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ORIGINAL PAPER

High level expression of a functionally active cholera toxin B: rabies glycoprotein fusion protein in tobacco seeds

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Abstract A synthetic DNA construct containing cholera toxin B subunit, genetically fused to the surface glycoprotein of rabies virus was expressed in tobacco plants from a seed specific (*legumin*) promoter. Seed specific expression was monitored by real-time PCR, GM1-ELISA and Western blot analyses. The fusion protein accumulated in tobacco seeds at up to 1.22% of the total seed protein. It was functionally active in binding to the GM1-ganglioside receptors, suggesting its assembly into pentamers in seeds of the transgenic plants. Immunoblot analysis confirmed that the ~80.6 kDa monomeric fusion polypeptide was expressed in tobacco seeds and accumulated as a ~403 kDa pentamer. Evaluation of its immunoprotective ability against rabies and cholera is to be examined.

Keywords Anti-cholera–rabies vaccine antigen · Edible vaccine · Fusion protein · Seed specific promoter · Transgenic plant

Abbreviations

ALP	Alkaline phosphatase
ctxB	Cholera toxin B subunit gene
CtxB	Cholera toxin B subunit
ER	Endoplasmic reticulum
hptII	Hygromycin phosphotransferase gene
i.p.	Intraperitoneal
rgp	Rabies glycoprotein gene
G protein	Rabies glycoprotein
TSP	Total seed protein

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Introduction

Commercial production of pharmaceutical proteins is currently limited to bacteria, yeast, insect or animal cell lines (Yusibov and Rabindran 2008). However, these systems have their own limitations. Bacterial cells do not show a large variety of post-translational modifications, important for the functions of mammalian proteins. Yeast and insect cells are glycosylate proteins but in manners different from those in mammals. Mammalian cell line-based expression of pharmaceutical proteins requires large investment and is also prone to contaminations by infectious agents, unsafe for human applications (Chen et al. 2005; Yusibov and Rabindran 2008; Tiwari et al. 2009). Transgenic plants overcome these limitations and thus offer enormous potential for the production of biopharmaceuticals. More than 100 recombinant proteins are estimated to have been expressed in transgenic plants (Chen et al. 2005; Tiwari et al. 2009). Expression of recombinant proteins can be targeted in different tissues of plants, depending on the promoter used. Different pharmaceutical proteins have been expressed in the seeds of tobacco (Tackaberry et al. 1999; Ramírez et al. 2001; Scheller et al. 2006), arabidopsis (Nykiforuk et al. 2006), rice (Nochi et al. 2007;

Hashizume et al. 2008; Oszvald et al. 2008), maize (Lamphear et al. 2002), soybean (Moravec et al. 2007), pea and wheat (Stöger et al. 2000). A major advantage of expressing industrially important proteins in seeds is that, the foreign proteins retain biological activities for a long duration at ambient temperature. Low protease activities and low moisture content of mature seeds allow their long-term storage and easy transportation. Besides being a good source of functionally active, stable proteins, the seeds offer the additional promise for direct delivery of antigens through edible route.

Oral administration of edible vaccine antigens is sometimes subjected to degradation in the stomach due to low pH and activities of gastric enzymes (Daniell et al. 2001). Another serious limitation in clinical application of oral vaccine is the development of immunotolerance, especially when repeated doses are required to maintain beneficial effects. However, a more pragmatic strategy is to deploy plant seeds as a preferred expression system and use these as a source of purified antigens to be delivered through mucosal route. The efficacy of such mucosal vaccination can be augmented by co-administering the antigens or fusing them with a strong mucosal adjuvant (Streatfield 2006; Moravec et al. 2007; Tiwari et al. 2009). It can enhance the immune response and also reduce the antigen amount required for vaccination. The *Vibrio cholerae* toxin B subunit (CtxB) and *Escherichia coli* heat labile enterotoxin B subunit (LTB) are potent mucosal immunogens and adjuvants (Nashar et al. 1993). Both bind directly to the GM1-ganglioside receptor molecules on M cells even after fusion with foreign antigens (Cuatrecasas 1973). Hence, these can improve the uptake of fused antigens by M cells and enhance immune response. The carrier enteric antigen additionally serves to modulate immune response against watery diarrhea (Yasuda et al. 2003).

Rabies is an acute contagious infection of the central nervous system, caused by rabies virus, which enters the body through the bite from an infected animal. Approximately 55,000 human and millions of animals die every year from rabies worldwide (Dodet and Bureau 2007). High incidence of rabies virus infection is reported from the developing countries, more often in children. Effective anti-rabies vaccines are available in the market but are very expensive and require cold chain for transportation. In the south and southeast Asian region alone, rabies vaccination is estimated to cost 25 million US dollars annually (Chulasugandha et al. 2006). The objective of the present study was to design the fusion construct of cholera toxin B subunit (*ctxB*) and rabies glycoprotein (*rgp*) genes under the control of seed specific promoter and examine its suitability for high level expression in tobacco seeds.

Materials and methods

Construction of chimeric *ctxB–rgp* fusion for high level expression in seeds

Fusion of c*txB–rgp* double stranded DNA was designed by modifying the natural sequence of the B subunit of *Vibrio cholerae* 0139 strain 1854 (accession no. BAA06291) and glycoprotein of rabies virus ERA strain (EMBL:RHRBGP, accession no. J02293). A 63 bp native signal sequence of *ctxB* was presented at 5′ end of the gene. Both the genes were fused with 24 bp $(Gly\text{-}pro)_4$ hinge region. Plant-preferred translation initiation context TAAACAATG (Sawant et al. 2001) and codons were used in designing the genes. The putative transcription termination signals (AAUAAA and its variants), mRNA instability elements (ATTTA) and potential splice sites were eliminated and long hairpin loops were avoided. An 18 nucleotide long sequence encoding for six amino acids, serine–glutamic acid–lysine–aspartic acid–glutamic acid–leucine (SEKDEL) was introduced at 3′ end of the gene for retaining fusion protein in the lumen of endoplasmic reticulum (ER). All gene manipulations were performed following protocols in Sambrook and Russell (2001). Parameters followed for the construction of fusion gene are summarized in Table 1. The fusion was cloned in pBluescript SK^+ cloning vector and named PB5 (Fig. 1a). Chickpea *legumin* promoter (accession no. Y13166) 1,076 bp upstream of the translation start site was PCR amplified using promoter specific primers (Chaturvedi et al. 2007). A fragment of approximately 2.179 kbp *ctxB–rgp–nos* terminator was digested from the vector PB5 and ligated downstream of the promoter. The full gene construct was cloned between *Pst*I and *Eco*RI sites in pBluescript SK^+ cloning vector (Stratagene, USA) (Fig. 1b). The fragment (*legumin–ctxB–rgp–nos*) was digested using *Bam*HI and *Sal*I restriction enzymes and cloned in plant expression vector pCAMBIA1300. The clone was confirmed by sequencing on the 96 capillary DNA analyzer (3730XL, Applied Biosystems, USA). The vector carrying *legumin–ctxB–rgp–nos* gene was named p1340 (Fig. 1c).

Tobacco transformation, selection and growth

Agrobacterium tumefaciens strain EHA101 containing the virulence helper plasmid pEHA101 (Hood et al. 1986) was transformed with p1340 by electroporation using a Gene-Pulser device (Bio-Rad, USA). Tobacco (*Nicotiana tabaccum* cv. Petit Havana) transformation and regeneration were carried out by leaf disc method (Horsch et al. 1985). The hygromycin resistant T_0 plantlets were transferred to soil in the green house for growth to maturity. The seedlings germinating in the presence of 100 mg/l hygromycin

Table 1 Parameters followed for designing genes for high level expression of chimeric fusion antigen in plants

Parameter	Rabies glycoprotein gene (*rgp*)		Cholera toxin B subunit gene (*ctxB*)	
	Native[a]	Designed	Native[b]	Designed
GC content (%)	47.5	53.6	32.4	50.5
AT content (%)	52.3	46.3	67.4	49.4
TA ending codons	30	4	12	0
CG ending codons	11	3	5	2
Hair pin loops with ΔG below −4.0 kcal/mol	22	0	11	0
Polyadenylation and RNA instability sequence	12	0	11	1
Length of reading frame (bp)	1,572	1,515	372	372
Translational initiation context	ATGGTTCCTCAG	–	ATGATTAAATTA	TAAACAATGGCTAGCTCC
Additional 3′ codons	–	Six codons for SEKDEL	–	–

[a] Glycoprotein of rabies virus ERA strain (EMBL:RHRBGP, accession no. J02293)

[b] *Vibrio cholerae* 0139 strain 1854 (accession no. BAA06291)

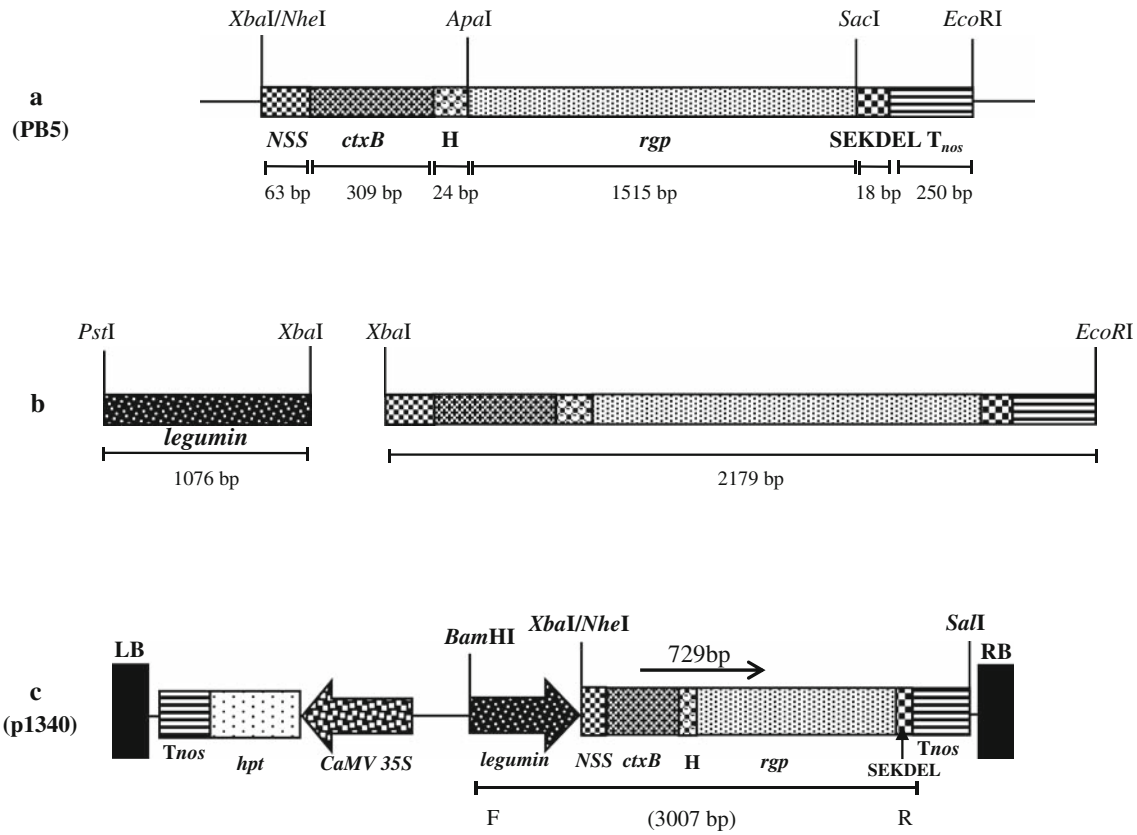

Fig. 1 Cloning of *legumin–ctxB–rgp* expression cassette in plant transformation vector pCAMBIA 1300 to obtain p1340. **a** The *ctxB* and *rgp* genes fused with 24 bp hinge (*H*) sequence encoded for four repeats of glycine—proline. Native signal sequence (*NSS*) of *ctxB* presented at the 5′ end of the gene. The ER retention sequence encoded for SEKDEL introduced at 3′ end of the fusion gene. The fusion cloned in pBluescript SK^+ cloning vector and named PB5. **b** A *ctxB–rgp–nos* fragment digested from PB5 and ligated at the down stream of seed specific *legumin* promoter and cloned in pBluescript SK^+. **c** Complete cassette digested with *Bam*HI and *Sal*I and cloned in pCAMBIA 1300. Restriction enzyme sites, size of fusion components, position of the probe (729 bp) and the positions of the primers (3,007 bp) used to amplify the sequence are shown

were scored to analyze the segregation of *hygromycin phosphotransferase* (*hptII*) transgene in the progeny.

Polymerase chain reaction (PCR) and Southern blot analysis in T_1 plants

Total genomic DNA was isolated from transgenic and wild type plant leaves using the DNeasy Plant Maxi kit (Qiagen) and quantified fluorometerically (DyNA Quant™ 200, Hoefer, Pharmacia Biotech). PCR analysis for detection of the *legumin–ctxB–rgp* gene was carried out using the gene specific (Leg-F 5′-CCA TAA CTG CAG CTC GAG ATG CAT TTT TTT ATT CTC AAT ACA TTG CT-3′ and SEKDEL-R 5′-ATC ACA ACT CAT CCT TCT CGG A-3′) primers. The position of primers is indicated in Fig. 1c. 100 ng genomic DNA was used as the template and the PCR reaction conditions were as 1 cycle of 5 min at 94°C followed by 30 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 1 min 30 s with a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 0.8% agarose gel.

Six PCR positive plants were subjected to Southern blot hybridization analysis. A fragment of 729 bp was amplified from *ctxB–rgp* fusion by using (forward) 5′- GAC CTT TGC GCC GAG TAC CAC AAC ACT CAA ATC TAC ACT CTC AAC GAC-3′ and (reverse) 5′-GAT CGG CCA CGG ATG GGG AAA TGA TGA CGA GGG ACT CCT TAG TAG T-3′ primers. The position of the primer used for probe synthesis has been marked in Fig. 1c. The PCR product was radiolabelled with α P^{32}-dCTP and used as probe. The 729 bp amplified fragment was used as a positive control. Southern blot hybridization was carried out following Tiwari et al. (2008). Since there was no *Nhe*I cleavage site within the selected *ctxB–rgp* probe, the number of hybridizing fragments indicated the number of insertion events.

Analysis of *ctxB–rgp* transcript by real-time PCR

Quantification of *ctxB–rgp* transcript in developing T_2 tobacco seeds was done by real-time PCR with an ABI Prism 7000 sequence detection system (Applied Biosystems, USA). Total RNA from the developing seeds (15 days after flowering) was extracted in six transgenic and one wild type tobacco plants by using Spectrum™ Plant Total RNA kit (Sigma, USA) following manufacturer's instructions. Two μg RNA was used for cDNA synthesis using Power Script™ RT (Clonetech, USA) according to the manufacturer's instructions. All minor-groove binder (MGB) probes and primers used in the expression study were designed by Primer Express™ (Applied Biosystems, USA) software. The transcript for *ctxB–rgp* was determined by using TaqMan probe 5′-FAM-TCC CAG AGA TGC AGT CC-MGB-3′ and primers (Forward) 5′-CCC GAC GGC AAC GTT TT-3′ and (Reverse) 5′-CAG AGC TTT CGA GCA ACT CCA T-3′. The estimated quantity of *ctxB–rgp* transcript was normalized with respect to *ubiquitin* as an internal (housekeeping gene) control in the same sample. For *ubiquitin*, TaqMan probe 5′-VIC-ACC TTG GCT GAC TAC AA-MGB-3′ and primers (Forward) 5′- GAA GCA GCT CGA GGA TGG AA-3′ and (Reverse) 5′-CCA CGG AGA CGG AGG ACA A-3′ were used. The results were analyzed in terms of % expression of *ctxB–rgp* relative to % expression of *ubiquitin* in the same sample.

Quantification of CtxB-G protein fusion by GM1-ELISA

The presence and quantitative expression of pentameric fusion protein in transgenic tobacco seed, leaf, stem and flower was monitored by monosialoganglioside-dependent enzyme linked immunosorbent assay (GM1-ELISA) as reported by Mishra et al. (2006) with some modifications. Tissues (1 g) from transgenic and wild type tobacco plants were crushed in liquid nitrogen and homogenized in 3 ml extraction buffer (100 mM Tris–Cl pH 8.0, 150 mM NaCl, 2 mM DTT, 0.05% plant protease inhibitor cocktail (PPIC; Sigma, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, 150 mM sorbitol and 5 mM EDTA). Rabbit raised anti-cholera toxin antibody (Sigma, USA) was used at 1:6,000 followed by 2 h incubation with alkaline phosphatase (ALP) conjugated goat anti-rabbit IgG (Sigma, USA) at 1:30,000 dilutions. The plates were read at 405 nm and the CtxB-G protein expression level was quantified on a linear standard curve plotted with purified bacterial CtxB (Sigma, USA) protein. The absorption value of the wild type plant was subtracted before determining the concentration of the fusion protein.

The stability of the recombinant protein in the transgenic seeds was monitored by GM1-ELISA. The fusion protein expression in 1 year stored (room temperature and 4°C) and freshly collected seeds were compared. Each experiment was repeated twice with triplicate samples.

Immunoblot analysis of fusion protein

Forty μg total seed protein (TSP) of selected transgenic and wild type plants was electrophoresed on 10% denaturing gel and transferred to iBlot™ PVDF membrane (Invitrogen, USA) by electroblotting following the manufacturer's instructions. Non-specific interactions were blocked by incubation of the membrane in 5% non-fat dry milk (NFDM) in TBS buffer (20 mM Tris–Cl, pH 7.5 and 500 mM NaCl) for 1 h at room temperature with gentle agitation on a rotary shaker (40 rpm), followed by washing

in TBS buffer for 5 min with gentle agitation. The CtxB-G protein fusion was detected by using rabbit anti-cholera toxin at 1:1,000 as primary antibody. The ALP conjugated goat anti-rabbit IgG at 1:5,000 dilution was used as the secondary antibody. The membrane was developed by ALP color development kit (Bio-Rad, USA) as per manufacturer's instruction.

For the detection of the pentameric form of CtxB-G protein fusion, 6% SDS-PAGE was used. Unboiled (non-reduced) samples were loaded without adding DTT in sample loading buffer (Areas et al. 2004). The gel was run at constant 30 V for at least 5 h, blotted into PVDF membrane by electroblotting on Hoefer Transphor™ apparatus with cooling. Human anti-rabies serum (Kam-*RAB*, Kamada Ltd., Beit-Kama, Israel) at 1:1,000 (primary antibody) and ALP conjugated goat anti-human IgG (Banglore Genie, India) at 1:5,000 (secondary antibody) used to detect pentamer of fusion protein. The membrane was developed as mentioned above.

Results

Development of transgenic tobacco plants

Thirty T_0 transgenic tobacco lines were selected on hygromycin containing medium. All the transgenic lines were phenotypically normal. Six progenies exhibited segregation ratio close to 3:1 on hygromycin containing Hoagland medium. These were analyzed by PCR and Southern blot hybridization. As expected, the 3,007 bp fragment was PCR amplified from all six T_1 plants (Fig. 2a). No amplification was noticed in the wild type plant.

The transgene integration events were analyzed by Southern blot analysis. A 729 bp radiolabelled probe designed from *ctxB–rgp* fusion region was hybridized with genomic DNA of the transgenic plants (Fig. 2b). All six T_1 plants revealed single copy gene insertion while no hybridization signal was detected in the wild type plant (Fig. 2b).

Quantification of *ctxB–rgp* transcript

Quantitative estimation of the *ctxB–rgp* transcript in transgenic tobacco seeds was carried out by real-time PCR. The % expression of the *ctxB–rgp* transcript in the seeds of transgenic plants was estimated from the number of cycles required for amplification as compared to that of *ubiquitin* taken as internal control. The % expression of *ctxB–rgp* transcript relative to % expression of *ubiquitin* transcript in the six transgenic plants can be seen in Fig. 3. Among the selected transgenic lines, the maximum 4.57% *ctxB–rgp* transcript expression was noticed in A/14. The highest

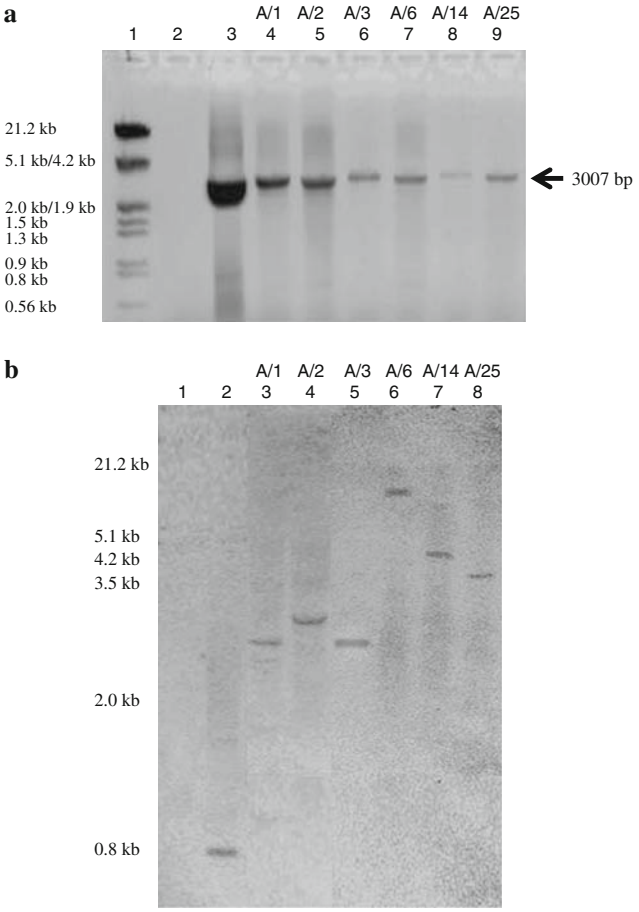

Fig. 2 Detection of stable transgene integration in genomic DNA isolated from six T_1 transgenic tobacco plants. **a** Agarose gel electrophoresis shows lambda DNA *Hin*dIII and *Eco*RI markers (*lane 1*), no amplification in wild type plant (*lane 2*), a 3,007 bp PCR amplification band from *legumin–ctxB–rgp* region in the positive control p1340 vector (*lane 3*) and transgenic plants (*lane 4–9*). **b** Southern blot hybridization of *Nhe*I digested genomic DNA from wild type plant (*lane 1*), transgenic plants (*lane 3–8*), and the 729 bp amplicon of *ctxB–rgp* fusion used as positive control (*lane 2*)

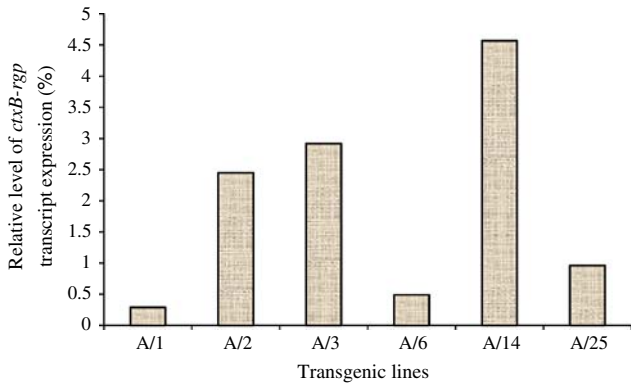

Fig. 3 Quantitative analysis of *ctxB–rgp* fusion transcript in seeds of six T_2 developing tobacco plants by real-time PCR. Level of *ctxB–rgp* fusion transcript was normalized with reference to *ubiquitin* taken as an internal control. *Bars* denote % expression of *ctxB–rgp* relative to % expression of *ubiquitin* in the same sample

expressing transgenic line (A/14) showed exponential increase in signal (due to amplification) from the 24.79 cycle (Supplementary Table 1S).

Quantification and immunoblot analysis of CtxB-G protein fusion

GM1-ELISA was performed on crude protein extracts prepared from seed, leaf, stem and flower tissues of the thirty transgenic lines to examine the expression of fusion protein. All tissues except for seed, showed no expression of fusion protein (Fig. 4a). The highest expression of CtxB-G protein fusion was observed in line A/14 (1.22% of TSP). The six transgenic lines showing single copy insertion, exhibited high level (0.45–1.22% of TSP) CtxB-G protein fusion expression. The fusion protein content in 1 year stored and freshly harvested seeds of A/14 transgenic line did not show any significant difference (Fig. 4b).

Immunoblot analysis confirmed the integrity of fusion protein expression in seeds. The seeds of the highest expressing line (A/14) were analyzed under denatured and non-reduced (unboiled) conditions. A ~80.6 kDa band representing the monomeric fusion (~66 kDa glycosylated G protein + 14.6 kDa glycosylated CtxB) was detected under denatured condition (Fig. 5a). In the non-reduced samples, an expected ~403 kDa (polypeptide pentamer) molecular mass of CtxB-G protein fusion was observed by G protein antibody (Fig. 5b). Wild type (control) plants did not show any immunoreactivity.

Discussion

The aim of the present study was to seek proof of the concept of high expression of a functional anti-cholera-rabies fusion antigen in tobacco seeds. The rabies G protein

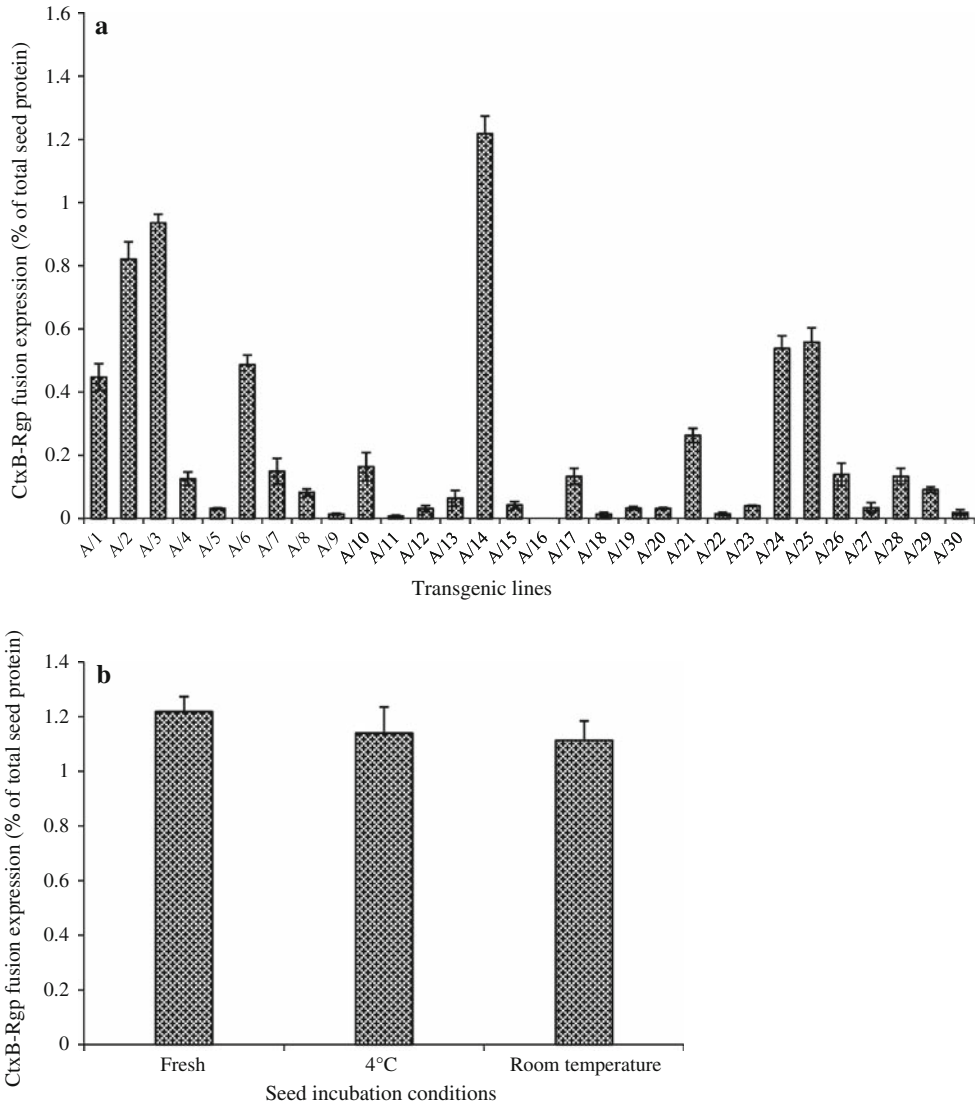

Fig. 4 Quantitative expression of the CtxB-G protein fusion by GM1-ELISA. **a** CtxB-G protein fusion expression in T_2 seeds of thirty independently transformed transgenic lines. Expression of fusion protein in flower, leaf and stem tissues not shown. **b** Fusion protein expression stability in fresh seeds (line A/14) and seeds stored for 1 year under different storage conditions. Activities in transgenic lines are indicated with the bars representing percent of TSP ± SD

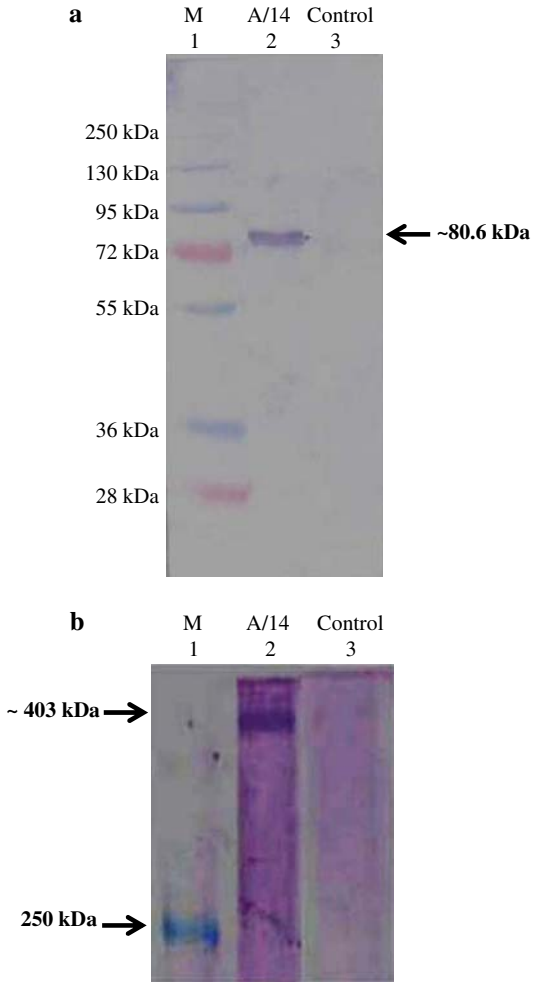

Fig. 5 Western blot analysis of CtxB-G protein fusion expression in T_2 seeds of tobacco plant. Total seed protein of A/14 line was loaded under denatured (**a**) and non-denatured (**b**) conditions. The expected molecular size bands are seen in *lane 2* while these are absent in the wild type control plant (*lane 3*). *Lane 1* shows protein molecular weight markers (*M*)

was fused with the CtxB to take advantage of both seed-specific and adjuvant-activated expressions. The use of CtxB as a carrier molecule can modulate immune response against cholera, besides rabies. A seed based rabies vaccine antigen expression system may potentially be more promising for commercial production of the antigen or its use as a mucosal or edible vaccine. The *legumin* promoter used in the present study achieved a high level of CtxB-G protein fusion expression in seeds. None of the tobacco transgenic lines showed any expression in flower and other vegetative plant parts. The results are in agreement with behavior of other globulin seed storage protein promoters (Bustos et al. 1989; Shirsat et al. 1989; Itoh et al. 1993). The level of expression of CtxB-G protein corresponds with the transcript level in different transgenic lines as evident from real-time PCR data. As expected, different transgenic lines showed variation in the level of expression of CtxB-G protein because of differences in the position of insertion of the fusion gene (Hobbs et al. 1990; Matzke and Matzke 1998; Day et al. 2000). Besides the use of a seed specific *legumin* promoter, the modification in native *rgp* and *ctxB* genes and inclusion of the ER retention sequence at 3′ end may have also contributed to achieving the high level (>1% of TSP) expression of the fusion protein observed in the present study in tobacco seeds. Several previous studies suggest that the avoidance of polyadenylation, mRNA destabilizing sequence and the use of plant-preferred codons improve yield of recombinant proteins in plants (Perlak et al. 1991; Mason et al. 1998; Chikwamba et al. 2002). The accumulation of stable and functional proteins in seeds as noticed by us, is crucial to molecular farming of proteins in plants. The ER is favorable location for post-translational modification of active heterologous proteins. ER retention is achieved through the C-terminal SEKDEL peptide, which mediates the retrieval of its tagged protein. A number of reports have demonstrated the positive effect of ER retention on the yields of several immunoglobulins and vaccine antigens which require chaperone assistance, oligomer formation, disulfide bond formation and/or co-translational glycosylation (Arakawa et al. 1998; Stöger et al. 2000; Moravec et al. 2007). We have earlier reported the designing of rabies G protein with ER retention peptide and its expression in tobacco leaves (Ashraf et al. 2005). The leaf expressed G protein showed glycosylation and, when given by intraperitoneal (i.p.) route, provided immunoprotection against the virus challenge in mice.

The G protein was selected in the present study because it is considered as the major antigen conferring protective immunity to rabies (Cox et al. 1977; Morimoto et al. 2001; Nel et al. 2003). In earlier studies, the rabies G protein has been expressed either alone or in combination with rabies nucleoprotein (N protein) in tobacco and spinach leaf tissue (Modelska et al. 1998; Yusibov et al. 2002; Ashraf et al. 2005). These studies demonstrated successful immunoprotection through i.p. and oral administration. Several other vaccine antigens have been expressed in leaf tissues. Leaves form an abundant tissue in plants but it is difficult to purify the desired protein from them due to high proteolytic activities and the presence of phenolic compounds and pigments (Stevens et al. 2000; Stoger et al. 2005; Benchabane et al. 2008; Tiwari et al. 2009). Moreover, implementation of good manufacturing practices is more difficult as large volume of biomass has needs to be handled. Further, the leaves can not be stored for a long time under room temperature. Arango et al. (2008) recently reported *CaMV35S* promoter regulated rabies N protein antigen expression (1–5% of total soluble fruit protein) in tomato plants and performed mice immunoprotection assay. Only i.p. immunized mice showed weak protection

against virus challenge while the orally immunized mice were not protected. They suggested the lack of mucosal adjuvant in oral administration of antigen as the possible reason for diminished immune response. Thus, the development of appropriate formulations for rapid absorption in buccal mucosa, as attempted in this study may be one of the solutions to such problems. Hooper et al. (1994) demonstrated that mice orally immunized with the N protein in the presence of the CtxB conferred partial protection against a rabies virus.

A number of proteins have been genetically fused to the C-terminus of the CtxB and expressed in different plants tissues in earlier studies. For instance, human insulin expressed in potato tuber and leaf (Arakawa et al. 1998), rotavirus enterotoxin protein (NSP4) (Arakawa et al. 2001; Kim and Langridge 2003) and anthrax lethal factor protein (LF) (Kim et al. 2004) in potato tuber, B chain of human insulin ($InsB_3$) in tobacco leaf (Li et al. 2006) and surface protective antigen (SpaA) of *Erysipelothrix rhusiopathiae* in tobacco hairy root (Ko et al. 2006). In each case, the fusion protein retained functional activity with respect to pentamerization and GM1 binding. The stable pentamer formation of the CtxB-G protein fusion in tobacco seeds was confirmed in our study by Western blot analysis. The expected ~403 kDa (pentameric ~14.6 kDa glycosylated CtxB + ~66.0 kDa glycosylated G protein) band was observed. The monomer of bacterial CtxB has molecular mass of 11.6 kDa (Cuatrecasas 1973; Cai and Yang 2003; Dawson 2005) and the G protein is ~60.0 kDa (Yelverton et al. 1983; Lathe et al. 1984). We have earlier reported that the plant expressed CtxB had significantly higher molecular mass of ~14.6 kDa (Mishra et al. 2006) because of glycosylation while the G protein was ~66.0 kDa (Ashraf et al. 2005), as in the case of native viral protein.

The present study demonstrated steady high level expression of the CtxB-G protein fusion in tobacco seeds. Considering high protein yield per unit area, antigen sufficient for vaccinating millions of individuals may become available from one acre crop by expressing the antigen in seeds of peanut (Tiwari et al. 2009). The peanut regeneration and genetic transformation was successfully achieved in our laboratory (Tiwari et al. 2008; Tiwari and Tuli 2008, 2009). Further studies regarding its expression in peanut seeds and utility of the peanut expressed antigen in mice immunoprotection assay are in progress.

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