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Generation of protective immune response against anthrax by oral immunization with protective antigen plant-based vaccine

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ABSTRACT

In concern with frequent recurrence of anthrax in endemic areas and inadvertent use of its spores as biological weapon, the development of an effective anthrax vaccine suitable for both human and veterinary needs is highly desirable. A simple oral delivery through expression in plant system could offer promising alternative to the current methods that rely on injectable vaccines extracted from bacterial sources. In the present study, we have expressed protective antigen (PA) gene in Indian mustard by *Agrobacterium*-mediated transformation and in tobacco by plastid transformation. Putative transgenic lines were verified for the presence of transgene and its expression by molecular analysis. PA expressed in transgenic lines was biologically active as evidenced by macrophage lysis assay. Intraperitoneal (i.p.) and oral immunization with plant PA in murine model indicated high serum PA specific IgG and IgA antibody titers. PA specific mucosal immune response was noted in orally immunized groups. Further, antibodies indicated lethal toxin neutralizing potential *in-vitro* and conferred protection against *in-vivo* toxin challenge. Oral immunization experiments demonstrated generation of immunoprotective response in mice. Thus, our study examines the feasibility of oral PA vaccine expressed in an edible plant system against anthrax.

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1. Introduction

Anthrax, a disease caused by *Bacillus anthracis* has gained prominence in the recent times due its potential implication as bio-warfare agent. Apart from the bioterrorist attacks, natural outbreaks continue to occur in many parts of the world especially in Central Asia, South America and Africa. The disease in nature affects animals but humans also contract the disease from animals or animal products. Recently, incidences of anthrax in wildlife from various national parks have also come in to light (Fasanella et al., 2010; Hudson et al., 2008). The situation demands a pragmatic vaccination approach suitable for both human and veterinary purposes under natural circumstances or during iniquitous bioterror events.

The present human and veterinary anthrax vaccines rely on rather old-fashioned methods. The veterinary anthrax vaccine developed in 1930's by Sterne is essentially an attenuated, non-encapsulated, toxigenic strain of *B. anthracis*, while the licensed human vaccine predominantly contains 'protective antigen', the main immunogenic component of the tripartite anthrax toxin. Both the vaccines have potential side effects. The ancient veterinary anthrax vaccine waned in its potency and showed discrepancies in virulence leading to occasional death of animals (Brossier et al., 1999; Shakya et al., 2007). In both these vaccines, the presence of residual virulence lead to local and systemic reactions in the subjects (Wang and Roehrl, 2005). These vaccines being injectable make vaccine delivery process tougher for medical professionals particularly in cases of affected livestock and wild animals and contribute to systemic immunity alone. Oral vaccines may provide significant respite in such circumstances by simplifying the vaccine delivery and activating both mucosal as well as systemic immunity.

Several heterologous expression systems such as bacterial, viral or plant systems have been employed for oral delivery of vaccines (Aloni-Grinstein et al., 2005; Baillie et al., 2008; Bielinska et al., 2007; Brey, 2005). Plant-based vaccines due to their

Abbreviations: BAP, benzyl amino purine; BC, before challenge titers; CFU, colony forming units; CT, cholera toxin; DMEM, Dulbecco's modified eagle medium; ELISA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; IBA, indole-3-butyric acid; i.p., intraperitoneal; LeTx, lethal toxin; LF, lethal factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-5 diphenyltetrazolium bromide; MS media, Murashige and Skoog media; MWCO, molecular weight cut-off; NAA, α-naphthalene acetic acid; NBT, nitroblue tetrazolium; PAGE, poly acrylamide gel electrophoresis; PBS, phosphate buffered saline; 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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natural bio-encapsulation ensure protection from digestive enzymes and enhance immune response by gradual release of the antigen in to the gut system (Twyman et al., 2005; Walmsley and Arntzen, 2003). Due to additional advantages concerning safety and cost-effectiveness these vaccines are fast replacing other systems. Various antigens successfully expressed in plants have proved to be efficacious (Ma et al., 2005). Plant-based vaccines can be a boon to the rural population of the developing world, which often cannot afford current vaccines. Additionally, livestock and the wildlife outbreaks of the disease in endemic areas can be most effectively prevented by including the vaccine in feed or fodder meal.

Our initial investigations concerning plant-based vaccine began with expression of PA in tobacco and tomato. We found that PA expressed in these crops generated lethal toxin neutralizing antibodies by intraperitoneal (i.p.) immunization in murine model (Aziz et al., 2002, 2005). In order to generate a comprehensive vaccine suitable for both veterinary and human needs we have expressed PA in mustard crop by *Agrobacterium*-mediated transformation. Mustard crop is commonly used as leaf and stem vegetable and also as fodder meal for cattle in different parts of the world. The versatility of the crop for both human consumption and animal feed makes this crop very lucrative for expression of PA gene. Due to availability of standard transformation protocols, efficient regeneration, large biomass, prolonged stability and safe storage of antigen in seeds, mustard crop can serve as a potential host for the expression of anthrax antigens. One of the major bottle necks with the first generation plant derived vaccines that target desired gene to nucleus has been low expression levels thus, soliciting the need for the expression of PA gene in chloroplasts. Chloroplast transformation technology marked by multifold expression levels, multigene processing and lack of environmental dissemination of transgenes due to maternal inheritance qualifies an ideal system for the expression of antigens. Vaccine candidates for various diseases expressed by chloroplast transformation proved to be immunogenic in animal models (Daniell et al., 2009). Our earlier studies demonstrated that i.p. immunization with PA expressed in plastids could generate toxin neutralizing antibodies (Aziz et al., 2005). In another study, we have evaluated PA(dIV) as a potential candidate for developing anthrax vaccine (Gorantala et al., 2011). Although PA(dIV) molecule holds significance with respect to post-exposure prophylaxis and proved to be efficacious, the importance of full length PA in vaccination cannot be undermined due to better protection levels and extensive research carried out in various animal models (Brown et al., 2010; Chawla et al., 2009; Ivins et al., 1995; Pitt et al., 2001). In the present study, we examined if protection could be achieved by oral immunization of PA expressed in mustard and tobacco. Thus, our study explores the possibility of utilizing *Brassica juncea* as a potential host for the development of an effective plant-based vaccine against anthrax. Also, our study provides scope for extending prospective investigations towards plastid transformation in an edible crop.

2. Materials and methods

2.1. Cloning of PA in chloroplast transformation vector

Protective antigen gene was amplified from pXO1 plasmid of *B. anthracis* (Sterne 34 F2 strain) and cloned in pCHV-RKB (Gen Bank accession number HQ130724) plastid transformation vector in *Not*I and *Xho*I restriction sites (Fig. 1A). Forward primer, 5′-AAA AGG AAA AGC GGC CGC AGG AGG TTT ATA TGG AAG TTA AAC AGG AGA ACC GG-3′ and reverse primer, 5′-CCG CTC GAG TTA GTG ATG GTG ATG GTG ATG TCC TAT CTC ATA GCC-3′ were used for amplification. A ribosomal binding site (GGGAG), spacer (TTTAT), initiation codon (ATG) was included in the forward primer. Transformed colonies were selected on spectinomycin 100 μg/L media. Positive clones were subjected to restriction digestion for confirmation and sequenced for authentication of the clone.

2.2. Plant transformations

For transformation of *B. juncea*, the plasmid pBIN-*pag* (Binary vector with protective antigen gene), was procured from lab stock (Aziz et al., 2002). The plasmid has constitutive CaMV 35S promoter for gene expression and *npt*II (*neomycin phosphotransferase*) gene, for kanamycin selection. *B. juncea* var. Varuna seeds were procured from Indian Agricultural Research Institute (IARI). Five-day-old hypocotyl explants cut approximately 1 cm in size were incubated at 24 °C, 100 rpm for 16 h in RM [MS liquid + B1N1 {BAP (1 mg/L) + NAA (1 mg/L)}]. Agroinfection was carried out using log-phase tertiary culture containing pBIN-*pag* at $OD_{600} \sim 0.3$ for 25–30 min and co-cultivated at 24 °C for 16 h in dark on a rotatory shaker at 50 rpm in a conical flask. The explants were then washed with RM supplemented with augmentin (250 mg/L) for 3 h and plated on selection medium [MS + B1N1 + $AgNO_3$ (20 mM) + augmentin (250 mg/L) + kanamycin (40 mg/L)]. The shoots that emerged from the transformed explants were transferred to the rooting medium [MS + IBA (2 mg/L)]. The plants thus obtained were subjected to hardening in agropeat in green house with appropriate conditions of photoperiod (200 lux, 16 h light and 8 h dark) and maintained at 23 ± 1 °C.

Chloroplast transformation of tobacco was performed on 3–4 week leaf explants with gold coated microcarriers as described earlier using particle gene gun [PDS-1000/He Bio-Rad (Hercules, CA, USA)] (Svab and Maliga, 1993). Briefly, sterile tobacco leaves were placed on abaxial side on osmoticum [MS media + 0.5 M sorbitol and 0.5 M mannitol] for 24 h prior to bombardment with pCHV-RKB-PA plasmid DNA coated microprojectiles. After bombardment, the leaf tissue was incubated in dark for 16 h at 25 °C on regeneration media (RM) [MS media + 0.1 mg/L NAA + 1 mg/L BAP]. The explants were then cut to 5 mm^2 pieces and transferred to selection medium [RM + 500 mg/L spectinomycin]. The shoots obtained after 3–4 weeks were subjected to several rounds of selection to ensure that all the plastids are uniformly transformed. Shoots thus emerged were rooted in MS media with spectinomycin (500 mg/L) for 6 weeks. The plants were then transferred carefully to soil containing agropeat and maintained in green house.

2.3. Molecular analysis of transgenic plants

DNA from putative transgenic mustard and transplastomic tobacco leaves was isolated using Qiagen DNeasy Plant Mini Kit (QIAGEN, Germany) as per the manufacturer's protocol. Mustard DNA samples were subjected to PCR using (i) PA gene specific primers (ii) forward primer specific to CaMV 35S promoter and reverse primer internal to PA gene and (iii) *npt*II (*neomycin phosphotransferase* II) gene specific primers.

PCR analysis of transplastomic plants was done using specific primers to PA gene. Further, site specific integration of PA gene in these plants was assessed using forward primer specific to internal site of PA gene and reverse primer specific to the flanking sequence derived from plastid genome.

Transgene integration in to the genome of transgenic mustard plants and transplastomic plants was evaluated by southern blot hybridization. Genomic DNA was extracted from leaf tissues of wild type tobacco or mustard and the transplastomic lines or transgenic mustard plants using Qiagen DNeasy Plant Mini Kit (QIAGEN, Germany) as per the manufacturer's protocol. Genomic DNA (10 μg) from transgenic mustard plants was digested with *Eco*RI enzyme and genomic DNA (4–5 μg) from transplastomics was digested with *Hind*III and *Mlu*I. DNA was resolved on 0.8% agarose gel, transferred to positively charged nitrocellulose membrane by

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