrated successful expression of antigens against cholera [32], tetanus [33], anthrax [34,35], plague [36], ameobiasis [30], canine parvovirus [37], HIV [38] and malaria [39] in chloroplasts. These studies have opened new avenues for development of effective plant based vaccines. The present study explores the feasibility of PA(dIV) expression in plant system by nuclear and chloroplast transformation. The study also compares the protective efficacy of plant expressed PA(dIV) with that of recombinant domain IV i.e.: rPA(dIV) derived from expression in *E. coli*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli strains, DH5α and XL Blue were used for the maintenance and propagation of plasmids. Luria Bertani (LB) + ampicillin (100 mg/l) or kanamycin (50 mg/l) or spectinomycin (100 mg/l) was used to grow bacteria depending on the antibiotic resistance gene in the constructs. Agrobacterium strain GV2260 (procured from lab stock) was cultured in yeast extract medium (YEM) supplemented with kanamycin (50 mg/l) or rifampicin (10 mg/l). All the room temperature chemicals and plant tissue culture media components and hormones were procured from Sigma–Aldrich (USA). Murashige and Skoog (MS) media was purchased from Himedia Laboratories (India).

2.2. Cloning of PA(dIV) gene in pCAMBIA vector

pCAMBIA-1303 vector purchased from Cambia (Australia) was used for cloning PA(dIV) gene. The vector is marked by a constitutive CaMv 35S promoter, *npt*II (neomycin phosphotransferase) gene for bacterial selection, *hpt*II (hygromycin phosphotransferase) gene

for the selection of plant transformants. PA(dIV) fragment (595–735 aa) of protective antigen gene was (PCR)-amplified from *B. anthracis* with forward primer: 5′-CGG GAA GAT CTG TTT CAT TAT GAT AGA AAT AAC ATA G-3′, reverse primer: 5′-CCG AAG ATC TAC TCC TAT CTC ATA GCC-3′ and cloned in pCAMBIA vector. The resulting construct pCAM1303PA(dIV) (Gen Bank accession number HQ130723) was mobilized into *Agrobacterium tumefaciens* GV2260 by freeze thaw method.

2.3. Cloning of PA(dIV) gene in pCHV-RKB chloroplast transformation vector

PA(dIV) fragment was cloned in a chloroplast vector pCHV-RKB (Gen Bank accession number HQ130724). The sequences derived from plastid genome (110,000–112,444 bp) facilitate the integration of foreign gene by an event of homologous recombination. PA(dIV) was amplified from the genomic DNA of *B. anthracis* sterne strain with gene specific primers. Forward primer: 5′-AAA AGG AAA AGC GGC CGC AGG AGGTTT AT ATG TTT CAT TAT GAT AGA AAT AAC ATA GCA GTT GGG-3′, reverse primer: 5′-CCG CTC GAG TTA GTG ATG GTG ATG GTG ATG TCC TAT CTC ATA GCC-3′. Forward primer was designed to introduce sequence for ribosome binding site RBS (GGGAG), a spacer (TTTAT), an initiation codon AUG and restriction site for NotI enzyme at 5′ end. The reverse primer included an XbaI restriction site. The putative clone [pCHV-RKB-PA(dIV)] (Gen Bank accession number HQ130725) was confirmed by restriction digestion and automated DNA sequencing. The expression of PA(dIV) was initially verified in *E. coli* by SDS PAGE, followed by immunoblot detection using polyclonal anti-PA antibodies.

2.4. Cloning expression and purification of PA(dIV) in pET-28a vector

PA(dIV) gene was PCR amplified from genomic DNA of *B. anthracis* sterne strain and cloned in pET-28a vector (Novagen) in BamHI and HindIII sites (Gen Bank accession number HQ130722). For purification of PA(dIV), the plasmid was transformed into BL21 (λDE3) cells. Cells were induced at $O.D._{600} \sim 0.8$ with 1 mM isopropyl β-D-thiogalactoside (IPTG) and incubated at 37 °C on rotary shaker. PA(dIV) was purified under denaturing conditions with Ni^{2+}-NTA affinity chromatography. Purified samples were analyzed on 15% SDS PAGE and electroblotted. Fractions containing the purified protein were pooled and extensively dialyzed against 10 mM HEPES (pH 8.0) buffer containing 10% glycerol.

2.5. Plant transformations

Agrobacterium mediated transformation was carried out by method described previously with slight modifications [40]. MS media supplemented with 0.5 mg/l of 1-napthalene acetic acid (NAA) and 2.5 mg/l of 6-benzylaminopurine (BAP) [regeneration medium (RM)] along with 25 mg/l of hygromycin and 500 mg/l of cefotaxime was used as selection media. All the putative transgenic plants were maintained in aseptic conditions with appropriate photoperiod 16 h light and 8 h dark at 26 °C.

Chloroplast transformation of *Nicotiana tabaccum* with pCHV-RKB-PA(dIV) was carried out with biolistics [Gene gun PDS-1000/He Bio-Rad (Hercules, CA, USA)] as described by Aziz et al. [35]. The regenerated shoots were subjected to further rounds of selection to achieve homoplasmy, finally transferred to rooting media and maintained with same procedures and conditions as described above for nuclear transformants. Wild type untransformed leaf explants maintained on selection media served as negative control while explants without selection served as positive control throughout the experiments.

2.6. Molecular analysis of putative transformants

Both, nuclear and chloroplast transformants were further analyzed for the presence of PA(dIV) gene. Genomic DNA was isolated from putative transgenic plants by CTAB method [41] or Qiagen kit according to the manufacturer's protocol. PCR was carried out with PA(dIV) gene specific primers to ascertain transgene integration. Site-specific integration and homoplasmy in transplastomic plants was ascertained by choosing one primer from the homologous sequence of the plastid genome and other from PA(dIV) gene itself. Further, southern blot hybridization was carried out as described by Aziz et al. [35]. Nuclear transgenic plant genomic DNA was digested with NcoI enzyme while DNA from transplastomic plants was digested with PstI enzyme and further hybridized with biotin labeled gene specific probe. Detection of hybridization signal was carried out with chemiluminescence detection system (Pierce Biotechnology, USA).

2.7. Extraction of total soluble protein (TSP) and immunoblot detection

Total soluble protein (TSP) was extracted from the leaf material ground in liquid nitrogen in HEPES buffer supplemented with 20% glycerol as described previously [40]. 2.5 ml of extraction buffer (EB) was added for each gram of leaves. The protein samples (boiled or unboiled) were resolved on 15% SDS and then transferred to nitrocellulose membrane for 12 h at 20 V. Primary antibody (polyclonal anti PA antibody raised in rabbit) at a dilution of 1:500 and 1:5000 was used for nuclear and chloroplast derived samples, respectively. Membrane was then probed with alkaline phosphatase conjugated secondary antibody at a dilution of 1:10,000 and developed using NBT–BCIP substrate.

2.8. Estimation of PA(dIV) in TSP samples

PA(dIV) expressed in transgenic plants (nuclear and chloroplast) was quantified with ELISA as described previously [35]. Serial dilution of rPA(dIV) (1–0.2 μg) and TSP (1–16 μg) extracted in phosphate buffered saline (PBS) was coated 100 μl/well in 96-well round bottom ELISA plate (BD Pharmingen, USA) and incubated for 1 h at 37 °C and overnight at 4 °C. After blocking, incubations with primary (anti PA antibody) and HRP conjugated anti rabbit IgG secondary antibody (Sigma), tetramethylbenzidine (TMB) substrate (BD Pharmingen, USA) was added (100 μl/well) for detection. The reaction was finally stopped with 1 M H_3PO_4 and the plate was read at 450 nm using ELISA reader (TECAN Group Ltd., Männedorf, Switzerland). Standard curve obtained with purified PA(dIV) samples was used to derive the amount of PA(dIV) present in TSP samples. All the reactions were carried out in triplicates.

2.9. Mice immunizations

Inbred BALB/c mice of 4–6 weeks age with an average weight of 18–20 g were procured from National Institute of Nutrition (NIN), Hyderabad, India. All the experiments were carried out in accordance with the regulations of Indian Animal Ethics Committee (IAEC) of Jawaharlal Nehru University (JNU).

Initially 2 groups of mice (NTdIV Nu) (TSP from nuclear transformed plants) and WT (TSP from wild type) comprising of 6 mice each were injected thrice 2 weeks apart intraperitoneally with TSP (containing 5 μg of PA(dIV)) derived from nuclear transgenic plants. Alhydrogel (20%, v/v) was used as an adjuvant.

Subsequent immunization experiments were performed in 9 groups of BALB/c mice, each group consisting of 5–6 mice. Two routes of immunization either oral or intarperitoneal were carried out with plant expressed PA(dIV) or recombinant purified

PA(dIV) from *E. coli* [rPA(dIV)]. For intraperitoneal injections, 10 μg of rPA(dIV) or TSP concentrated to contain 10 μg of PA(dIV) was adsorbed 20% (v/v) of alhydrogel and immunized (100 μl of sample per site). The TSP was passed through centricon 5 kDa MWCO spin filter to concentrate the plant protein and remove contaminants. For oral immunization, 10 μg of rPA(dIV) or TSP concentrated to contain 10 μg of PA(dIV) mixed with 5 μg of cholera toxin (CT) or without CT was immunized by gavaging. All the mice that were to be orally immunized were deprived of food 8 h prior to and 1 h after immunization. 0.4 mg of omeprozole (Ranbaxy Labs Ltd., India) was given 1 h prior to gavaging for neutralization of gastric acids.

Immunizations i.p. or oral were carried out on day 0, 7, 14, 21, and 28. A final booster of 10 μg of purified rPA(dIV) was given by i.p./oral on day 175 in all groups except the control groups PBS/WT/WT + CT. Control mice received TSP from wild-type plant material with or without CT. Blood and fecal matter were collected 7 days after each immunization. Sera and fecal pellets were stored at −80 °C for further analysis. Immunizations were performed as tabulated in Table 1.

2.10. Serum and fecal sample preparation

Immediately after collection of the blood from the retro-orbital plexus of the mice, the serum was separated and stored at −20 °C until further analysis. Fecal pellets for each group of mice were weighed and dissolved in PBS buffer supplemented with 0.25 mM protease cocktail inhibitor and 1 mM PMSF. PBS 10 μl, was added for every mg of dry fecal matter. The samples were vortexed, soaked overnight at 4 °C and then centrifuged at 17,000 × *g* for 20 min. The supernatant obtained was used to determine the IgA antibody levels.

2.11. Estimation of antibody titers and isotyping

The presence of PA(dIV) specific antibodies in the sera and fecal matter of immunized mice was determined by ELISA as described previously [35]. Microtiter plates were coated with 0.5 μg of rPA(dIV) diluted in PBS and incubated at 4 °C overnight. The serum obtained at various immunization points was serially diluted five folds for IgG antibody titers and two folds for determination of IgA antibody levels and incubated for 1 h. Peroxidase conjugated rabbit anti-mouse IgG /IgG1/IgG2a/IgA (Santa Cruz Biotechnology, Inc., USA) was used as secondary antibody. End point titers were expressed as the maximum dilution of the sample giving an absorbance at least twice as wild-type background. The results are presented as the reciprocal of the mean antibody titers done in triplicates for each sample.

2.12. Lethal toxin neutralization assay

The titer of the toxin neutralizing antibody in immune sera or feces was determined based on the ability of the antibody in the sera or fecal matter to inhibit the cytotoxicity of the lethal toxin. The assay was performed as described previously [25] with slight variations. Briefly, J774A.1 murine macrophage cells grown in Dulbecco's modified eagle medium (DMEM, Sigma) containing 10% inactivated fetal bovine serum (FBS) (BD Biosciences, USA) were plated at 3×10^4 cells/well in 96-well titer plate. Cells were incubated at 37 °C in CO_2 incubator for 16 h. Serial dilutions of antisera were made in DMEM in separate microtiter plates to which PA 250 ng/ml and LF 100 ng/ml was added. The media was removed from the cells and the serum toxin mixture was added at 100 μl/well and incubated at 37 °C in CO_2 incubator for 4 h. The serum toxin mixture was then removed, 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide) was added and then incubated for 45 min. Solubilization buffer [0.5% (w/v) SDS and 25 mM HCl in 90% (v/v) isopropanol] was added at 100 μl/well. Cells receiving no lethal toxin were used to determine 100% cell viability. Lethal toxin neutralizing titer [NC50] was defined as the dilution resulting in 50% protection of the cells. A positive control that resulted in 50% protection of the cells as derived from the standard curve was also included in the experiment.

2.13. Spore uptake assay

B. anthracis sterne 34F2 spores were prepared as described earlier [42]. The vegetative bacilli that remained after spore preparation were heat killed at 65 °C for 30 min and viable spore titer was determined by counting cfu after plating various dilutions of spores according to the method described previously [18]. J774A.1 murine macrophage cells at 1×10^5 were infected with the opsonized spores at a multiplicity of infection (MOI) 1:1 (macrophage to spore ratio). The cells were harvested after defined time intervals (1, 1.5 and 3 h) and plated on BHI agar to determine viable bacterial cfu.

2.14. Toxin challenge

The acapsular *B. anthracis* sterne strain vegetative cells were used to challenge the mice as described previously [43]. The mice were challenged i.p. with 10^8 cfu/0.2 ml of *B. anthracis* sterne strain vegetative cells.

3. Statistical analysis

All data were expressed as mean ± SD and are representation of at least 2 different experiments. Student's *t* test was done to derive *p* values and $p < 0.05$ was considered statistically significant. Titers among the groups were analyzed by two way ANOVA followed by Bonferroni post tests. Sigmaplot 11.0 and Graph pad prism version 5.0 (Graphpad Software, Sandiego, CA) softwares were used to perform statistical analysis.

4. Results

4.1. Evaluation of PA(dIV) clones

All the constructs, pCAM-PA(dIV); pCHV-RKB-PA(dIV) (Fig. 1A and B); pET28a-PA(dIV) were confirmed by PCR analysis with PA(dIV) gene specific primers and restriction digestion. The *prrn* promoter (plastid ribosomal RNA promoter) one of the strongest promoters evaluated till date drives the *aad A* (aminoglycoside 3″-adenylyltransferase) gene that confers resistance to spectinomycin [44]. Upstream elements; the ribosome binding site sequences, a spacer and a start codon was added for regulation of PA(dIV) gene. T*psba* 3′ UTR was added to confer stability to mRNA transcripts (unpublished data). Owing to the similarity of *E. coli* and plastid transcription machineries, the expression of PA(dIV) was initially checked in *E. coli* before commencing the plastid transformation experiments. Immunoblot detection depicted a 16.5 kDa band thus, confirming the expression of pCH-RKB-PA(dIV) in *E. coli* (data not shown). With respect to PA(dIV) expressed in *E coli*, protein pET-28aPA(dIV) when purified under denaturing conditions afforded > 95% purity and resulted in 28 mg/l yield. Purified PA(dIV) protein from pET28a-PA(dIV) when subjected to immunoblot detection with polyclonal anti-PA antibodies raised in rabbit demonstrated single 17.5 kDa band without any cross reactivity. The purified protein rPA(dIV) was found to be stable for at least 6 months at 4 °C. All the constructs and hosts used in this study have been tabulated in Table 1 (supplementary data).

Table 1

Group	Description	Mode of immunization	Schedule of immunization	Final booster 10 μg rPA(dIV) mode
[rPA(dIV) + CT]	Recombinant domain 4 from *E. coli* + cholera toxin	Oral gavage	0, 7, 14, 21, 28	Oral gavage
[rPA(dIV) + Al]	Recombinant domain IV + alhydrogel	Intraperitoneal injection (i.p.)	0, 7, 14, 21, 28	i.p.
[NT-PA(dIV) + CT]	Tobacco domain IV TSP + CT	Oral gavage	0, 7, 14, 21, 28	Oral gavage
[NT-PA(dIV)]	Tobacco domain IV TSP	Oral gavage	0, 7, 14, 21, 28	Oral gavage
[NT-PA(dIV) + Al.]	Tobacco domain IV TSP + alhydrogel	i.p.	0, 7, 14, 21, 28	i.p.
[WT + CT]	Wild type TSP + CT	Oral gavage	0, 7, 14, 21, 28	Nil
[WT]	Wild type TSP	Oral gavage	0, 7, 14, 21, 28	Nil
[PBS]	Phosphate buffered saline	i.p.	0, 7, 14, 21, 28	Nil

4.2. Generation of transgenic plants and molecular analysis

The shoots obtained after 3 weeks in nuclear transformants, when analyzed for the presence of PA(dIV) gene by PCR demonstrated an amplicon of 420 bp. Transgene integration was confirmed by southern blot analysis (Figs. 2A and 3A). Further, a 16 kDa protein was obtained as assessed by immunoblot detection which confirmed the expression of PA(dIV) in transgenic plants (Fig. 4A). All the plants either transformed with vector alone or the wild-type plants did not show any amplification or protein band when analyzed by PCR, southern blot and western analysis respectively (Figs. 2A, 3A and 4A). ELISA results demonstrated an expression levels 0.3–0.8% of total soluble protein.

With respect to plastid transformation with pCHV-RKB-PA(dIV) an amplicon of 450 bp PA(dIV) was observed when amplified with gene specific primers (Fig. 2B). To strengthen our results and confirm the site specific integration of the gene in the plastome, PCR amplification with a forward primer specific to chloroplast genome and the reverse primer specific to PA(dIV) gene was performed. An amplicon of the expected size 2.5 kb was obtained in transplastomic samples (Fig. 2C). Southern blot analysis with gene specific probe showed transgene integration at specific site (Fig. 3B). Enzyme PstI cuts once each in the *aad* A region and the intergenic region. Immunoblot detection of TSP from PA(dIV) transplastomic plants when probed with anti-PA antibodies resulted in a distinct 16.5 kDa band while TSP from WT plants did not display any protein band (Fig. 4B). PA(dIV), when quantified by ELISA showed expression levels of 3.7–5.3% TSP in mature leaves. Mature leaves demonstrated higher expression levels of PA(dIV) protein as compared to young and old leaves. Expression levels as low as 1.5–3.3% was seen in old or young leaves (Fig. 5). A quantification protein of leaf is given in Table 2 (supplementary data).

4.3. Generation of serum IgG immune responses

Mice immunized with TSP from pCAM-PA(dIV) plants 2 weeks apart (3 immunizations) by intraperitoneal immunization generated very low IgG titers of 10^3. Toxin lethal neutralization titers of the order 10^1 were detected in this group (data not shown). These results prompted us to change the dosage and immunization schedule to enhance the immune response. Hence, an immunization schedule that would be suitable for i.p. and oral immunizations was planned. Immunization with plastid expressed PA(dIV) and recombinant purified PA(dIV) was assessed by measuring antibody titers after oral and i.p. immunization in mice. Mice were immunized on day 0, 7, 14, 21, 28. Sera and fecal matter was collected prior to

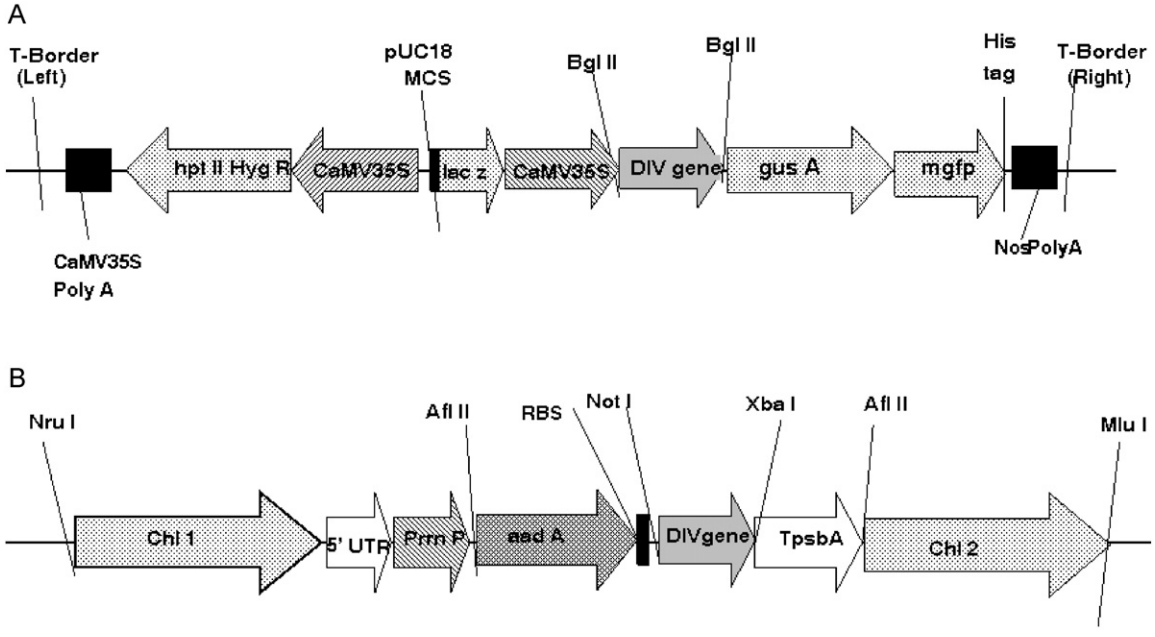

Fig. 1. (A) Schematic representation of plasmid 1303-PA(dIV). Domain IV of protective antigen gene (*pag A*) was cloned in BglII site driven by Cauliflower mosaic virus (CaMV) 35S promoter. The construct also contains *hygromycin phosphotransferase* gene (*hptII*), glucorinidase A (*gus A*) gene, modified green flouroscent protein *(mgfp5)* gene for selection of transformants. Other elements of the construct include CaMV poly A, Nopaline synthase poly A terminus, *β-galactosidase* gene for screening bacterial colonies and right and left T border sequences for efficient transfer of the construct in to the plant cell. (B) Domain IV gene was cloned in plastid transformation vector pCHV-RKB. Flanking sequences were derived from plastid intergenic sequences chl1 (110,000–111,234 bp) and chl2 (111,235–112,444 bp) for integration in to the plastid genome. PA(dIV) gene was cloned in NotI and XbaI sites of the multiple cloning site with ribosome binding site RBS and a spacer. The constitutive plastid 16 S rRNA promoter was used to drive the gene *aminoglycide adenyltransferase* A (*aad A*) that confers resistance to spectinomycin. 5′UTR and 3′UTR were used to promote the stability of the transcript.

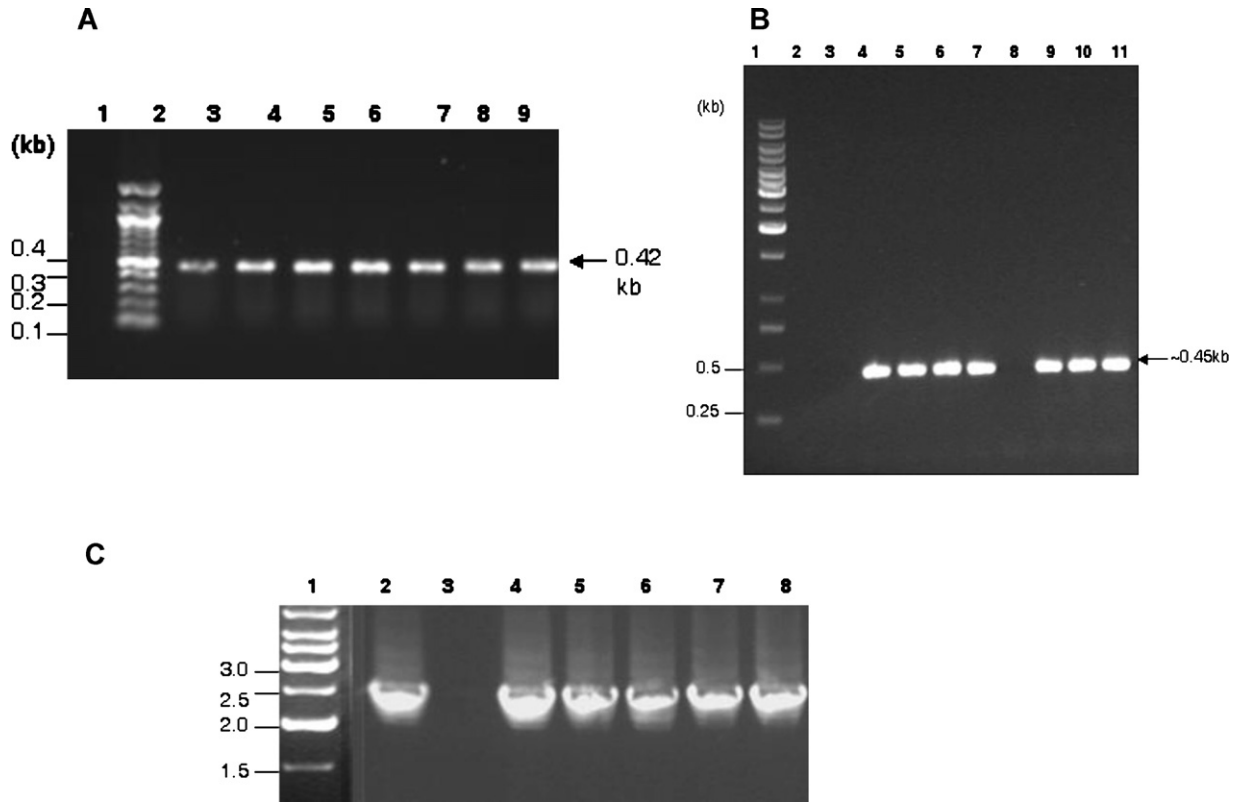

Fig. 2. PCR analysis of putative transgenic tobacco plants. Genomic DNA extracted from transgenic leaves was used as template for amplification. (A) Amplification from putative tobacco plants transformed by agrobacterium mediated transformation. PCR carried with full length PA(dIV) gene specific primers led to 0.42 kb amplicon (lanes 3–9). Lane 1 shows amplification from wild-type tobacco plants. (B) PCR from transplastomic tobacco plants. Lanes 4–11, 0.45 kb amplicon was obtained from PA(dIV) transplastomic tobacco plants (amplicon includes the RBS site + PA(dIV) sequence and His tag) and lane 2, amplification from WT plants. Lane 3, no amplification from the putative transgenic plant. (C) Site specific integration of PA(dIV) gene in the plastome. An amplicon of 2.5 kb was obtained when PCR was carried out with primer P1 specific to plastid flanking region (chl2) and primer P2 specific to PA(dIV) region.

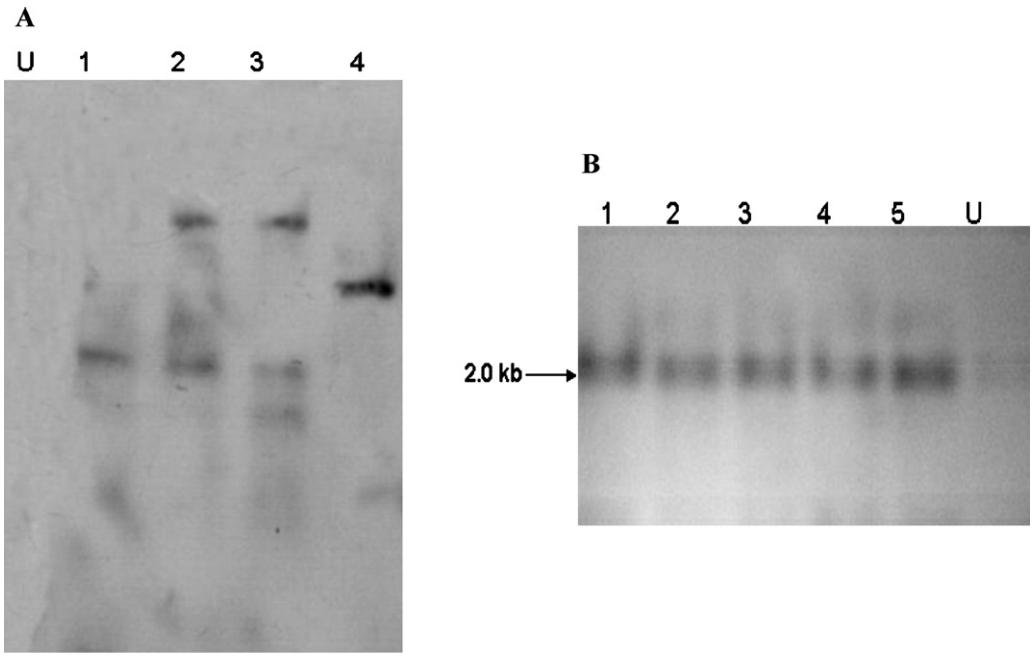

Fig. 3. Southern blot analysis of transgenic plants. Genomic DNA (10 μg) was digested with NcoI for nuclear plants and PstI for plastid transformed plants DNA was separated on 0.8% agarose gel and blotted on a positively charged nitrocellulose membrane and hybridized with biotin labeled PA(dIV) probe. NcoI cuts once within the T-DNA. PstI cuts once in the *aad*A region and after the intergenic region 2. (A) Nuclear transgenic plants. 1–3, transgenic plant samples; U, untransformed tobacco plant sample; 4, positive control plasmid with PA(dIV) gene. (B) Transplastomic plants. 1–4, transplastomic plant samples; U, untransformed tobacco sample. Hybridization signal was obtained at 2.0 kb.

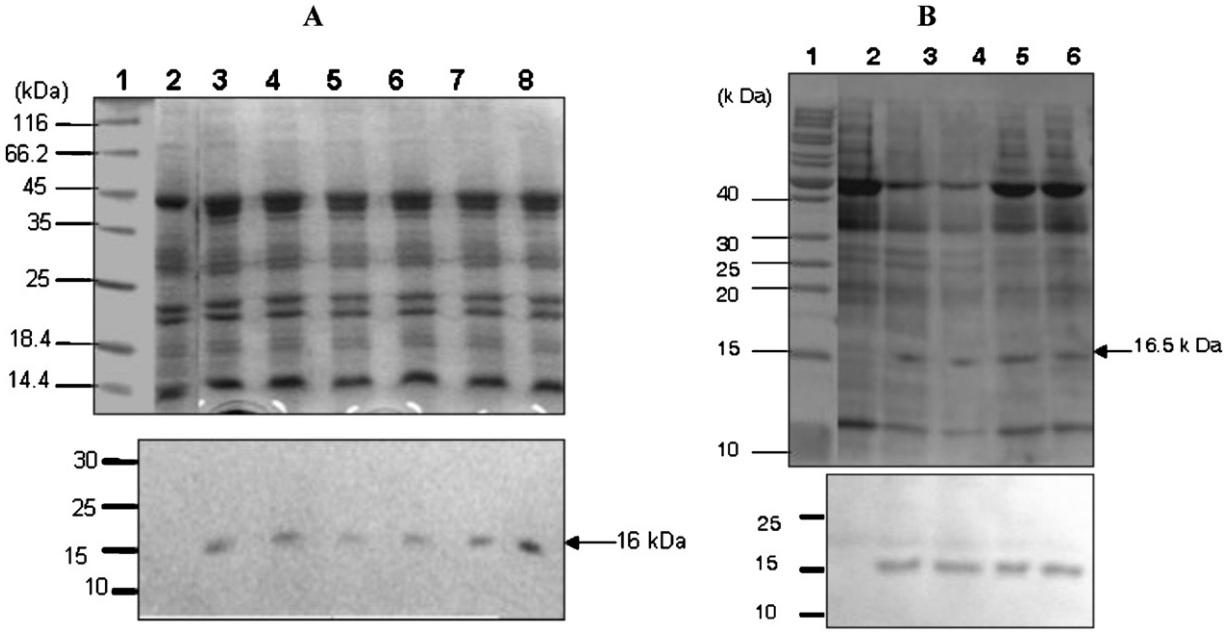

Fig. 4. SDS–PAGE and Western blot analysis demonstrating the expression of PA(dIV) in transgenic plants. (A) Total soluble protein extracted from transgenic tobacco plants transformed by agrobacterium mediated transformation. Coomassie blue stained picture of TSP from transgenic plants (upper). A 16.0 kDa protein band was obtained by western blot (lower) from transgenic plants confirms the expression of PA(dIV). Lane 1, precision marker; lane 2, TSP from wild type plants; lanes 3–8, TSP from transgenic plants. (B) Western blot analysis of TSP extracted from transplastomic PA(dIV) plants. Coomassie stained gel showing expression of PA(dIV) protein 16.5 kDa (upper). Western blot showing the expression of PA(dIV) in transplastomic plants. Lane 1, precision marker; lane 2, TSP from untransformed tobacco plants; lanes 3–6, samples from transplastomic plants.

priming and one week after each immunization. After 2 weeks of first immunization, induction of IgG was seen in all the immunized groups. Further, there was progressive increase in antibody titers in all the groups with each dose of immunization (supplementary data). Serum antibody titers reached peak by the 5th bleed in all groups ($p < 0.001$). The antibody titers detected after the final booster were either higher than or similar to those obtained at 5th bleed. Highest antibody titers were detected in adjuvanted recombinant [rPA(dIV)] groups i.e.: [rPA(dIV) + Al] up to 2.2×10^5 and 2.4×10^5 ($p < 0.001$[5th and BC]), followed by [rPA(dIV) + CT] ($p < 0.001$[5th and BC]). Antibody titers in these groups were further enhanced after final booster dose but the titers obtained were not significantly different from the titers obtained at 5th bleed ($p > 0.05$). Significant serum antibody levels of $>10^4$ were also detected in the groups [NT-PA(dIV) + Al] ($p < 0.001$[5th and BC]) and [NT-PA(dIV) + CT] ($p < 0.05$ at 5th and $p < 0.001$BC). The titers obtained in orally immunized [rPA(dIV)] and [NT-PA(dIV)] groups were not statistically significant by 5th bleed ($p > 0.5$). However, [rPA(dIV)] titers were significant ($p < 0.5$) when the last booster dose [rPA(dIV)] (10 μg) was given. Comparisons among the groups by ANOVA demonstrated that antibody titers among the groups [rPA(dIV) + CT] and [NT-PA(dIV) + Al] were not statistically different ($p > 0.05$) by 5th and last bleed. Titers were similar among the groups [rPA(dIV)] and [NT-PA(dIV) + CT] by 5th bleed and in [rPA(dIV)] and [NT-PA(dIV)] by 5th bleed and last immunization (Fig. 6).

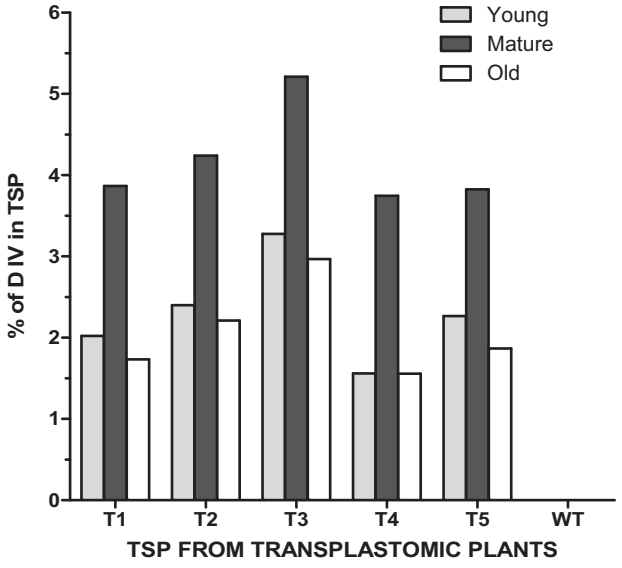

Fig. 5. Quantification of PA(dIV) in transplastomic plants from young, mature and old leaves as obtained by ELISA. Percent of TSP was calculated by the comparison of the absorbance with respect to a standard plot derived from purified PA(dIV) by ELISA. The amount of PA(dIV) derived from mature leaves was higher as compared to young and old leaves. Highest expression levels ranging from 3.7 to 5.3% was recorded in mature leaves. The expression levels obtained in various transgenic plants were not significantly different. There was no expression of PA(dIV) in wild type plants.

Further, the type of immune response generated was evaluated as it plays a significant role in acquiring the protection. The pattern of IgG subtype response predicts the type of protective immune response induced. All the immunized groups demonstrated a higher IgG1 antibody titer over IgG2a (Fig. 7) thus predicting a polarized Th2 type response. Our results are consistent with previous studies that have also shown that immunization with PA or PA(dIV) induces a strong Th2 type response [22,45].

4.4. Generation of serum IgA and mucosal IgA response in orally immunized mice groups

Serum anti-PA(dIV) IgA antibodies were detected at 5th bleed and before challenge with the toxin. Antibody levels were higher in the groups in which CT adjuvant was added. Highest serum IgA titers was detected in the group [rPA(dIV) + CT], [NT-PA(dIV) + CT] ($p < 0.001$) followed by [rPA(dIV)] ($p < 0.01$) at 5th bleed and last

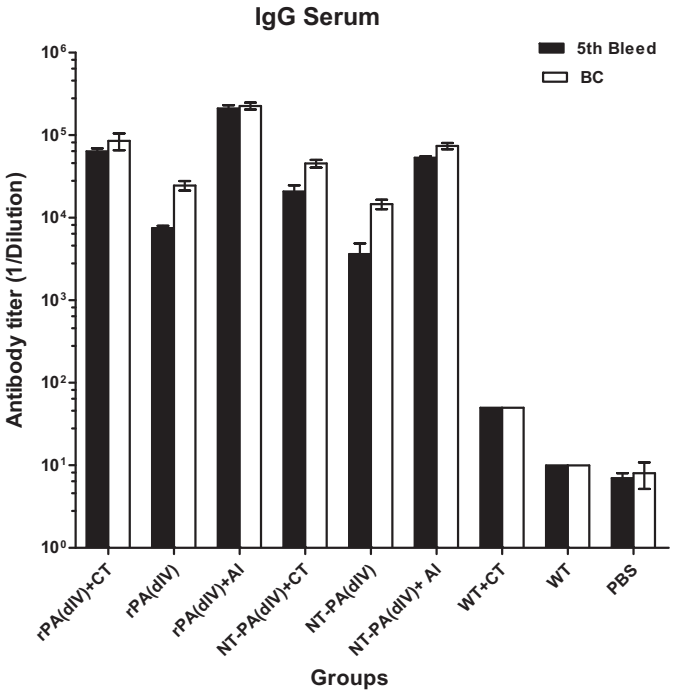

Fig. 6. PA specific Serum IgG immune responses in mice immunized with recombinant PA(dIV) or plant expressed PA(dIV). Groups of mice were immunized intraperitoneally or orally on day 0, 7, 14, 21 and 28. A final booster was given on day 175 with 10 μg of recombinant purified protein by i.p./oral immunization. Antibody titers analyzed at 5th bleed and on day 189 representing week 27 or before challenge (BC) with *B. anthracis* are represented in the above graph. Data are representative of 3 independent experiments done in triplicates. Standard deviations (SD) were calculated on log-transformed titres. [rPA(dIV) + CT], recombinant domain IV + cholera toxin; [rPA(dIV)], recombinant domain IV; [rPA(dIV) + Al], recombinant domain IV + alhydrogel; [NT-PA(dIV) + CT], tobacco domain IV TSP + CT; [NT-PA(dIV)], tobacco domain IV TSP; [NT-PA(dIV) + Al.], tobacco domain IV TSP + alhydrogel; CT, cholera toxin; WT + CT, wild type + CT; WT, wild type; and PBS, phosphate buffered saline represented control groups.

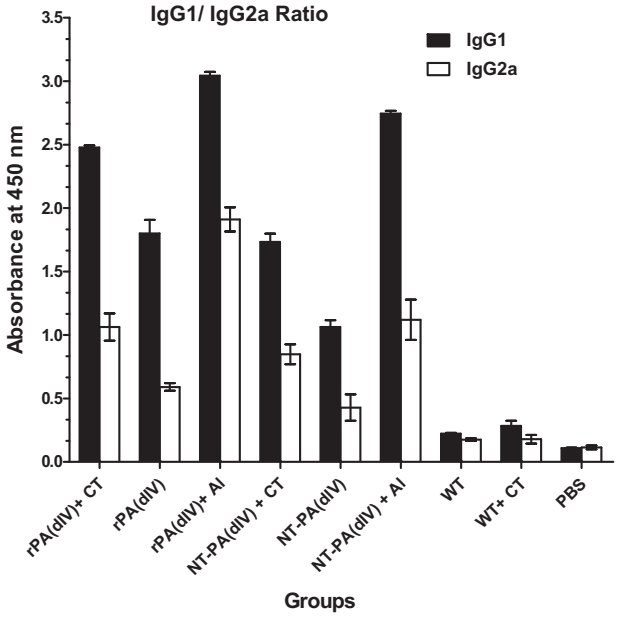

Fig. 7. Specific IgG1 and IgG2a antibody response in various groups of mice immunized with recombinant or plant expressed PA(dIV). IgG1 and IgG2a antibody levels of 5th bleed were measured at a serum dilution 1:100 by ELISA. Absorbance was recorded at 450 nm. The bar graphs represent data of three independent experiments done in triplicates. Data represents mean absorbance ± SD. [rPA(dIV) + CT], recombinant domain IV + CT; [rPA(dIV)], recombinant domain IV; [NT-PA(dIV) + CT], tobacco domain IV TSP + CT; [NT-PA(dIV)], tobacco domain IV TSP; [NT-PA(dIV) + Al.], tobacco domain IV TSP + alhydrogel; CT, cholera toxin; WT + CT, wild type + CT; WT, wild type; and PBS, phosphate buffered saline represented control groups.

bleed and [NT-PA(dIV)] ($p < 0.05$) by 5th bleed and ($p < 0.01$) by last bleed. The titers among the groups [rPA(dIV)] and [NT-PA(dIV)] were not significantly different from each other ($p > 0.05$) by both 5th and last bleed. There was no significant increase in serum IgA titers in any of the immunized groups by the last bleed (Fig. 8A).

IgA antibody that is secreted at the mucosal surfaces (sIgA) plays an important role in conferring significant protection against the pathogen. Oral immunization leads to protective immune responses at the mucosal surfaces. sIgA released at the gut surface can be detected by measuring the IgA antibody levels in fecal extracts. Anti PA sIgA responses were detected in only in groups [rPA(dIV) + CT] [NT-PA(dIV) + CT] at the 5th bleed (Fig. 8B). Remaining groups that were immunized orally did not show any measurable levels of anti PA sIgA antibody. Sera and fecal extracts from all the control mice did not depict any antibody titer. IgA titers could not be detected in any of the immunized groups by the last bleed before challenge in the fecal extracts.

4.5. Generation of robust lethal toxin neutralizing antibodies

Lethal toxin (LeTx) induced mortality of J774A.1 cells can be prevented by the neutralizing activity of anti-PA antibodies. Sera from immunized mice when assessed for the toxin neutralizing potential by *in vitro* toxin neutralization assay were able to prevent LeTx mediated killing. Neutralizing antibody titers with an NC 50 of $>10^3$ were generated in the groups [rPA(dIV) + Al] [rPA(dIV) + CT]. In groups immunized with plant expressed PA(dIV) or rPA(dIV) the NC 50 titers of $>10^2$ were detected. A comparison of neutralization titers at 5th and last bleed indicated an increase in NC 50 titers with the final boost in all the experimental groups although; the rise was not statistically significant. NC 50 titers of all weeks have been reported in supplementary data. Mucosal neutralizing antibodies could not be detected in any of the immunized groups though there was an increase in O.D. in the groups [rPA(dIV) + CT] [NT-DIV + CT]. Sera from the negative control groups [WT]/[WT + CT]/[PBS] did not generate any LeTx neutralizing antibodies (Fig. 9).

4.6. PA(dIV) antisera affect B. anthracis spore uptake and germination

To examine if rPA(dIV) plays any role in inhibiting spore uptake and germination, spore uptake assay was carried out using rPA(dIV) antisera as opsonin for *B. anthracis* spores. The spore uptake after opsonization with recombinant PA(dIV) antisera and plant PA(dIV) antisera increased up to 5 and 3 folds respectively as compared to non-opsonized control (Fig. 10A). The spore uptake percentage was found to be 50% and 26% with spores opsonized with rPA(dIV) + Al and NTPA(dIV) + Al antisera. The percentage of spore germination was determined after heat treatment of harvested cell aliquots. In the macrophages infected with opsonized spores, the germination percentage was 76% of total spore uptake whereas that of control macrophage infection was only 50% at 1 h of infection. But eventually, intracellular germination of spores decreased after 1.5 and 3 h of infection in both the experimental groups (Fig. 10B). These results demonstrate that PA(dIV) antisera is able to function as opsonin and also has an inhibitory effect on germination of spores within macrophages after increased infection time.

4.7. Immunized mice survive lethal toxin challenge

All the immunized mice were challenged with a lethal dose of *B. anthracis*. A dose of 10^8 cfu/200 μl was found to be completely

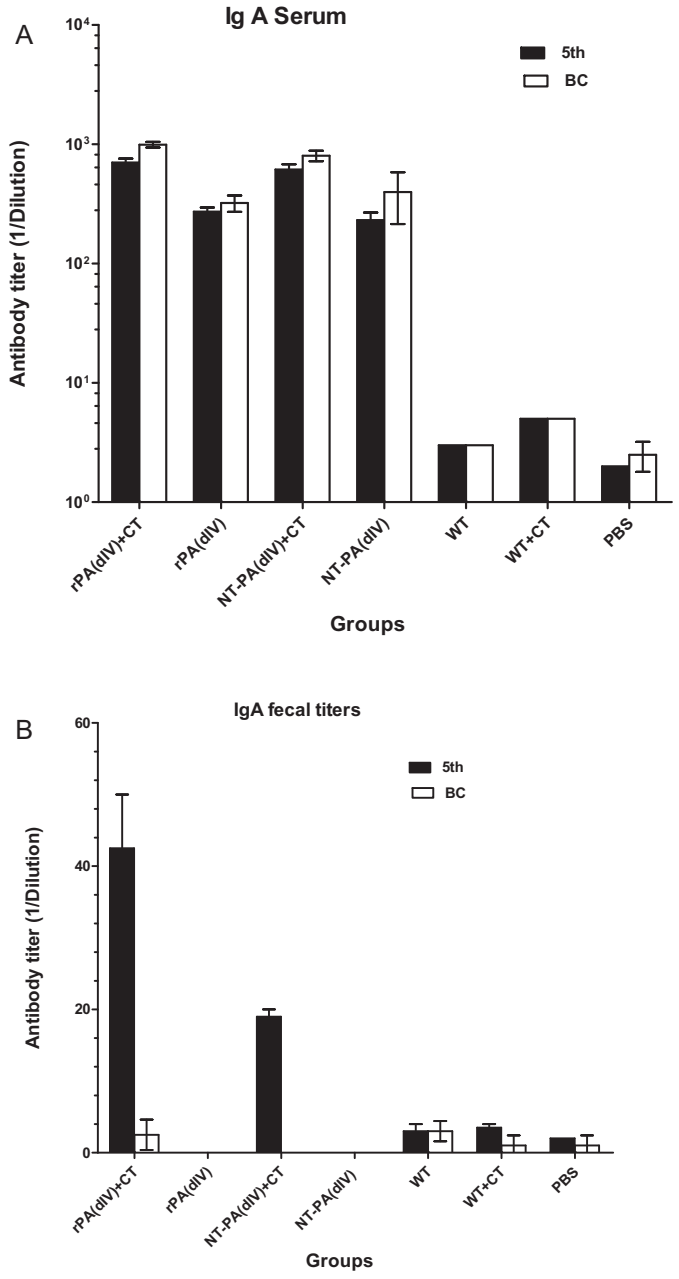

Fig. 8. PA specific serum IgA/fecal IgA response in various groups of mice immunized orally. (A) Sera collected at 5th bleed and before challenge (BC) with *B. anthracis*, was analyzed by ELISA. Data represents titer obtained in 3 independent experiments done in triplicates. Standard deviation (SD) was calculated on log-transformed titers and represented as mean ± SD. Highest IgA serum titers were obtained in adjuvanted groups. [rPA(dIV) + CT], recombinant domain IV + CT; [rPA(dIV)], recombinant domain IV; [NT-PA(dIV) + CT], tobacco domain IV TSP + CT; [NT-PA(dIV)], tobacco domain IV TSP; CT, cholera toxin; WT + CT, wild type + CT; WT, wild type; and PBS, phosphate buffered saline represented control groups. (B) Fecal samples collected at 5th bleed and before challenge with were analyzed by ELISA. Data represents titer obtained in 3 independent experiments done in triplicates. Fecal IgA titers were detected only in adjuvanted groups immunized orally. The data obtained is represented as mean ± SD. [rPA(dIV) + CT], recombinant domain IV + CT; [rPA(dIV)], recombinant domain IV; [NT-PA(dIV) + CT], tobacco domain IV TSP + CT; [NT-PA(dIV)], tobacco domain IV TSP; CT, cholera toxin; WT + CT, wild type + CT; WT, wild type; and PBS, phosphate buffered saline represented control groups.

lethal to BALB/c mice with a mean time death (MTD) of 62–75 h. All the challenged mice were followed up to 14 days. Immunized mice demonstrated survival percentages ranging from 0 to 100% in the groups immunized with PA(dIV). Recombinant groups [rPA(dIV) + Al] and [rPA(dIV) + CT] demonstrated highest survival

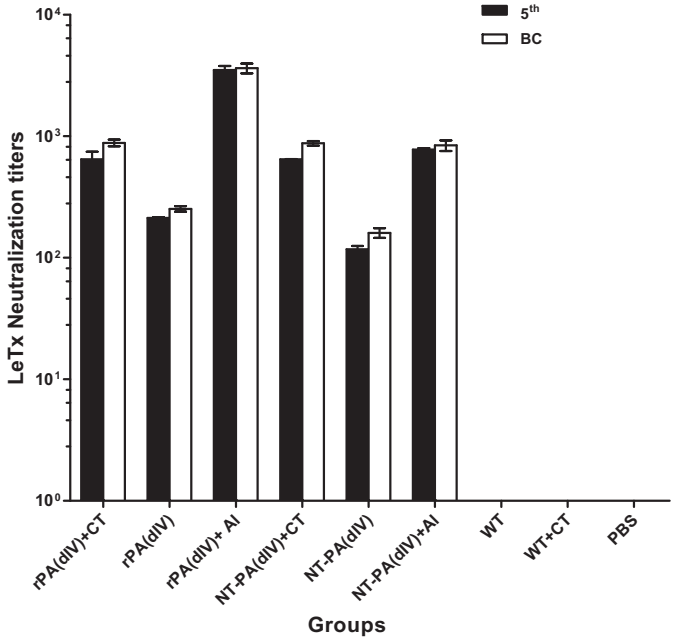

Fig. 9. *In vitro* lethal toxin neutralization assay. NC 50 LeTx neutralization titers in mice immunized with various PA(dIV) combinations. Sera analyzed for toxin neutralization potential at 5th bleed and before challenge are represented in the above graph. Serial dilutions of serum were made and added to the J774A.1 cells pre-incubated with LeTx. Cell viability was assessed by using MTT dye. Bars represent the antibody dilution at which 50% of the cells were viable. Standard deviation (SD) was calculated on log-transformed titers. Data represents 3 independent experiments done in triplicates. [rPA(dIV) + CT], recombinant domain IV + CT; [rPA(dIV)], recombinant domain IV; [rPA(dIV) + Al], recombinant domain IV + alhydrogel; [NT-PA(dIV) + CT], tobacco domain IV TSP + CT; [NT-PA(dIV)], tobacco domain IV TSP; [NT-PA(dIV) + Al.], tobacco domain IV TSP + alhydrogel; CT, cholera toxin; WT + CT, wild type + CT; WT, wild type; and PBS, phosphate buffered saline represented control groups.

percentages of 100% and 80% as on 14th day post challenge recording. Mice immunized with plant derived PA(dIV) demonstrated 60% survival with alhydrogel or CT. The groups in which adjuvant was not added succumbed to death although, there was time delay in death. Mice immunized with WT/WT + CT/PBS only completely succumbed to death by 3rd day. The graphical representation of percent survival is depicted by Kaplan–Meier curves (Fig. 11).

5. Discussion

In pursuit of developing a safe anthrax vaccine that can cater to the needs of large population at risk, we have expressed PA(dIV) in *planta* and *E. coli*. Further, protective immune response of PA(dIV) expressed in these systems was investigated in mice. Our study demonstrated that PA(dIV) expressed in *planta* and *E. coli* upon oral and intraperitoneal immunization in mice is able to generate protective immune response against *B. anthracis* toxin challenge.

Pioneering studies with respect to anthrax plant based vaccines focused on the expression of PA in tobacco and tomato by agrobacterium mediated transformation [40]. Subsequent studies reported the expression of PA in tobacco by chloroplast transformation and further evaluated the immunological response in mice [34,35,46]. But, there have been certain reservations about the usage of PA molecule for vaccination therapy. Presently, we do not know much about the interaction of toxins with PA, which is delivered by immunization during anthrax infection. Qualms about reintroduction of toxin subunit in population already affected by anthrax limit the implication of PA vaccines to individuals during or shortly after infection [47].

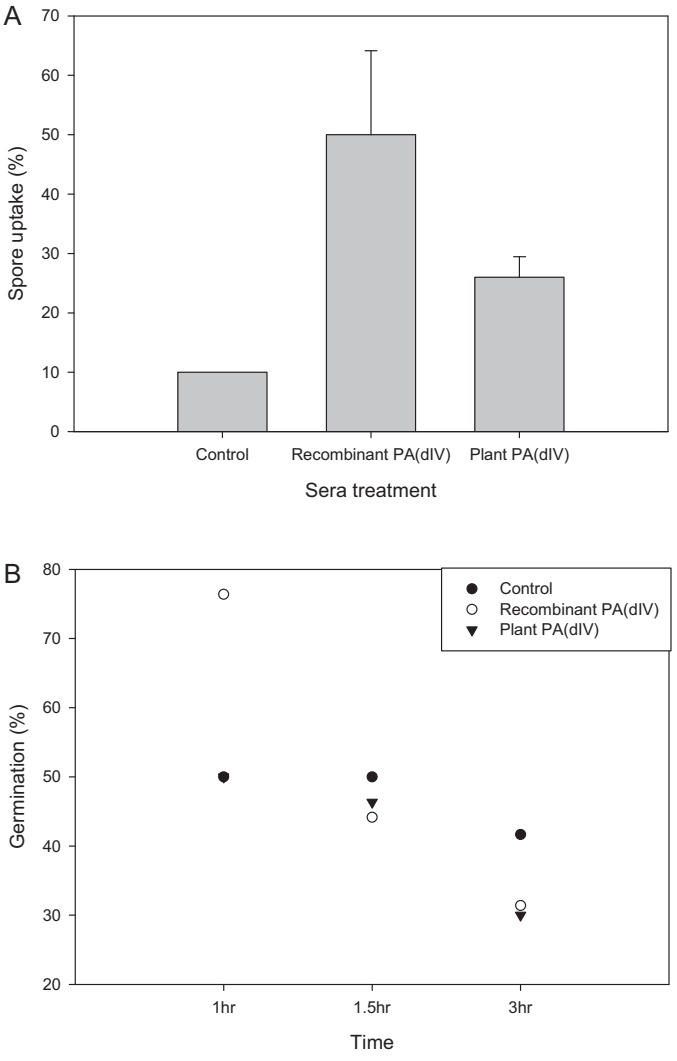

Fig. 10. Spore uptake assay. Macrophage cell line J774A.1 infected with *B. anthracis* sterne 34F2 spores were analyzed for spore uptake and germination. Spores were opsonized with antisera and cfu was determined after infection of macrophages and removal of vegetative bacilli. In control experiment, the spores were not opsonised with antisera prior to infection of macrophages. (A) Uptake of spores treated with PA (dIV) antisera and untreated spores. (B) Germination percentage of treated spores at different time intervals. All the experiments were done independently and in triplicates.

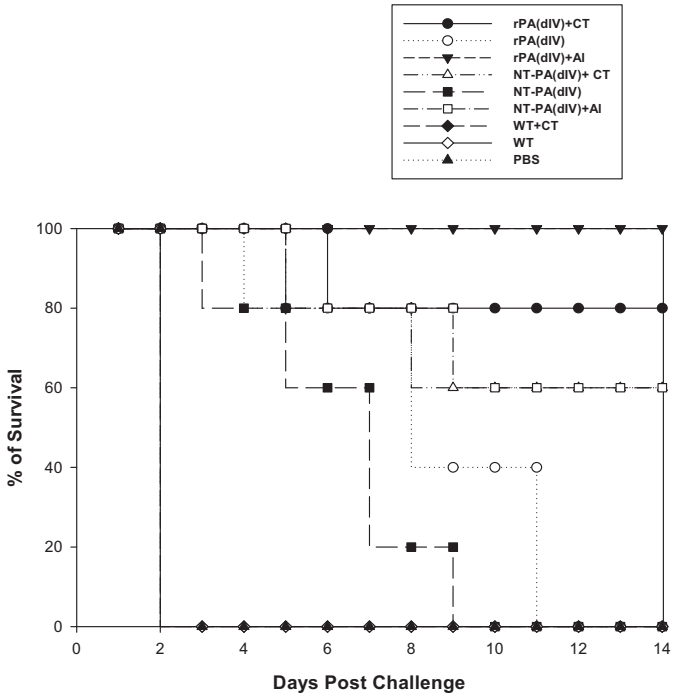

Fig. 11. Immunization with PA(dIV) in various groups provides efficient protection against challenge with *B. antharcis*. All the mice immunized were challenged with 10^8 cfu of *B. anthracis* by intraperitoneal injection. All the control mice groups PBS/WT/WT + CT succumbed to death with MTD of 3 days. Average percent survival is represented by Kaplan–Meier curves. [rPA(dIV) + CT], recombinant domain IV + CT; [rPA(dIV)], recombinant domain IV; [rPA(dIV) + Al], recombinant domain IV + alhydrogel; [NT-PA(dIV) + CT], tobacco domain IV TSP + CT; [NT-PA(dIV)], tobacco domain IV TSP; [NT-PA(dIV) + Al.], tobacco domain IV TSP + alhydrogel; CT, cholera toxin; WT + CT, wild type + CT; WT, wild type; and PBS, phosphate buffered saline represented control groups.

The significance of post-exposure prophylaxis in anthrax has been highlighted recently by CDC [48]. At present antibiotics are the only means for a post exposure therapeutics approved by Food and Drug Administration (FDA). The exhaustive dosage schedule of ciplofloxicin (60 days twice daily), adverse side effects and relapse of the disease upon discontinuation of antibiotics in some cases demands for alternative approaches [49]. As a part of trial under investigational new drug procedures, anthrax vaccine administered along with short antibiotic course demonstrated optimum protection after anthrax infection [1]. Since, PA(dIV) molecule is not a part of functional toxin unit and does not interact with LF and EF, it could offer a safer alternative to current approaches. A recent study done in animal model showed that PA(dIV) when administered in conjunction with antibiotics could enhance the protective effects of ciprofloxacin [50]. The present study emphasizes on development of a [PA(dIV)] plant based vaccine against anthrax and suggests that the molecule could also be used for a post exposure prophylaxis therapy against anthrax infection.

The success of preliminary experiments pursued with PA strengthened the idea of furthering the concept towards generation of PA(dIV) plant based vaccine against anthrax. Studies with respect to PA(dIV) plant based vaccines were limited to transient viral mediated expression in tobacco and spinach [51,52]. Although, the viral expression in plants promotes high expression levels safety concerns regarding the viral vectors limit its practical application [26]. Stable recombinant expression of PA(dIV) by nuclear or chloroplast transformation could provide safe and reliable means for vaccine development.

In the present study, initially PA(dIV) was expressed in tobacco by agrobacterium mediated transformation method. Low and varied levels of PA(dIV) expression (0.3–0.8% of TSP) in these plants indicated the need to improve expression levels. In order to enhance expression levels, chloroplast transformation was carried out in tobacco with pCHV-RKB-PA(dIV). Expression levels up to 5.3% of TSP were obtained in these plants. Apart from the ploidy levels of the plastid genome (approx. 10,000 genome copies per cell), the high AT rich PA(dIV) sequence (71.8% AT content) was favored by the plastid genome, thus contributing to increased yields. Consistent with the previous findings [46] mature leaves showed highest percentage of PA(dIV) as compared to young and old leaves. This variation in expression could be attributed to the presence of higher number of chloroplasts in mature leaves.

Most of the studies pertaining to anthrax vaccination have focused on systemic immunity alone [11,12,15]. However, recently several researchers have highlighted the significance of mucosal immunity to activate the primary host defense mechanisms and effectively encounter the pathogen at the portal of entry itself [53]. Taken in to consideration that the anthrax disease man-

ifests in 3 different forms; the cutaneous, the gastrointestinal, the inhalational that primarily make way for the pathogen *via* mucosal lining it would be essential to activate mucosal immune responses. Studies have been pursued with PA based oral, nasal or transcutaneous immunization in mice [54–59]. Previous investigations concerning mucosal immunization with PA(dIV) were based on *Salmonella* vectors [59,60]. Although, intranasal immunization with these vectors showed efficient protection against *B. anthracis* aerosol spore challenge, there was no protection upon oral immunization. These studies indicated the need for the generation of effective mucosal response against anthrax. Oral immunization is an important means towards generation of mucosal immunity, as the gut provides largest surface area to effectively elicit mucosal response [61]. We envisage that a plant based vaccine could provide effective means for oral vaccination therapy against anthrax.

To investigate the protective immune response of purified recombinant *E. coli* and plant based PA(dIV) vaccine, we performed i.p. and oral immunizations in mice model. Repeated immunizations were carried out in various groups of mice. With respect to plant based oral vaccines various studies have indicated that multiple dosage schedules offered better immune response [62–64]. Thus, in the present study we chose to adopt similar strategy. Prolonged dosage schedule may not be a serious issue in perspective of oral vaccines as they are easy to administer, could provide respite from jabs, local injection site reactions such as reddening, erythema, or allergic reactions and most importantly provokes systemic and mucosal immunity which is otherwise unachievable with present injectable vaccines. Robust IgG antibody titers > 10^5 were obtained in groups [rPA (dIV) + CT] and [rPA(dIV) + alhydrogel], while the groups [NT-PA(dIV) + CT], [rPA(dIV)] and [NT-PA(dIV) + alhydrogel] generated immunological titers > 10^4. Over all adjuvanted groups depicted higher antibody titers as compared to non adjuvanted counterparts. Several studies have stressed the need for incorporation of a strong adjuvant for generation of efficient immune response [46,56]. The low antibody titers with tobacco expressed PA(dIV) over the recombinant purified PA(dIV) can be attributed to impurities and alkaloids, or other proteins (proteases) present in tobacco TSP. Our findings are in agreement with the previous reports in which higher antibody titers were obtained with purified recombinant protein when compared to the plant TSP [35,46].

Protective immunity achieved is often attributed to the type of immune response generated. The class of IgG subsets induced after oral/i.p. immunization predict the type of humoral immune response generated. Th1 lymphocytes stimulate production of complement fixing IgG subsets (e.g. IgG2a) and cell mediated immune response (CMI). On the other hand, non-complement fixing IgG subtypes i.e.: IgG1 are stimulated by Th2 lymphocytes [65]. The production of increased IgG1 titers over IgG2a demonstrated a strong Th2 immune response. Immunization studies with PA [45] and PA(dIV) [22] have predicted a Th2 biased immune response. Also, protective immunity against anthrax toxin challenge has been associated with high IgGl levels. In agreement with these findings, all the groups immunized with rPA(dIV) or plant derived PA(dIV) (i.p. and oral) have shown a strong polarization towards Th2 response. Further, it may be derived that the type of immune responses stimulated by plant expressed PA(dIV) is similar to those stimulated by rPA(dIV). It may be noted that adjuvant incorporated also strongly influences the outcome of the type of immune response. Both, alum adjuvant and CT have demonstrated to induce a polarized Th2 type immune response [66,67]. In fact, it is evident from studies that mucosal adjuvant CT promotes essentially strong Th2 response with minimal or no IgG2a antibodies [68]. The groups in which CT was administered as mucosal adjuvant revealed very less IgG2a antibodies (Fig. 6) thus, corroborating with the previous findings.

A mucosal vaccine can be accounted to be successful only if it is able to induce protective immune response at the mucosal sites where it encounters the pathogen at the site of entry itself. The immune responses generated at the mucosal surfaces are marked predominantly by the production of secretory IgA (sIgA) [69]. The measurement of sIgA antibody induced by mucosal immunization could indicate the production of mucosal immune response [64]. Both, serum and fecal IgA titers were measured. Serum IgA titers were higher as compared to fecal IgA titers. Although, IgA titers were detected in all the serum samples derived from various groups of mice, analysis of fecal extracts from the immunized mice demonstrated IgA titers only in the groups [rPA(dIV + CT)] and [NT-PA(dIV) + CT]. In all the other groups no IgA titers were detected in fecal samples. It may be suggested that since CT is a potent mucosal adjuvant, the groups in which PA(dIV) was administered with CT demonstrated IgA antibody titer. Further, the production of sIgA antibody indicated the generation of mucosal immune response with PA(dIV) based vaccine against anthrax. Although, sIgA titers obtained in fecal extracts are good indicators of generation of mucosal immune response, they may not represent the actual amount of secretions at the gut mucosa [64]. This is because feces carry only the residual antibody secreted at the gut surface. Thus, the actual amount of sIgA secreted into the gut lumen may be higher than what we obtained from our study.

The functional significance of the antibody response generated in immunized mice was evaluated using *in vitro* toxin neutralization assay (TNA). Sera from immunized mice groups demonstrated neutralizing potential when combined with lethal toxin on mouse J774A.1 macrophage like cell line. The neutralizing titers obtained in the recombinant groups were higher than plant produced PA(dIV) with or without adjuvant. Sera derived from orally immunized groups also demonstrated LeTx neutralizing titers thus, confirming that mucosal immunization is adept at stimulating the systemic immune response as evidenced by other studies [56]. Further, neutralizing capacity of sera from mice immunized with plant PA(dIV) indicated the immunological potential of plant based PA(dIV) vaccine. Lethal toxin neutralization titers were not noticed with fecal extracts of immunized mice although, there was increase in O.D. in the groups [rPA(dIV) + CT] and [NTPA(dIV) + CT] when compared to the control groups. Although, immunological antibody IgA titers were recorded in immunized groups in serum and feces we could not establish any direct relation between IgA and protective immune response.

It is well known that *B. anthracis* spores germinate in macrophages [70]. Germination of spores within macrophages is a critical step in anthrax pathogenesis. Previous studies indicated that anti-PA antibodies stimulate spore uptake and inhibit germination in macrophage culture [71]. In order to assess if PA(dIV) has similar function, an *in vitro* spore uptake assay was performed. Our experiments indicated that PA(dIV) antisera is effective in increasing spore uptake by macrophages and also elicited anti-germinating effect after increasing time of incubation with treated spores. Therefore, immunization with PA(dIV) may curtail anthrax pathogenesis at the initial stages of infection itself. Plant derived PA(dIV) was also able to exhibit similar properties as evident from the assay. The discrepancy in spore uptake rate and germination percentage in both the groups could be correlated to low antibody titers in plant PA(dIV) immunized groups.

All the groups of mice when challenged with *B. anthracis* vegetative cells have demonstrated variable protection levels in different immunized groups. The group [rPA(dIV) + alhydrogel] produced highest protection level of 100%. The groups [NT-PA(dIV) + Al.] and [NT-PA(dIV) + CT] showed 60% survival rate. Studies have indicated that, TNA titers reflect the rate of protective immune response achieved. Concordant to these findings, we have noticed a tight correlation between the neutralizing titers and the survival

rates in mice after toxin challenge [72,73]. The groups in which high NC 50 titers were observed demonstrated better protection levels.

Taken together, our study demonstrates for the first time stable transformation of PA(dIV) in *planta* and further evaluates its immunological potential in mice. The protective immune response achieved in various groups provide scope for the development of PA(dIV) based vaccine against anthrax. Future studies may direct the research towards evaluation of PA(dIV) vaccine against full virulent spores. The development of an oral plant based vaccine against anthrax can promote effective systemic and mucosal immune response. Although, mucosal antibody response was noticed in some groups, the lack of neutralizing potential of these antibodies indicated the need for further improvement. We believe that further optimization in dosage of the antigen, adjuvant and oral delivery of antigen expressed in an edible crop could lead to protective mucosal immune responses. Studies have shown that proteins expressed in *planta* in edible parts and consumed as such provided better immune response as compared to oral delivery of soluble proteins [63]. Therefore, prospective investigations need to focus on development of an edible vaccine against anthrax. This may be achieved through expression in chloroplast of edible plant species. Currently, chloroplast transformation is restricted mostly to tobacco due to availability of transformation vectors and optimized transformation protocols. Although, chloroplast transformation technology has been expanded to some of the edible crop species [74–76], issues concerning the expression levels in edible parts and fertility of the plant have prevented from wide adaptation of this technology. With growing knowledge of plastid genome sequences and efficient transformation methods for various edible plants; an ideal vaccine against anthrax may soon be available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.03.082.

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