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Receptor Mediated Oral Delivery Of Bioencapsulated Green Fluorescent Protein Expressed In Transgenic Chloroplasts

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RECEPTOR MEDIATED ORAL DELIVERY OF BIOENCAPSULATED GREEN FLUORESCENT PROTEIN EXPRESSED IN TRANSGENIC CHLOROPLASTS

by

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ABSTRACT

The skyrocketing costs of prescription medicine in developed countries and their lack of availability in developing countries are the most challenging problems of human health. Primary reasons for such high cost are fermentation-based production, expensive purification methods, the need for low temperature storage and transportation and the delivery through sterile injections. Most of these expenses could be minimized or eliminated when therapeutic proteins are expressed and orally delivered via plant cells.

Chloroplasts have the machinery to fold complex and biologically active eukaryotic proteins in the soluble chloroplast stromal compartment. Protein expression through chloroplast transformation system offers a number of advantages over nuclear transformation such as a high level of transgene expression (up to 47% of the total soluble protein), due to the presence of 10,000 copies of the transgene per cell, which is uniquely advantageous for oral delivery of adequate amounts of the therapeutic protein or vaccine antigen. It is also an environmentally friendly approach due to effective gene containment and lack of transgene expression in pollen since the chloroplast genome is maternally inherited.

To study receptor-mediated oral delivery of therapeutic proteins using the transmucosal carrier cholera toxin B subunit (CTB), a *CTB-GFP* fusion protein separated by a furin cleavage site was expressed via the tobacco chloroplast genome and used as a visible marker. Site specific integration of the transgene was confirmed by PCR analysis. Southern blot analysis confirmed homoplasmy. Immunoblot analysis confirmed the expression of both the monomeric as well as the pentameric forms of CTB-GFP in

transgenic plants. Expression levels of upto 21.3% were obtained and the functionality of the CTB-GFP pentamers was confirmed by an *in vitro* GM1 binding assay. GFP was seen in the intestinal mucosa, liver and spleen of mice orally fed with CTB-GFP expressing leaves, while CTB was detected only in the intestinal cells. Intestinal macrophages and dendritic cells stained positive for both the CTB as well as GFP. These results suggest successful cleavage of the foreign protein from the transmucosal carrier and its delivery to various organs. These investigations should facilitate the development of a novel cost-effective oral delivery system for plant-derived therapeutic proteins.

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INTRODUCTION

One of the most challenging problems of human health management is the high cost of prescription drugs in developed countries and their lack of availability in developing countries. For example, interferon (IFN) alpha 2b is used for the treatment of viral diseases such as hepatitis C as well as for certain cancers. However, interferon treatment for four months costs $26,000 in the United States where more than forty five million Americans do not have health insurance (Daniell 2004b). Several hundred million people in developing countries are infected with hepatitis but the daily income of one third of the world's population is less than $2 per day (Daniell 2004b). The high cost of prescription drugs is due to a number of reasons including fermentation based production (each fermenter costs several hundred million dollars to build), expensive purification and *in vitro* processing methods (such as column chromatography, disulfide bond formation costing about 60% of production cost, Petridis, 1995), the need for storage and transportation at low temperature and delivery via sterile injections requiring the involvement of hospitals and highly qualified health professionals (Daniell 2004b). Therefore, new approaches to minimize or eliminate most of these expenses are urgently needed.

Transgenic plants to produce biopharmaceuticals

Transgenic plants offer many advantages over other methods of production of biopharmaceuticals. As discussed previously, the low cost of production of biopharmaceuticals due to elimination of the need for expensive purification and *in vitro*

processing reduces the prices of these products thus making them available to people who need them the most. Since the proteins are encapsulated in plant cells, it eliminates the necessity of the presence of a cold chain to get to the consumer (Daniell 2004b). Protection of the protein via bioencapsulation also provides heat stability (Mason 2002, Arntzen 2005). Plant based biopharmaceuticals are also safer since plant-derived products are less likely to be contaminated with human pathogenic microorganisms than those derived from animal cells because plants in general do not act as hosts for human infectious agents (Giddings 2000). The generation of systemic and mucosal immunity (Mason 1998) or induction of oral tolerance (Arakawa 1998) on oral delivery of biopharmaceuticals or vaccine antigens is another important advantage.

Chloroplast Genetic Engineering

The concept of chloroplast genetic engineering was first conceived in the mid 1980's with the introduction of isolated intact chloroplasts into protoplasts (Daniell and McFadden 1987). The development of the biolistics method of transformation made it feasible to transform plastids without the need to isolate them (Klein *et al.*1987). In 1991, the *C. reinhardtii* chloroplast genome was transformed with the *aad*A gene conferring spectinomycin or streptomycin resistance (Goldschmidt- Clermont 1991). This became a major breakthrough since different varieties of plants could be transformed using *aadA* as the selectable marker. In 1993, stable integration of the *aad*A gene into the tobacco chloroplast genome was demonstrated (Svab and Maliga 1993). Initially, when the transgenes were introduced via the chloroplast genome, it was believed that foreign genes could be inserted only into transcriptionally silent spacer regions of the chloroplast

genome (Zoubenko *et al*. 1994). The second major breakthrough came in 1998 when Daniell *et al*. (1998) advanced the concept of inserting transgenes into functional operons and transcriptionally active spacer regions. This approach facilitated the insertion of multiple genes under the control of a single promoter, enabling the coordinated expression of transgenes (DeCosa *et al*. 2001). The trnI-trnA intergenic region is transcriptionally active because of the read-through transcription of the upstream 16S rRNA promoter capable of transcribing six native genes downstream (Dhingra *et al*. 2004).

Transformation of tobacco chloroplasts

Tobacco, a non-food/ feed crop is ideal for transformation because of its ease for genetic manipulation. Another advantage of tobacco is that it is an excellent biomass producer (in excess of 40 tons fresh leaf weight/acre based on multiple mowings per season) and a prolific seed producer (up to one million seeds produced per plant), thus making it very beneficial for large-scale commercial production of vaccines and therapeutic proteins. For example, one acre of cultivar can produce 400 million doses of anthrax vaccine (Watson *et al*. 2004). It has been estimated that the cost of production of recombinant proteins in tobacco leaves will be 50-fold lower than that of *Escherichia coli* fermentation systems (Kusnadi 1997). Tobacco has been used for hyper-expression of vaccine antigens for cholera (Daniell *et al*. 2001), anthrax (Watson *et al*. 2004), plague (Daniell *et al*. 2005), hepatitis C (Bhati 2005), rotavirus (Kalluri 2005), amoebiasis (Chebolu 2005) and tetanus (Tregoning *et al.* 2003). Various biopharmaceuticals such as human serum albumin (Fernandez *et al*. 2003), human somatotropin (Staub *et al*. 2000),

interferon-GUS fusion proteins (Leelavathi and Reddy 2003), magainin, a broad spectrum topical agent, systemic antibiotic, wound healing stimulant and a potential anticancer agent (Degray *et al*. 2001), interferon alpha (Daniell *et al*. 2004a), insulin and insulin- like growth factor (Daniell *et al*. 2004) have also been expressed. Monoclonal antibodies such as Guy's 13, a monoclonal antibody against *Streptococcus mutans* which protects against dental carries has also been expressed in the chloroplast (Daniell *et al*. 2001b). Xylanase, an industrially important enzyme, when expressed through the nuclear transgenic plants showed cell wall degradation and affected plant growth, while expression via chloroplasts showed no such effects (Leelavathi *et al.* 2003, Daniell *et al*. 2004d).

Introduction of agronomic traits via chloroplast engineering

Chloroplast genetic engineering technology has been used to generate useful traits such as insect resistance, herbicide resistance, drought tolerance, and so on. Insect resistance has been achieved by expressing insecticidal proteins from *Bacillus thuringiensis* (Bt); the Cry2Aa2 protein was expressed at levels up to 47% of total leaf protein which to date is the highest expressed foreign protein in transgenic plants (DeCosa *et al*. 2001). Herbicide resistance was achieved against glyphosate, a broad spectrum herbicide that non-selectively kills weeds by inhibiting a nuclear-encoded chloroplast localized enzyme, 5-enolpyruvylshikimate- 3-phosphate synthase (EPSPS), in the shikimic acid pathway of plants and microorganisms that is required for the biosynthesis of aromatic amino acids (Daniell *et al*. 1998). The antimicrobial peptide MSI-99, an analog of magainin has been expressed in the chloroplast genome of

transgenic tobacco up to 21.5% TSP (Degray *et al*. 2001). MSI-99 offers protection against prokaryotic organisms due to its high specificity for negatively charged phospholipids found mostly in bacteria. Extracts from MSI-99 transformed plants inhibited growth of *Pseudomonas aeruginosa*, a multi-drug resistant bacterium, which acts as an opportunistic pathogen in plants, animals and humans. MSI-99 is also biologically active against *Pseudomonas syringae*, a major plant pathogen (DeGray *et al*. 2001, Devine and Daniell 2004, Daniell *et al*. 2004a). Drought tolerance was generated by expressing the yeast trehalose phosphate synthase (TSP1) gene in tobacco plants (Lee *et al*. 2003).

Chloroplast engineering of important crops

Chloroplast genetic engineering technology is currently applied to other useful crops such as potato, tomato, carrot, cotton and soybean by transforming different plastid genomes (Sidorov *et al*. 1999, Ruf *et al*. 2001, Kumar *et al*. 2004a, Kumar *et al*. 2004b, Dufourmantel *et al*. 2004). Successful transformation of food crops can enable the expression of vaccines and therapeutic proteins in these plants, making edible vaccines and proteins a reality. There are certain limitations to this however, such as inadequate tissue culture protocols, lack of selectable markers and a difficulty in expressing transgenes in non-green tissues (Daniell *et al*. 2004d).

Carrot plastid genome has been transformed using the non-green tissue as explants and regenerated via somatic embryogenesis (Kumar *et al.* 2004a). Overexpression of betaine aldehyde (BA) in carrot was carried out to engineer salt tolerance. The toxic betaine aldehyde is converted to non-toxic glycine betaine by the

chloroplast BADH enzyme. Glycine betaine serves as an osmoprotectant and confers salt tolerance. The transgenic calli obtained from cultured cells expressing BADH are green in color which distinguishes them from the untransformed cells which are yellow. Since somatic embryos of carrot are derived from a single cell and multiply via recurrent embryogenesis, the resultant transgenic plant is obtained from a single source of origin. Carrot is also an ideal plant to be used as an edible vaccine since it can be eaten raw which preserves the structural integrity of the vaccine antigen.

Other than carrot, chloroplast genetic engineering of various other plants has also been achieved. Kumar *et al*. recently transformed the cotton plastid genome. The transgenic seeds obtained were resistant to kanamycin selection whereas the untransformed seeds were not (Kumar *et al.* 2004b). Similarly, the first successful development of transgenic soybean plants was achieved by Dufourmantel *et al*. (Dufourmantel *et al*. 2004). The successful plastid transformation of these crop plants was suggested to be due to the use of 100% homologous plastid DNA sequences in the species-specific vectors. Even though the concept of universal vector was proposed several years ago, the use of species-specific vectors has demonstrated successful plastid transformation (Daniell *et al*. 2004d).

Advantages of chloroplast engineering over nuclear expression system

One of the major drawbacks of the nuclear expression system is the possibility of transmission of the transgene to the surrounding nontransgenic plants via cross pollination. This is one of the primary reasons for resistance to GM crops in Europe. The chloroplast genome on the other hand is maternally inherited (Nagata 1995). Thus the

technique is environmentally safe; making chloroplast transgenic plants an excellent choice as biofactories to produce vaccines and biopharmaceuticals on a large scale (Daniell 2002). The maternal inheritance of chloroplast genome in plants is achieved during the male pollen development of generative cells which form the sperm cells. During the process of microspore mitosis, all the plastids get distributed to the vegetative cells and the generative cells are devoid of plastids. In some species, the generative cells get a few plastids which degenerate during maturation. Another type of maternal inheritance seen in several cereal crops occurs during the fertilization process, during which the sperm nucleus alone is transmitted into the egg cell while the plastid genome is removed either just prior to or during the process of fertilization (Hagemann 2004).

Another drawback of the nuclear expression system is the poor levels of protein expressed in the plants. In order for production of proteins in plants to be commercially feasible, expression levels greater than 1 % of the total soluble protein must be achieved (Kusnadi *et al*. 1997). Chloroplast genetic engineering has shown a very high level of expression of the foreign protein (DeCosa *et al*. 2001), making this approach commercially viable for the production of biopharmaceuticals on a large scale. The high expression levels are also very important when delivering vaccines orally i.e. when using the concept of edible vaccines, since adequate quantities of the vaccine antigen can be delivered due to the high expression levels. The high expression levels are due to the presence of 10,000 copies of the chloroplast genome in a single cell. A typical plant leaf cell contains about 100 chloroplasts and each chloroplast further harbors approximately 100 copies of the same genome. This implies that a single gene is represented by at least

10,000 copies in a single plant cell. The copy correction mechanism ensures that introduction of the transgene into any one of the inverted repeat regions of the chloroplast genome gets integrated into the other region as well, thus further increasing the number of transgenes per cell. This makes it quite appealing to introduce a transgene into the chloroplast genome and obtain high levels of expression, taking advantage of the high copy number (Daniell *et al* 2004c). Unlike random nuclear integration, chloroplast integration is site specific. Thus the problems of gene silencing and position effect due to random integration of the transgene are not seen in chloroplasts. It is possible to insert a foreign gene into the site-specific transcriptionally active spacer region between the functional genes of chloroplast genome using two flanking regions via homologous recombination (Daniell 2001). Chloroplast transformation vectors are thus designed with homologous flanking sequences on either side of the transgene (Daniell *et al*. 1995) and introduced into the chloroplast genome of plant cells via particle bombardment (Sanford *et al* 1993) or into the protoplasts by the process of PEG treatment (Golds *et al* 1993). Chloroplasts also possess the ability to accumulate any foreign proteins in large amounts that could otherwise be harmful if they were in the cytoplasm. Trehalose, a pharmaceutical industry preservative was toxic when accumulated in cytosol where as was non toxic when compartmentalized in plastids by chloroplast expression system (Lee *et al* 2003).

Unlike in nuclear transformation, multigene engineering in a single transformation event is possible in chloroplasts since chloroplasts can process polycistronic RNAs arranged in an operon (Daniell et al 2002). Under the control of

strong 16 s ribosomal RNA gene promoter (Prrn) located upstream of the transgene, read through transcription occurs which facilitates the transcription of the foreign gene or genes flanked by 3' and 5' untranslated regions. This approach has been highly successful in expressing proteins in an operon system downstream of the aad*A* gene after inserting the cassette into trnA–trnI intergenic region of the chloroplast vector (Quesada *et al* 2005). The *cry* operon from *Bacillus thurengiensis* (Bt), coding for the insecticidal protein delta-endotoxin, introduced into the chloroplast genome showed an expression of about 47% of the total soluble protein (DeCosa *et al*. 2001). A native bacterial operon without codon optimization was engineered successfully in transgenic chloroplasts. Two bacterial enzymes that confer resistance to two different forms of mercury poisoning (mercuric ion reductase (merA) and organomerurial lyase (merB)) were expressed as an operon in transgenic chloroplasts, and conferred resistance to very high levels of mercury and highly toxic organomercurial compounds (Ruiz *et al* 2003).

Advantages of chloroplast engineering over *E. coli* expression system

A major disadvantage of the *E. coli* expression system is the high cost of fermentors required as well as expensive *in vitro* processing and purification steps needed to properly fold and purify a transgenic protein. Recombinant proteins such as insulin for instance require very expensive *in vitro* processing which accounts for about 60% of the cost of production in *E. coli* (Petridis 1995).

Chloroplasts, on the other hand, have the machinery to correctly process and fold eukaryotic proteins in the soluble stromal compartment (Daniell 2004b). Chaperonins present within chloroplast are believed to aid in the folding and assembly of non native

prokaryotic and eukaryotic proteins (Daniell *et al* 2001, Daniell *et al* 2005). The light signal sensed by chlorophyll is transferred via the photosynthetic electron flow to proteins called thioredoxins, which are very efficient in thio-disulfide interchanges with various protein disulfides (Ruelland and Miginiac-Maslow, 1999). The Protein Disulfide Isomerase (PDI) system consisting of chloroplast polyadenylate-binding proteins that specifically bind to the 5'UTR of the *psb*A mRNA and are modulated by redox status through PDI (Kim and Mayfield, 1997), is another mechanism of control of disulphide bond formation.

Another advantage of chloroplast genetic engineering over *E. coli* expression system is the ability to hyperexpress the transgene using a light regulated promoter. The psb*A* 5'UTR consists of essential light regulated translation elements that are target sites for enhancing the translation. The light driven photosynthetic electron transport chain generates an electrochemical gradient across the thylakoid membranes, the redox states of specific electron carriers, and stromal ADP/ATP ratio which are sensed by the translation regulators. These regulators aid in the binding of specific proteins to the ribosome binding sites of 5'UTR psbA which enhances translation (Zerges 2004). Expression of human serum albumin HSA under the control of psb*A* 5'UTR was light dependent as was seen by the 11.1% of tsp expression levels obtained after 50 hours of continuous light. There was also a 2-4 fold decrease in expression after the 8 h dark period thus highlighting the importance of the light regulated promoter. The levels of expression of HSA in mature plants under the translational control of SD sequence showed very low amount of accumulation when compared to expression under the control of psb*A*

promoter and 5'UTR which resulted in a 500-fold increase in HSA accumulation as inclusion bodies in chloroplast which probably offered protection from proteolytic degradation (Fernandez-San Millan A *et al* 2003). Similarly, expression levels of Interferon alpha 2b was about 19% of TSP under continuous light illumination (Daniell *et al* 2004a).

Vaccine antigens expressed in transgenic chloroplasts

Apart from the general advantages of the chloroplast expression system, there are several special features that make the expression of vaccine antigens via the chloroplast genome very attractive.

1. Subunit vaccines are not toxic even when expressed at high levels
2. Bacterial genes have high AT content allowing for high expression in the chloroplast
3. Oral delivery of vaccines via plant cells enables the development of both systemic as well as mucosal immunity
4. Expression of the vaccine antigen in the plant cell protects the antigen by bioencapsulation

Vaccines that have already been expressed in the chloroplast include the Cholera toxin B-subunit (CTB), which does not contain the toxic component that is in CTA (Daniell *et al*. 2001a), the F1~V fusion antigen for plague (Singleton 2003), the 2L21 peptide from the Canine Parvovirus (CPV) (Molina *et al*. 2004), Anthrax Protective antigen (PA) (Watson *et al.* 2004), C terminus of *Clostridium tetani* (TetC) (Tregoning *et al*. 2003), LecA for *Entamoeba histolytica* (Chebolu 2005), NS3 for hepatitis C (Bhati

2005), NSP4 for rotavirus (Kalluri 2005). The functionality of these vaccine antigens was determined by both in vitro assays as well as in vivo studies. CTB for instance was effective in the GM1-ganglioside binding assay which indicates proper folding and formation of disulfide bonds to form pentamers (Daniell *et al*. 2001a). When mice were immunized intraperitoneally with the leaf extracts from chloroplast expressed CTB-2L21, the developed anti-2L21 antibodies were able to recognize VP2 protein from CPV (Molina *et al*. 2004). Anthrax PA83 expressed in transgenic tobacco chloroplasts elicited an immune response in the mice proving that plant derived PA is biologically similar to PA derived from *Bacillus anthracis* (Koya 2004). The C terminus of *Clostridium tetani* (TetC) was expressed at 25% TSP for AT rich and 10% TSP for GC rich sequences which shows that chloroplasts favor prokaryotic-AT rich sequences. TetC when administered intranasally produced both IgG and IgA antibodies and was immunoprotective against the toxin (Tregoning *et al*. 2003).

Bioencapsulation for protection of vaccine antigens in plant cells

Oral delivery of vaccines and biopharmaceuticals is essential for development of mucosal immunity as well as induction of tolerance. It also serves as a cost effective means of protein delivery eliminating the need for expensive in vitro processing, purification, cold chain and sterile injections. Plant cells provide the ideal vehicle for oral delivery primarily due to the phenomenon of bioencapsulation. The vaccines and therapeutic proteins are protected inside the cell wall of the plant cell hence preventing degradation by the stomach acid pH and digestive enzymes (Walmsley and Arntzen 2000, Yu and Langridge 2001). In the intestine, certain enzymes such as alpha,beta

hydroxylases create pores in the cell wall enabling the release of the transgenic protein into the intestinal lumen allowing for successful oral delivery. In human clinical trials performed with plant derived vaccines, plant cells have proven sufficient for vaccinogen protection against digestion, and the vaccinogen has induced systemic and mucosal immune responses without the aid of adjuvants (Tacket *et al*. 1998, Kapusta *et al*. 1999, Tacket *et al*. 2000, Walmsley and Arntzen 2000, Tacket *et al*. 2003, Tacket *et al*. 2004). Heat-labile enterotoxin B-subunit (LTB) from *E. coli* was expressed by nuclear transformation in tobacco (<0.01% Total soluble protein) and potato (0.19% TSP). The LTB expressed in potato was found to be immunoprotective when administered orally. Inspite of lower expression in tobacco these antigens were immunogenic (Haq *et al*. 1995, Mason *et al*. 1996, Tacket *et al*. 1998). The capsid protein of the Norwalk virus expressed in potato and tomato was immunogenic when administered orally (Mason *et al*. 1996, Richter *et al*. 2000, Tacket *et al*. 2000). The envelope surface protein of hepatitis B virus was expressed by nuclear transformation in tobacco, potato and lupin. They all had less than 0.01% fresh weight expression but were still immunogenic (not protective) when administered orally (Richter *et al*. 2000, Kapusta *et al*. 1999). This again brings us to the need to ensure high expression of therapeutic proteins in plants. IFN-alpha given orally has biological activity in humans and other animals (Bocci 1999). Plant derived edible vaccines have also been proven in commercial animal and native animal trials (Castanon *et al*. 2000, Tuboly *et al*. 2000). Chloroplast genetic engineering is currently being applied to crops amiable to oral vaccines such as potato, tomato, carrot and

soybean (Sidorov *et al*. 1999, Ruf *et al*. 2001, Kumar *et al*. 2004a, Dufourmantel *et al*. 2004).

CTB as a transmucosal carrier to orally deliver biopharmaceuticals

Cholera toxin (CT) belongs to the AB5 family of toxins and consists of an A subunit and a pentameric B subunit. Cholera toxin B subunit (CTB) is a non-toxic part of the enterotoxin produced by *Vibrio cholerae,* which causes acute watery diarrhea (rice water stools). CTB is a homo-pentamer of 5 identical subunits of 11.6 KDa that form a ring like structure and have the ability to bind selectively to oligosaccharide GM1 ganglioside receptors present on the intestinal epithelial cell surfaces (Sixma *et al*. 1991, 1992; 18 Lencer, 2001). GM_1 sorts the CT into lipid rafts and via a retrograde trafficking pathway to the endoplasmic reticulum, where the toxin unfolds and transfers its enzymatic subunit (CTA) to the cytosol, probably by translocation through the translocon $sec61_P$ (Lencer, 2001). CTB bound to the receptor GM_1 exits the ER and remains membrane bound in the basolateral surface of the cell. In this manner, CTB delivers a macromolecule conjugated to it across the intestinal lumen into the intestinal cell. CTB is also known to act as a mucosal adjuvant which enhances the antibody response when coadministered with unrelated antigens and increase antibody titers (Northrup and Fauci, 1972, Richards, 2001, Millar *et al*. 2001, Salmond *et al*. 2002).

RATIONALE AND APPROACH

The aim of this project was to create an efficient delivery mechanism for the oral delivery of therapeutic proteins expressed in transgenic chloroplasts. For this proof-of-concept project GFP was chosen as a visible marker. Since the chloroplast expression system has several unique advantages as discussed above, the method was selected to transform tobacco chloroplasts. CTB was chosen as a transmucosal carrier to efficiently deliver proteins across the intestinal lumen.

Accordingly, a CTB-GFP fusion protein, separated by a furin cleavage site, was expressed in tobacco chloroplasts. Furin, a member of prohormone-proprotein convertases (PCs Van den Ouwenland *et. al*,. 1990, Nakayama 1997) is a ubiquitously expressed enzyme found in the trans-Golgi network (TGN Bosshart *et al*.1994, Molloy 1994), endosomes, plasma membrane and extracellular space (Mayer 2004). Furin cleaves protein precursors with narrow specificity following basic Arg-Xaa-Lys/ Arg-Arg-like motifs (Henrich *et al*. 2003). The furin cleavage site between CTB and GFP would therefore facilitate intracellular cleavage of the target protein (GFP), thus enabling the delivery of the GFP across the intestinal cell into the circulation.

The CTB-furin-GFP transgene cassette was cloned into the universal chloroplast vector pLD-Ctv which has flanking regions from the tobacco chloroplast genome to enable homologous recombination. The resultant leaves of the transgenic plants were fed to female Balb/c mice which were then sacrificed. The liver, spleen and intestine of the mice were examined for the presence of GFP. To judge the efficiency of the transmucosal carrier, transgenic leaves expressing another construct, IFN-furin-GFP were also fed to

another group of Balb/c mice, which were also sacrificed. The liver, spleen and intestine of these mice were examined as well. As a negative control, untransformed leaves were fed to a third group of Balb/c mice.

MATERIALS AND METHODS

General Protocols

The ultra competent cells were prepared using rubidium chloride method (Kumar *et al.* 2004). The ultra competent cells are absolutely necessary for the transformation of bacterial cells with the plasmid. *E.coli* XL1-Blue MRF ab (Stratagene), a disabled non pathogenic, tetracycline resistant strain, has a history of safe laboratory use due to its inability to survive in the antibiotic environment and has been used to prepare the ultra competent cells. The *E.coli* glycerol stock was streaked on the LB agar plate containing 12.5 µg/ml tetracycline and incubated at 37 °C overnight. Single isolated colony was picked and grown in 5 ml of Psi broth (per liter- 5g Bacto yeast extract, 20g Bacto Tryptone, 5g magnesium sulfate, pH 7.6) containing 12.5 µg/ml tetracycline and incubated at 37 °C for 12- 16 hrs in a horizontal shaker at 225 rpm. Approx. 1 ml of the overnight culture was inoculated in 100 ml of Psi broth and was incubated at 37 °C for about 2 hours in a shaker at 225 rpm. The optical density (O.D) was checked at 550 nm after two hours and subsequently after each half hour or an hour depending on the O.D value. The culture was continued to grow until it reaches to 0.48 O.D. The culture was kept on ice for 15 minutes. The cells were pelleted by centrifugation at 3000g/5000 rpm for 5 minutes in a sorvall centrifuge. The supernatant was discarded and the pellet was resuspended in 0.4 volume (40 ml) of ice-cold TFB-I solution. The cells were re-pelleted at 3000g / 5000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in (0.04 volume) 4 ml of TFB-II solution and immediately iced for 15

minutes. This suspension was divided into 100 µl aliquots, then quick frozen in liquid nitrogen and stored at - 80 °C.

Transformation of the Competent E. coli XL1-Blue cells

The competent cells of one hundred µl aliquot were taken from -80 °C and immediately thawed on ice and transferred to a falcon tube. About one µl (100 ng) of plasmid DNA was added to the competent cells and was mixed by gentle tapping. The mixture was incubated on ice for 30 minutes with gentle tapping at after first 15 min. Then, the mixture was incubated at 42° C in a water bath for 90-120 seconds and then immediately put on ice for two minutes. Approx. 900µl of LB broth was added to cells and were incubated at 37° C for 45 minutes in a horizontal shaker at 225 rpm. The cells were pelleted by spinning at 13,000 rpm for 30 seconds. The eight hundred µl of supernatant was discarded leaving 100µl, followed by resuspending the cells. Two samples, 50µl and 100µl, of the suspension were inoculated on the agar plate with appropriate selection agent and spread with a glass rod.

Construction of chloroplast vector

The pLD-CTB-GFP construct was based on the universal chloroplast vector pLD (Fig.1) that has been used successfully in this laboratory (Daniell 2001a). CTB-GFP construct was engineered with a furin cleavage site, Pro-Arg-Ala-Arg-Arg, in between CTB and GFP. The constitutive 16 s rRNA promoter was used to drive transcription of the *aad*A and the CTB-GFP genes. The aminoglycoside 3'adenylyltransferase (*aad*A) gene conferring spectinomycin resistance was used as a selectable marker. The 5'-UTR

from *psb*A, including its promoter, was engineered to enhance translation of the CTB-GFP since it has several sequences for ribosomal binding sites. The 3'UTR region conferred transcript stability.

Bombardment and selection of transgenic plants

The Bio-Rad PDS-1000/He biolistic device was used to bombard pLD-CTB-GFP onto sterile *Nicotiana tabacum* cv. Petit Havana tobacco leaves, on the abaxial side as has been previously described (Daniell 1997). The bombardment media was prepared as described previously (Daniell 1997). For the bombardment, it is most important to maintain the aseptic conditions. For this all the essential equipments were sterilized. The stopping screens, macro carrier holders, forceps, Whatman filter paper, Kim wipes were autoclaved prior to bombardment. The macro carriers and the rupture discs were sterilized under hood by immersing them in 95% ethanol for 15 minutes followed by drying. Fifty µl of gold particles was placed in a micro centrifuge tube and 10 µl of DNA (1 µg/µl) were added. Fifty µl of 2.5M $CaCl_2$, 20 µl of 0.1M spermidine-free base were added sequentially to the mixture to ensure proper binding of DNA to the gold particles. Vortexing was done after addition of each component to ensure proper mixing of components and binding of DNA to the gold particles. The mixture was then vortexed for 20 minutes at 4 °C. Two hundred µl of absolute ethanol was added to the vortexed mixture at room temperature and followed by a quick spin at 3000 rpm in a microfuge for 30 seconds, supernatant was removed and this wash procedure was repeated four times. Finally, the gold particles were resuspended in thirty ul of 95% ethanol. The gold particles with DNA were placed on ice to be used in next two hours. Aseptic green

healthy leaves from *Nicotiana tabacum* var. *Petit havana* plant growing in jars containing MSO medium (30g sucrose, 1 packet of MS basal salt mixture, pH 5.8) and placed on a petri dish *(100 x 15)* containing RMOP media with no selection and a Whatman filter paper on the top of media. The RMOP media contains MS basal salt mixture (one pack), 30 grams of Sucrose, 100mg of myo-inositol, 1ml of benzylaminopurine (BAP: 1mg/ml), 100 µl of α -naphthalene acetic acid (NAA: 1mg/ml), 1ml of thiamine hydrochloride (1mg/ml), and Water (1 liter), with the pH adjusted to 5.8 using 1N KOH. About six grams of phytagar per liter was added to the media and autoclaved. The leaves were placed with the abaxial side upwards. The gene gun (Bio-Rad PDS-1000/He) was sterilized in the inside chamber with 70% ETOH prior to bombardment. The macro carriers were placed on the macro carriers holders. The gold particles lying on ice were vortexed and five µl of gold particles containing the DNA were placed on top of the macro carrier. Vortexing is an important step while placing the gold particles on the macro carriers. The rupture disc, stopping screens and macrocarrier holders containing the macrocarrier, and the leaf were put in place and secured to proceed with the bombardments. The gene gun and the vacuum pump were turned on, and the helium tank was turned to the open positions and the valve is turned on till the pressure reaches 1350 psi. The vacuum in the gene gun was allowed to build to 28 psi, and was then held briefly and then fired (the fire switch was held until the rupture disk broke at ~1100 psi). After the bombardment, the vacuum was released, and the petri dish with the leaf was taken out and covered. After the samples were finished they were covered with aluminum foil (to keep them dark) and incubated for 48 hours at room temperature (Kumar and Daniell

2004). The bombarded leaves were incubated in the dark for 24 hours and then placed on shooting media (RMOP) containing 500 µg/ml spectinomycin for two rounds of selection.

PCR analysis to test stable integration

DNA was isolated using Qiagen DNeasy Plant Mini Kit, from the transgenic shoots and PCR analysis was performed to confirm integration of the transgene in the inverted repeat regions of the chloroplast genome. DNA from a known transgenic plant was used as a positive control while DNA from a wild type *Petit Havana* plant was used as a negative control. To confirm integration of the transgene cassette into the chloroplast genome, PCR analysis was carried out using the primers 3P (5' GGAATTGAATTCCATATGTGTGAGAACAGA3') and 3M (5' AGAATTGCCTCTAGACTATTCTGAAAC 3'). To confirm integration of the gene of interest into the chloroplast genome the primers 5P (5'ATGTAGAAGTCACCATTGTTGTGC-3')and2M(5'-GACTGCCCACCTGAGAGC-GGACA-3') (Daniell et. al, 2001a) were used. The samples were denaturated for 5 mins at 95◦C followed by 30 cycles of the following temperatures: 95 ◦C for 1 min, 65 ◦C for 1 min, and 72 ◦C for 2 min and a 72◦C hold for 10 min after all 30 cycles were completed. After confirmation of transgenic plants, the shoots were then transferred to a rooting medium (MSO) with 500 µg/ml spectinomycin as a selective agent.

Southern blot analysis

Total plant DNA from different lines of transgenic plants and one wild type plant was digested overnight with *EcoR1,* separated on a 0.7% agarose gel at 45V for 8 hours. The gel was then depurinated by immersing it in 0.25M HCl (depurination solution) for 15 minutes (until the color of the dye became yellow), washed twice in dH_2O for 5 minutes, and then equilibrated in transfer buffer (0.4N NaOH, 1M NaCl, filled to 1 liter with water) for 20 minutes. The DNA was transferred overnight to a nitrocellulose membrane. The next day the membrane was washed with 2X SSC (3M NaCl, 0.3M Na Citrate, H_2O, the pH was adjusted with 1N HCl to 7 and water was added to 1L) for 5 minutes. Following, the membrane was allowed to dry on a Whatman paper for 5 minutes and then cross-linked using the Bio-Rad GS Gene Cross Linker at setting C3 (150 m joules). pUC-CT vector DNA was digested with *Bam*HI and *Bgl*II to generate a 0.8 kb probe which was used as a flanking probe as it contains the chloroplast flanking sequences for the trnI and trnA. (Daniell et al, 2005). GFP from pLD-CTB-GFP was used as a gene specific probe. After labeling the probe with P^{32}, hybridization of the membranes was done using Stratagene QUICK-HYB hybridization solution and protocol (Stratagene, La Jolla, CA). The membrane was washed twice as follows: 50ml of wash solution number 1 (2X SSC and 0.1% SDS) was poured and incubated at room temperature for 15 minutes. The liquid was discarded in the liquid waste container and the step was repeated. A second round of washes was performed twice by pouring 50ml of solution number 2 (0.1X SSC and 0.1% SDS) and incubating it for 15 minutes at 60 °C to increase the stringency. The liquid of these washes were discarded into the radioactive

liquid container. The membranes were placed into the plastic wrap, placed in the film cassette and then taken to the dark room. Using the safe light (red light), the X-ray film was placed into the cassette on top of the blot and the intensifier screen was placed on top of the X-ray film. The cassette with the blot and the film was placed into a black bag to protect against light and then incubated overnight at –80 °C. The next day the cassette was taken out from the –80 °C, allowed to thaw, and then moved to the dark room where the film was developed.

Western blot analysis

Approximately 100 mg of leaf tissue was ground in liquid nitrogen and resuspended in 500 µl of plant extraction buffer (0.1% SDS, 100mM NaCl, 200mM Tris–HCl pH 8.0, 0.05% Tween 20, 400mM sucrose, 2mM PMSF). After centrifugation at 13,000 rpm for 5 minutes, the supernatant containing the extracted protein was collected. 10 µl of the plant extract along with 10 µl of sample loading buffer containing BME were boiled, and then run on a 15% SDS–PAGE gel for 40 mins at 50V and then 2 hours at 80V. Unboiled samples along with samples loading buffer without BME were also loaded. The protein was then transferred to nitrocellulose membrane for 1 hour at 80V. After blocking the membranes with PTM (1×PBS, 0.05% Tween 20, and 3% dry milk) for 1 hour, rabbit anti-CTB primary antibody (Sigma) was added. Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) was used as a secondary antibody. The membranes were washed with PBS (140mM NaCl, 2.7mM KCl, 4mM Na_2HPO_4, and 1.8mM KH_2PO_4, pH 7.2) and the chemiluminescent substance was added. Later the X-

ray films were exposed to chemiluminescence and the films were developed in the film processor to visualize the bands.

Furin Cleavage Assay

Approximately 100 mg of leaf material was powdered in liquid nitrogen and resuspended in 500 µl of plant extraction buffer containing 15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, 5 mM $CaCl_2$, 0.5% Triton-X, 2-mercaptoethanol at pH 6.0 and pH 7.0. 1mM PMSF was added to some of the samples. After centrifugation at 13,000 rpm for 5 minutes, the supernatant containing the extracted protein was collected. The extract (20 µl) was incubated at 30°C for 4 h with 4 units of furin. A control group was also incubated at 30°C for 4 h without furin. After 4 hours, each sample was mixed with 20 µl sample loading buffer, boiled, and run on 12% SDS-PAGE gel for 45 minutes at 80V and then 2 hours at 100V. The Western blot analysis was performed as per the procedure outlined above. Chicken anti-GFP antibody (Chemicon) at a 1:3000 dilution was used as the primary antibody and alkaline phosphatase conjugated rabbit anti-chicken IgG (Chemicon) at a dilution of 1:5000 was used as a secondary antibody.

Estimation of total soluble protein

The total soluble protein of the leaf samples was estimated using the Bio-Rad Bradford Protein Assay. Leaf samples of transformed and untransformed plants (100 mg) were powdered in liquid nitrogen and resuspended in plant protein extraction buffer (15mM Na2CO3, 35mM NaHCO3, 3mM NaN3, pH 9.6, 0.1% Tween20, and 5mM PMSF). The standard curve was obtained by diluting Bovine Serum Albumin in plant

extraction buffer. 10 µl of each standard and sample were mixed with 200 µl of Diluted (1:4) Biorad dye reagent, loaded in microtiter plate wells, incubated at room temperature for 10 min and read on a plate reader at 630 nm.

ELISA

The CTB-GFP quantification was done using the enzyme linked immunosorbent assay (ELISA). The standards and test samples were diluted in coating buffer (15mM Na2CO3, 35mM NaHCO3, 3mM NaN3, pH 9.6). The standards, ranging from 20 to 320 ng, were made by diluting recombinant GFP in 1% PBS. The leaf samples were collected from plants exposed to regular lighting pattern (16 h light and 8 h dark), and protein was extracted using the plant protein extraction buffer described above. 100 µl of standard GFP dilutions and protein samples were bound to a 96-well plate overnight at 4°C. The background was blocked with fat-free milk in PBST for 1 hour at 37°C followed by washing with PBST and water. Primary antibody used was chicken anti-GFP antibody (Chemicon) diluted (1:3000) in PBST containing milk powder. Secondary antibody was HRP-conjugated rabbit anti-chicken IgG- secondary antibody (Chemicon) at a 1: 5000 dilution in PBST containing milk powder. For the color reaction, 100 µl of 3,3_,5,5_-tetramethyl benzidine (TMB from American Qualex) substrate was loaded in the wells and incubated for 10–15 min at room temperature. The reaction was stopped by addition of 50 µl of 2N sulfuric acid per well and the plate was read on a plate reader (Dynex Technologies) at 450 nm.

GM_1binding assay

To test the functionality of CTB-GFP expressed in chloroplasts, a CTB-GM_1binding assay was performed. 96 well plates were coated with 100 ul of monosialoganglioside- GM_1 (Sigma) (3.0 µg/ml in ELISA coating buffer) and incubated overnight at 4^{o}C. After washing with PBST and water, the standards and samples were incubated for 1 hour at 37^{o}C. The plate was blocked with 1% BSA in 1X PBS for 1 hour at 37^{o}C. Rabbit anti-CTB primary antibody (Sigma) and alkaline phosphatase (AP) conjugated goat anti-rabbit secondary antibody (Sigma) was used to detect the CTB binding to GM_1 receptor. The plates were washed with PBST and water, and 200 µl of the substrate PNPP was added to the wells and incubated in the dark at 37^{o}C for 20 minutes. The reaction was stopped by adding 50 µl of 3N NaOH and the plates were read on a plate reader (Dynex Technologies) at 405 nm.

Animal studies

Three groups of five week old female Balb/c mice were fed with CTB-GFP, Interferon alpha2B-GFP (IFN-GFP) and wild type (untransformed) plant leaf material. 350 mg of leaves were powdered in liquid nitrogen, mixed with peanut butter and fed to the mice which had been starved over night prior to this experiment. The mice were then gavaged for two more days, two times a day with 40 mg of leaf material per gavage, powdered with liquid nitrogen and mixed with 0.1M phosphate-buffered saline (PBS). Five hours after the last gavage the mice were sacrificed and perfused with 10 ml of PBS followed by 4% paraformaldehyde in PBS. Fresh frozen sections of the liver, spleen, ileum and jejunum were collected according to Samsam et al., (2003). Additional tissue

was removed and immersed in Tissue Tec freezing medium (Vector labs) and immediately frozen in nitrogen cooled isomethylbuthane (isopathane, Sigma). Fixed tissue was cryoprotected by passing through 10%, 20% and 30% sucrose solutions in PBS. Ten micrometer (μm) thick frozen sections of various tissues were then made using a cryostat.

Immunohistochemistry for GFP, CTB and immune cells

Immunohistochemistry was performed in order to show the presence of GFP and/or CTB in various tissues. The slides were first blocked with 10% BSA (bovine serum albumin) and 0.3% Triton-X 100. Chicken anti-GFP (Chemicon) or rabbit anti-CTB (Sigma) primary antibodies, at a concentration of 1: 500 and 1: 300 respectively in 1% BSA and 0.3% Triton-X, were used for GFP or CTB localization of the tissues. Those sections processed for HRP conjugated secondary antibodies were blocked with a mixture of Methanol / Hydrogen peroxide 30% (2:1 ratio) to block the endogenous peroxidases. The secondary antibodies were horseradish peroxidase (HRP) -conjugated anti-chicken IgG (Chemicon), or HRP-conjugated goat anti rabbit (Sigma). Tissue bound peroxidase was developed using the 3,3' diaminobenzidine (DAB) as a substrate to visualize the immunoreaction.

For macrophage localization of the tissues, rat monoclonal F4/80 antibody (Serotec) was used according to Berghoff et al., 2005. The secondary antibody was Alexa 555 conjugated Goat anti rat IgG (Molecular Probes). American hamster anti CD11c primary antibody and anti-hamster Alexa 546 conjugated secondary antibody (Molecular Probes) were used to visualize dendritic cells in the intestine and other tissues. FITC

labeled anti-chicken IgG was used as a secondary antibody in such immunofluorescence stainings to detect GFP in tissues.

RESULTS

GFP expression in transgenic plants

Figure 1 shows the transgenic and wild type plants. In B, the GFP expression of the transgenic plants can be seen under the UV light, which is not seen in the wild type (untransformed) plant (fig. 1A). Figure C shows wild type plant and D, the CTB-GFP expressing plant under a low magnification fluorescent microscope. Expression of GFP is clearly evident in D.

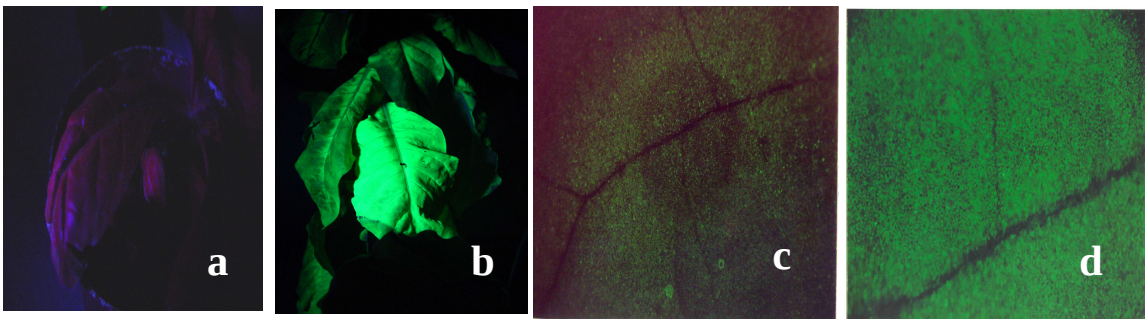


Figure 1: GFP expression in transgenic plants:

(a) Wild type (untransformed) plant seen under UV light.

(b) CTB- GFP expressing leaf showing fluorescence observed under UV light.

(c) Wild type leaf under a low magnification microscope.

(d) CTB-GFP expressing leaf showing fluorescence under a low magnification microscope.

Confirmation of transgene integration into chloroplast genome

Nicotiana tabacum cv. Petit Havana leaves were bombarded with the pLD-CTB-GFP vector and the leaves were grown on selective medium containing 500 mg/l spectinomycin. The resultant shoots could be chloroplast transformants, nuclear transformants or spontaneous mutants. Spontaneous mutation of the 16S rRNA gene, which confers resistance to spectinomycin in the ribosome, could allow plants to grow on spectinomycin without integration of the gene cassette which will result in the mutant shoot growth. The *aadA* gene in the gene cassette confers resistance to spectinomycin and hence the shoots with the integration of the gene cassette in either nuclear or chloroplast genome grow on the selection medium. True chloroplast transformants were distinguished from nuclear transformants and mutants by PCR analysis.The 3P primer lands on the native chloroplast genome upstream of the site of integration in the 16srRNA region, and the 3M primer lands on the *aadA* transgene producing a 1.65kb PCR product. This analysis ruled out the nuclear transformants since 3P primer would not anneal and the spontaneous mutants since 3M primer would not anneal.

To check for the presence of the transgene in the chloroplast, the 5P-2M PCR analysis was performed. The 5P primer lands on *aadA* gene and the 2M lands on the *trnA* coding sequence producing a 2.9kb PCR product with CTB-GFP. This confirmed the correct integration of the transgene in the inverted repeat regions of the chloroplast genome.

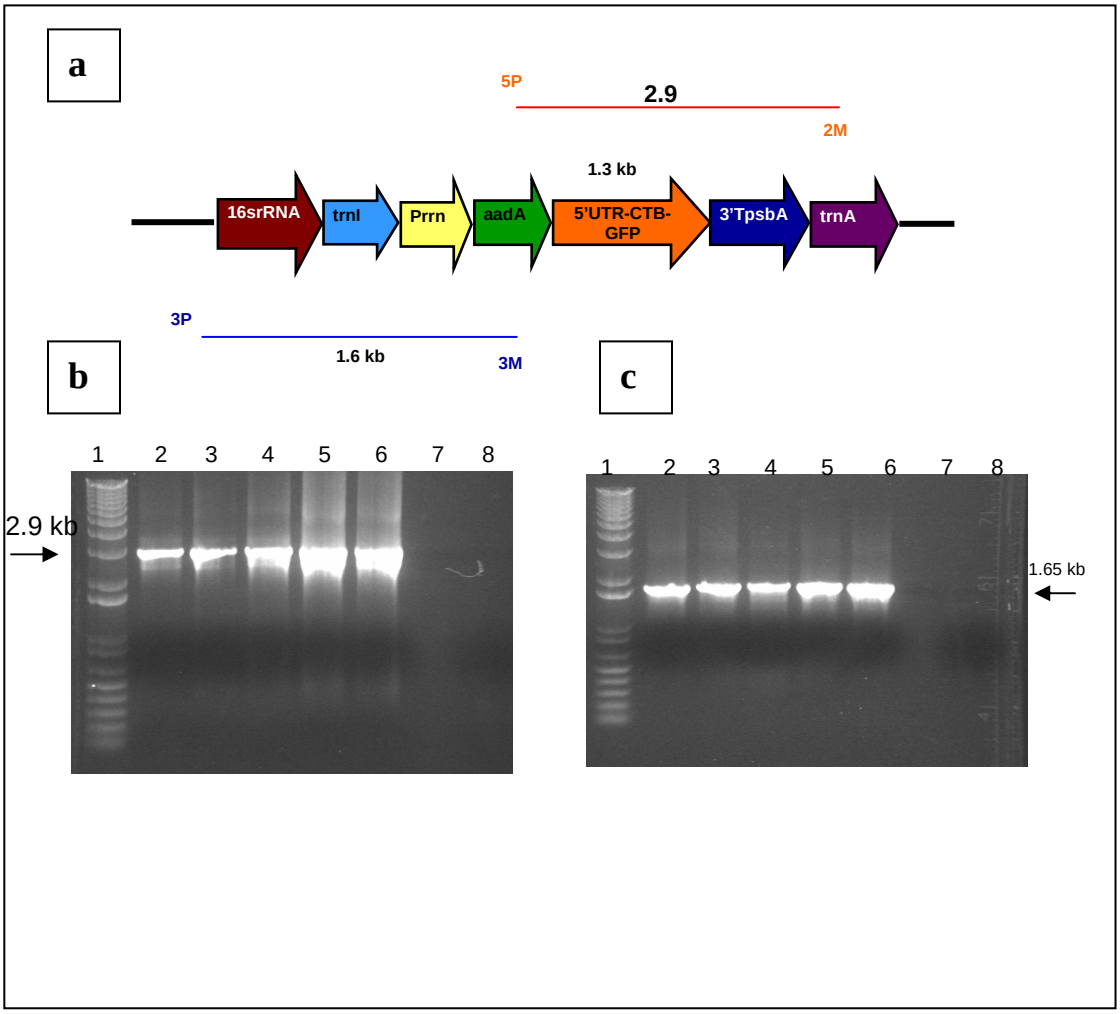


Figure 2: PCR analysis for the confirmation of transgene integration:

(a) Schematic representation of the transgene cassette.

(b) 5P/2M – These primers land on the *aadA* and *trnA* regions (flanking the CTB-GFP). A 2.9 kb PCR product was obtained from the PCR analysis of trasgenic plants.

(c) 3P/3M – The 3P primer lands on the native chloroplast genome and the 3M primer lands on the *aadA* gene. A 1.6 kb PCR product was obtained from the PCR

anlysis of the transgenic plants. **Lane 1**: 1kb plus ladder, **Lanes 2-5**: Transgenic lines of CTB-GFP, **Lane 6**: Positive Control, **Lane 7**: Empty, Lane 8: Wild Type Plant.

Southern blot analysis to investigate homoplasmy

To further confirm the integration of the transgene into the chloroplast genome and to determine if homoplasmy had been achieved, Southern blot analysis was performed. Total plant DNA was digested with the enzyme *EcoRI* and hybridized with a chloroplast flanking sequence probe (0.8kb). Wild type plants generated a 4.4 kb fragment and transgenic plants generated a 4.9 and a 2.2 kb fragments (Fig. 1D). All of the transgenic lines tested appeared to be homoplasmic (within the levels of detection) which means that all of the chloroplasts in the plant contained the transgene CTB-GFP. Total plant DNA digested with *EcoRI* was also hybridized with a gene specific probe (GFP). A 4.9 kb fragment was detected in the transgenic samples confirming the correct integration of the entire transgene in the correct spacer region in the chloroplast genome. No fragment was detected in the wild type plants using the gene specific probe.

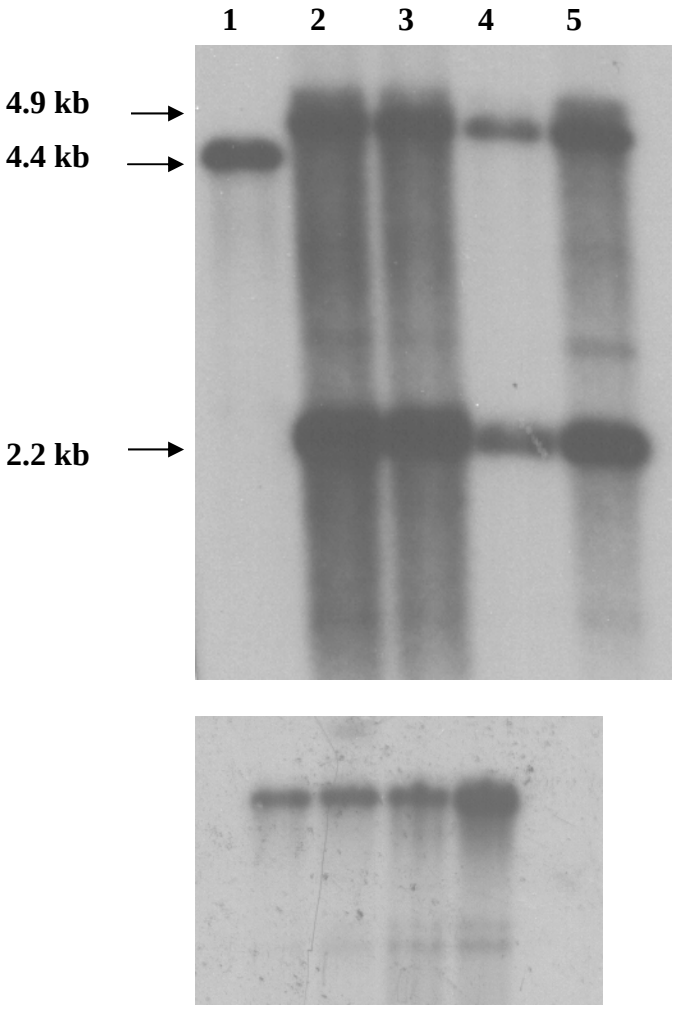


Figure 3A: Southern blot analysis

(a) Southern blot analysis with flanking sequence probe. **Lane 1**: Wild-type showing 4.4 kb fragment, **Lane 2 - 5**: Transgenic plants showing 4.9 and 2.2 kb hybridizing fragments.

(b) Southern blot analysis with gene specific GFP probe. **Lane 1 – 4**: Transgenic plants showing 4.9 kb fragment, **Lane 5**: Wild type plant

Immunoblot analysis of transgenic plants

Western blot analysis was performed to investigate the expression of the fusion protein CTB-GFP in transgenic tobacco chloroplasts (Fig. 4) using anti-CTB antibody. The pentameric form (188 kDa) was observed in the unboiled samples of the transgenic plants, while predominantly the monomeric form (37.6 kDa) was detected in boiled samples.

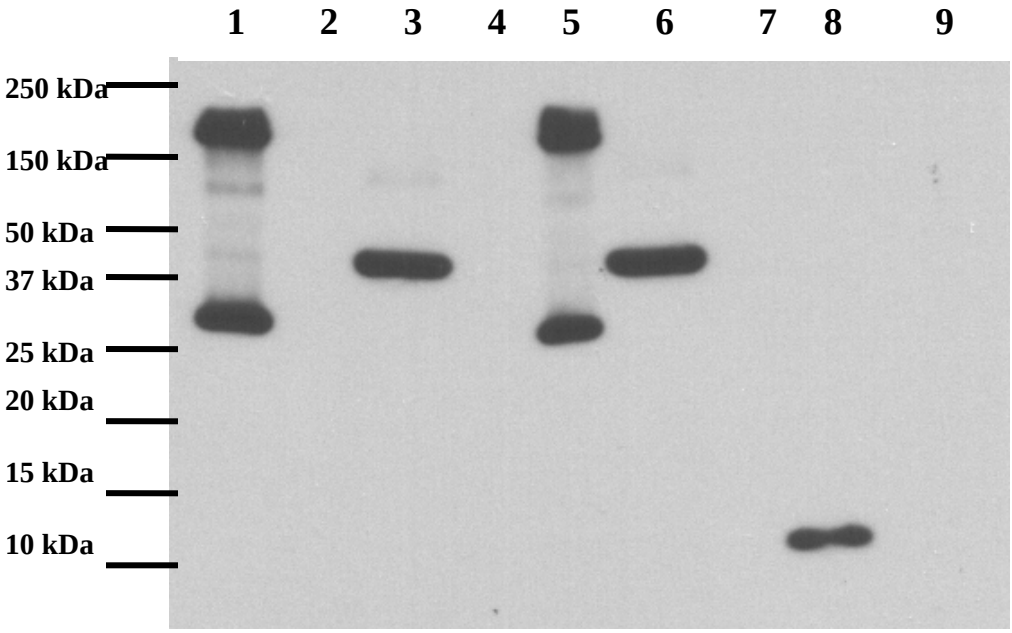


Figure 4: Immunoblot analysis of CTB-GFP using anti-CTB antibodies

Lane 1: Unboiled crude extract of transgenic line A, **Lane 3**: boiled crude extract of transgenic line A, **Lane 5**: Unboiled crude extract of transgenic line B, **Lane 6:** Boiled crude extract of transgenic line A, **Lane 8:** Purified CTB standard 200ng**, Lane 9:** Wild type plant crude extract, **Lane 2, 4, 7:** empty.

Furin Cleavage Assay

The protease furin is present in the constitutive secretory pathway and on the cell surface of virtually all cells. An *in vitro* furin cleavage assay was performed on the CTB-GFP expressing plant extract to show that the engineered cleavage site (Arg-Ala-Arg-Arg) was recognized by furin. As seen in Fig. 5, a 26 kDa polypeptide that corresponded with the recombinant GFP protein was observed in the samples that were incubated with furin, thus proving that furin was able to cleave CTB-GFP to release GFP. Furin cleavage occurred at both pH 6.0 and 7.0 in the samples with and without PMSF. There was still some protein that did not get cleaved, probably because the amount of enzyme was not sufficient to cleave all the CTB-GFP protein present in the plant extract. The incubation time of 4 hours may also have been insufficient. However, the presence of the cleaved GFP product in the samples incubated with furin confirms that the engineered furin cleavage site is functional. The introduction of furin consensus sequences at the B-chain/C-peptide and the C-peptide/A-chain interfaces of human proinsulin has been demonstrated to increase the processing of proinsulin to mature insulin in a wide variety of non-neuroendocrine cells, including fibroblasts, myoblasts, epithelial cells, and lymphocytes. As furin cleavage site is also recognised by the endopeptidases PC2 and PC3/1, it is likely that CTB-GFP fusion protein is cleaved more efficiently during the process of receptor mediated delivery.

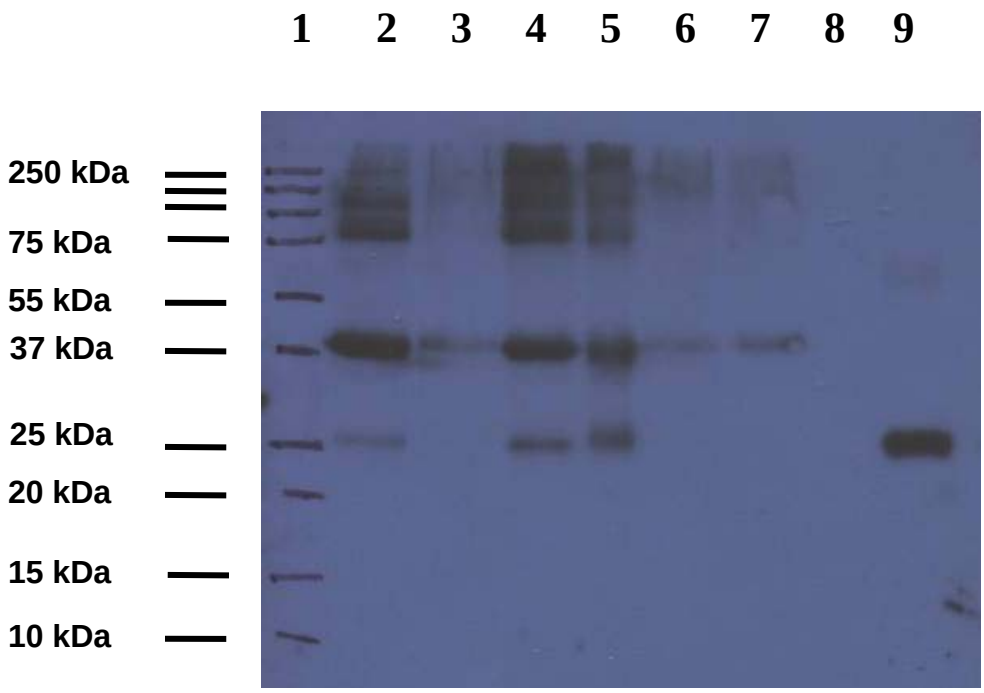


Figure 5: Furin Cleavage Assay

Lane 1: Marker, **Lane 2:** CTB-GFP pH 6.0 with furin, no PMSF, **Lane 3:** CTB-GFP no incubation, no furin, **Lane 4:** CTB-GFP pH 6.0 with furin and PMSF, **Lane 5:** CTB-GFP pH 7.0 with furin and PMSF, **Lane 6:** CTB-GFP pH 6.0 with PMSF, no furin, **Lane 7:** CTB-GFP pH 6.0 no PMSF, no furin, **Lane 8:** Blank, **Lane 9:** GFP standard

Quantification of CTB-GFP

To quantify the amount of CTB-GFP fusion protein in transgenic tobacco leaves, enzyme linked immunosorbent assay (ELISA) was performed (Fig. 6). A standard curve was obtained using different concentrations of recombinant GFP (Vector Labs). The amount of CTB-GFP in the transgenic plants was compared to the known concentrations

of the recombinant GFP (standard curve). Expression levels of CTB-GFP were upto 21.3% TSP.

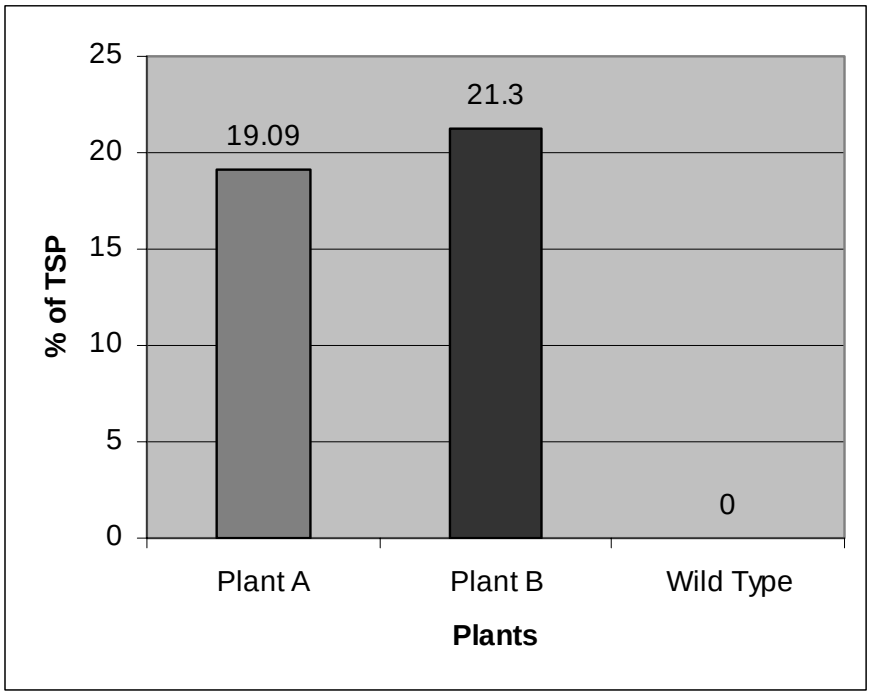


Figure 6. Quantification of CTB-GFP fusion protein expression levels in transgenic plants.

Expression levels in % total soluble protein (TSP) of CTB-GFP expressed in different transgenic lines. The CTB-GFP expression ranged from 19.09% to 21.3% of TSP.

GM_1binding assay

The functionality of chloroplast derived CTB-GFP was determined by its ability to bind to GM_1 in an *in vitro* GM_1binding assay (Fig. 7). Quantification with GM_1binding assay showed that pentamers of CTB-GFP were formed and expressed at levels of up to

3.5% of the total soluble protein in plants grown under natural light in the greenhouse and 2% in plants grown under artificial light in growth chambers. This confirms the correct folding and disulfide bond formation of CTB pentamers within transgenic chloroplasts because only the pentameric form of CTB can bind to GM_1.

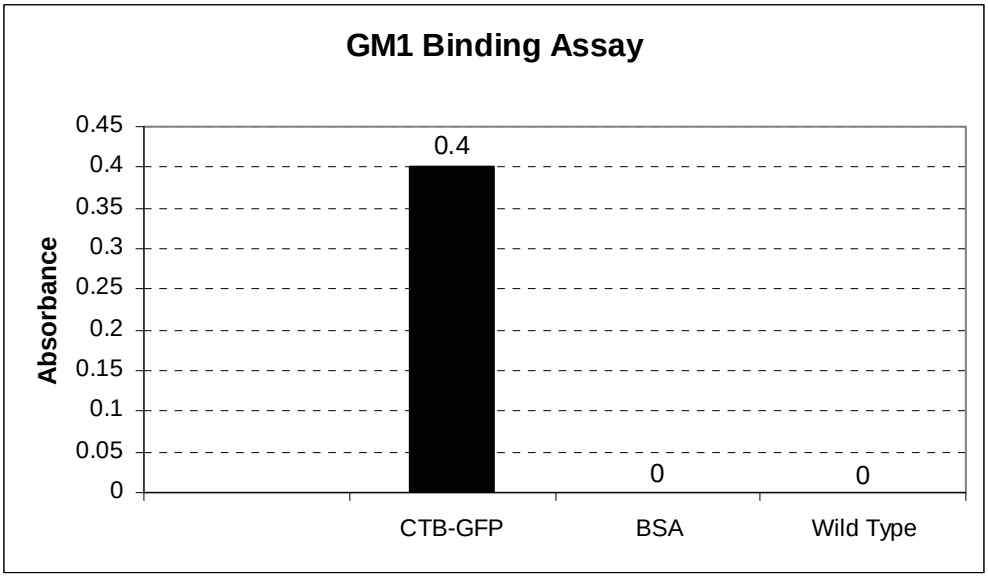


Figure 7. GM1 binding assay of CTB-GFP expressed in transgenic chloroplasts
Absorbance of CTB-GFP plant extract bound to GM1 as compared with absorbance of BSA and wild type plant extract.

Fluorescent microscopy to detect the presence of GFP in the tissue

Fixed tissue and fresh frozen sections of the liver, spleen, ileum and jejunum were made from the three groups of mice. Fluorescence microscopy showed the presence of GFP in intestinal mucosa and submucosa (Fig. 8A), as well as in the hepatocytes of the liver (Fig. 8D) of mice fed with CTB-GFP expressing plant leaf material. GFP was also

detected in various cells of the spleen of mice fed with CTB-GFP expressing plant leaf material (fig 8G).

In the mice fed with wild type (untransformed) leaf material, no GFP fluorescence was seen (Fig 8 B, E and H). In the mice fed with IFN-GFP expressing plant leaf material, no GFP was detected in the liver or spleen (Fig 8 F and I). In a few sections of the intestinal of IFN-GFP treated animals, some GFP was detected in both mucosa and submucosa (fig. 8C). Although GFP was transported across the lumen of the intestine probably via the M cells, it could not reach organs such as the liver or the spleen. This suggests that it is the transmucosal carrier which is responsible for the delivery of GFP across the intestinal lumen into the systemic circulation.

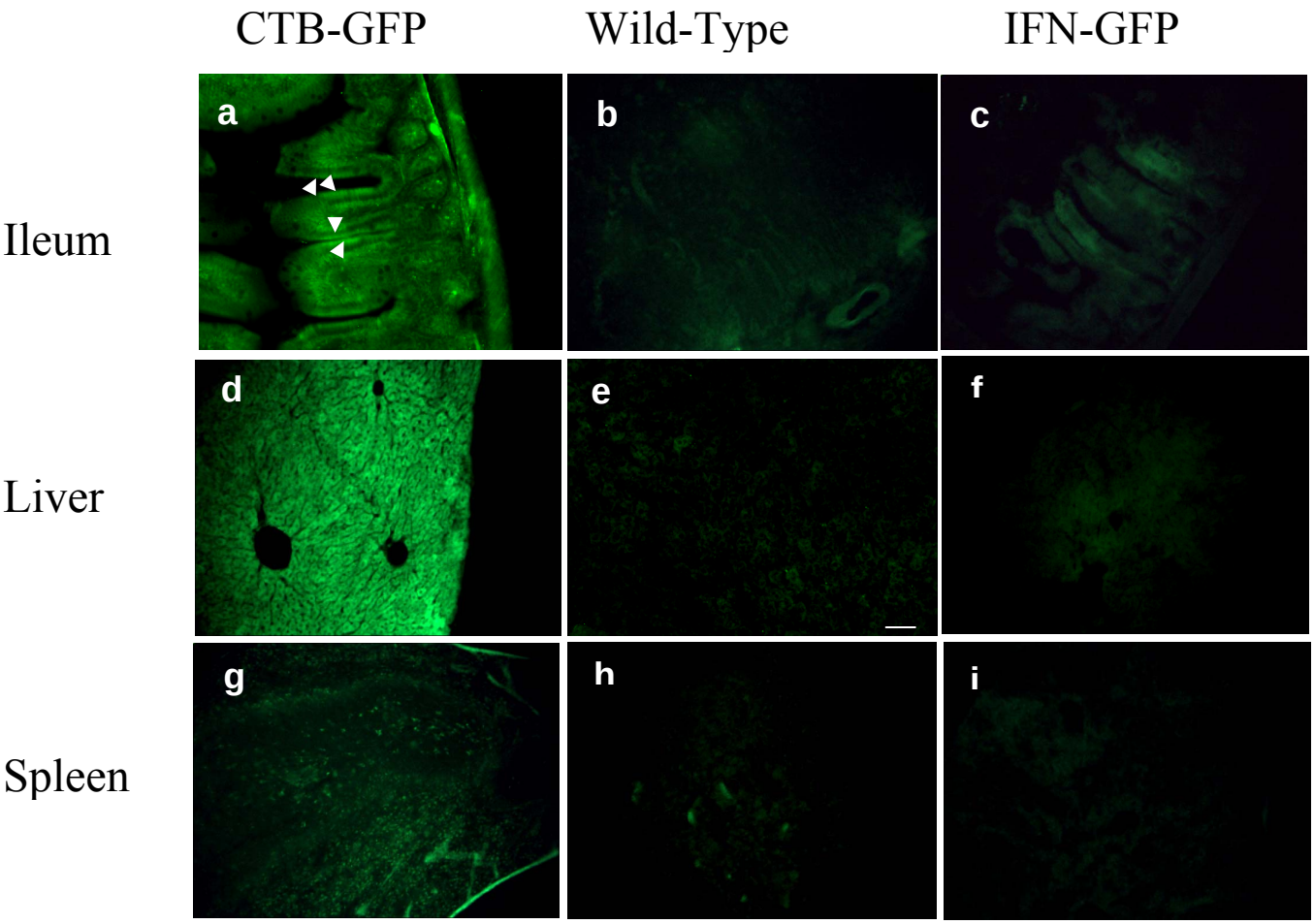


Figure 8: Cryosections of the intestine and liver of the mice fed with CTB-GFP or wild type plant leaves material.

(a) GFP in the ileum of a mouse following oral delivery of the CTB-GFP expressing plant leaf material. Arrows show numerous columnar cells of the intestinal mucous membrane which have up-taken the CTB-GFP. Various cells in the connective tissue beneath the epithelium also show the presence of GFP.

(b) Section of the ileum of a mouse fed by the wild type (untransformed) plant leaf material.

(c) Section of the ileum of a mouse fed by the IFN- GFP leaf material.

(d) GFP in hepatocytes of a mouse liver following oral delivery of CTB-GFP expressing plant.

(e) Section of the liver of a mouse fed by the wild type plant material.

(f) Section of the liver of a mouse fed by IFN- GFP expressing plant material.

(g) GFP in the spleen of a mouse following oral delivery of CTB-GFP expressing plant. Arrows show various splenic cells with GFP.

(h) Section of the spleen of a mouse fed by the wild type plant material.

(i) Section of the spleen of a mouse fed by IFN- GFP expressing plant material.

Scale bar: 50 μm

Immunohistochemistry

In order to confirm the fluorescent microscopy findings, immunostaining was performed with both CTB and GFP antibodies. In the intestine of the mice fed with CTB-GFP, anti-GFP antibody detected GFP inside the epithelial cells of the villi of the intestine, in the crypts, as well as in the submucosal tissue (Fig. 9 a,b,c) suggesting GFP uptake by lymphoid cells as well as the circulation. These results confirmed the previous microscopy findings (Figure 8) and showed the presence of GFP in various tissues, confirming that GFP was successfully delivered to blood when transgenic leaf material was orally fed to the mouse. GFP immunoreactivity was detected in the liver and spleen (Fig. 9 e,g) in a similar pattern to that seen with fluorescence microscopy of the native tissue (Fig. 8 d,g). In the case of the mice fed with wild type leaf material, no GFP was detected in any of the tissues (Fig. 9 f,h). In the mice fed with plants expressing IFN-

GFP, GFP was not detected in the liver or spleen cells. These results support the hypothesis that CTB can act as a transmucosal carrier, and orally deliver fused proteins via the intestinal cells.

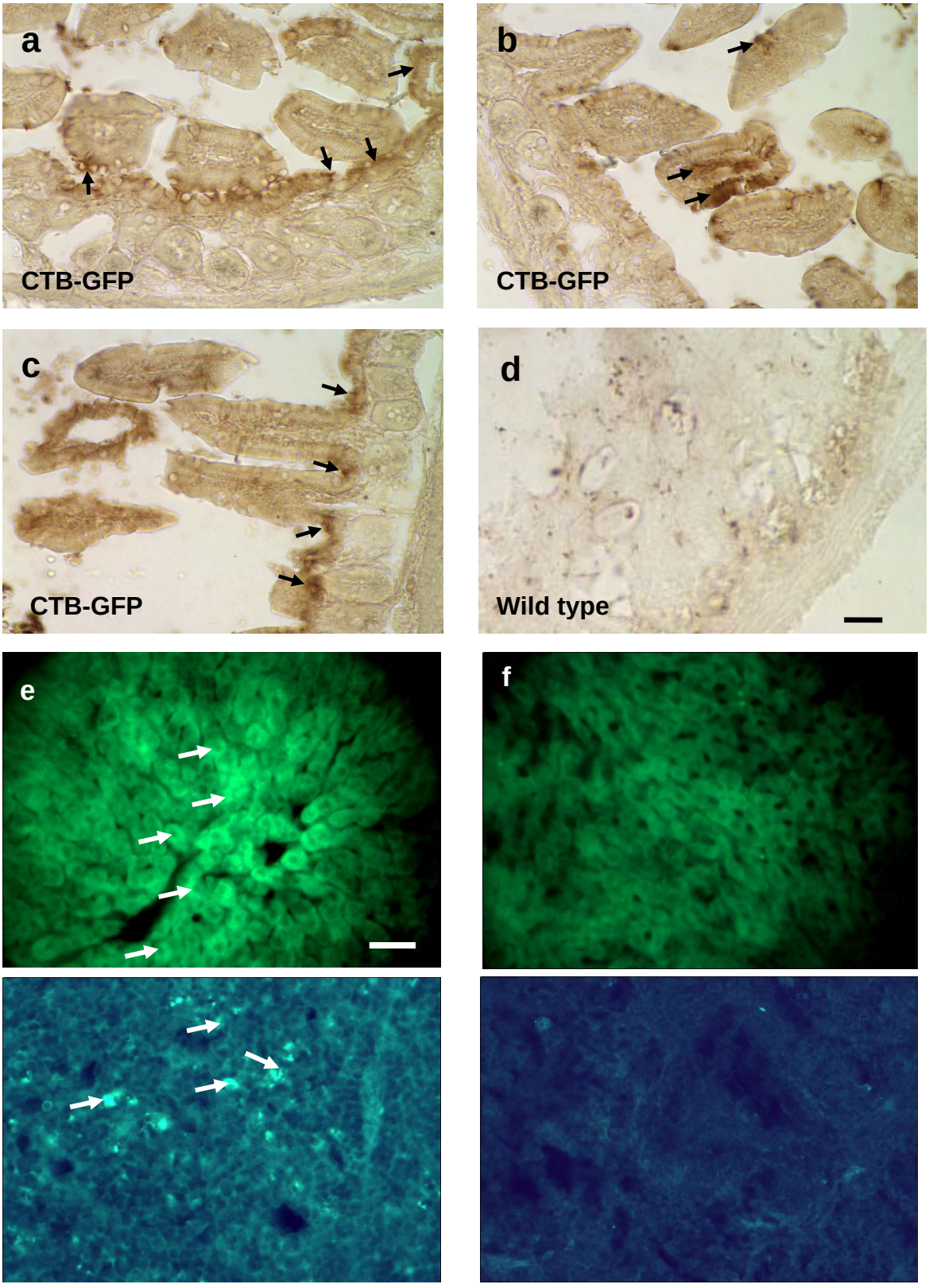

a
CTB-GFP
b
CTB-GFP
c
CTB-GFP
d
Wild type
e
f

Figure 9: Immunohistochemical localization of the GFP in mouse ileum, liver and spleen.

(a), (b) and **(c)** are sections of the ileum of the mice fed with CTB-GFP expressing plant leaf. Arrows indicate presence of GFP in the intestinal epithelium as well as cells of the crypts.

(d) Section of the ileum of a mouse fed with wild type (untransformed) plant leaf materials.

(e) GFP- immunoreactivity in hepatocytes (arrows) in a mouse fed orally by CTB-GFP expressing plant.

(f) Section of the liver from a mouse fed by wild type (untransformed) plant.

(g) GFP- immunoreactivity in the spleen of mouse fed orally by CTB-GFP expressing plant. Arrows indicate various cells with a higher GFP content.

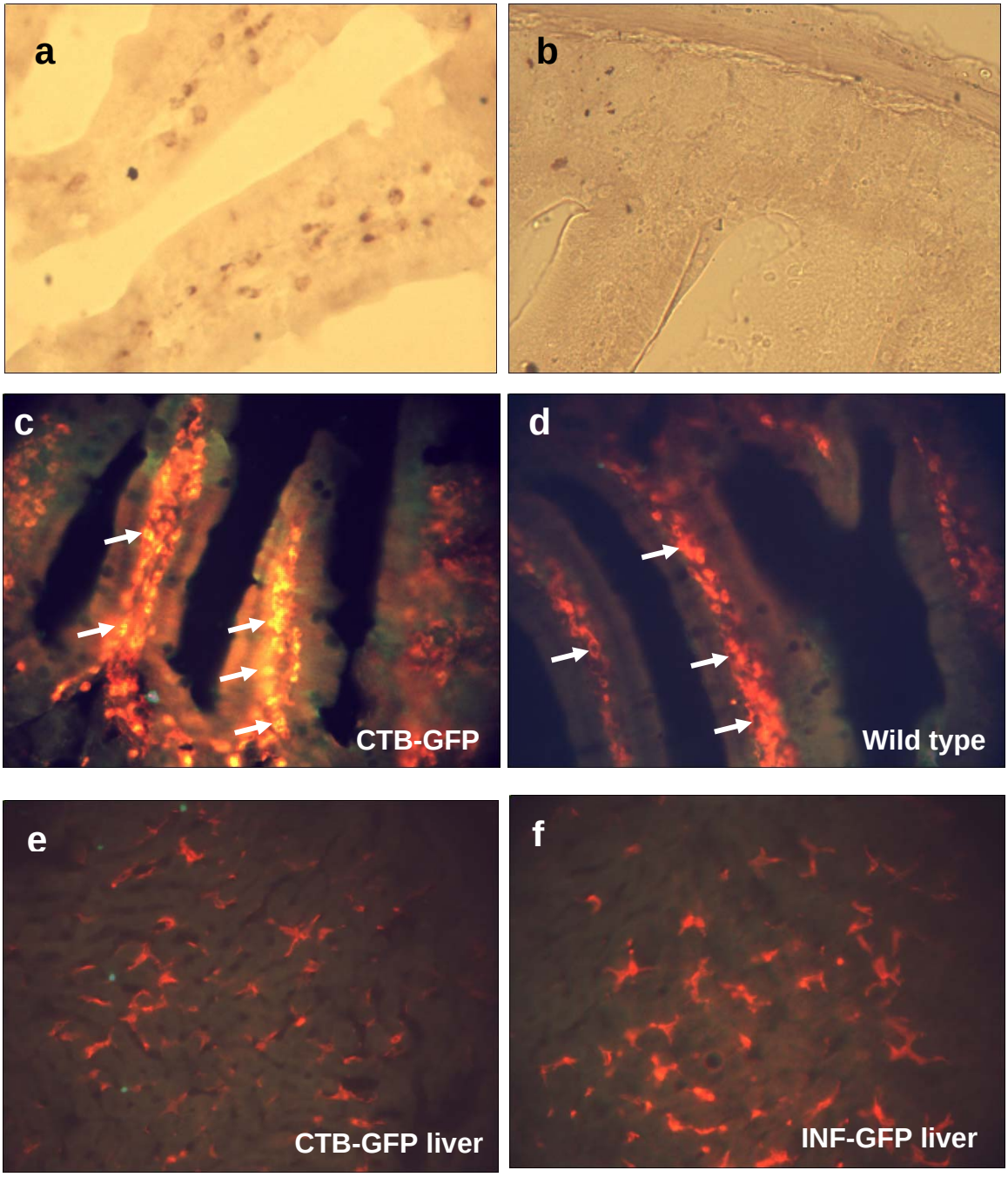
(h) Section of the spleen from a mouse fed by wild type (untransformed) plant.

Scale bar = 50 μm

In order to see the fate of CTB in the body, we performed immunohistochemistry using anti-CTB antibodies. CTB was detected in the intestinal cells as well as inside the villi (fig. 10 A) in the lamina propia and the submucosa. It was however, not detected in the liver (Fig. 10 D) or the spleen, indicating that GFP is cleaved away from CTB in the intestinal cell and that while GFP leaves the cell, CTB probably is translocated to the basolateral membrane of the cell.

To localize the GFP and/ or CTB in the gut associated lymphoid tissue (GALT) and other tissues double staining for antigen presenting cells such as macrophages or

dendritic cells was performed. A double staining with F4/80 antibody for macrophages showed CTB expression inside macrophages (Fig. 10 C). Fig. 10 G shows macrophages associated with GFP and fig. 10 I shows dendritic cells taking up the GFP. In either case, there are associations of GFP with these antigen presenting cells. Most of the macrophages were not associated with GFP, which is perhaps due to uptake by the blood and lymph circulation, while the CTB, is translocated to the basolateral membrane and is associated with macrophages.


a
b
c
CTB-GFP
d
Wild type
e
CTB-GFP liver
f
INF-GFP liver

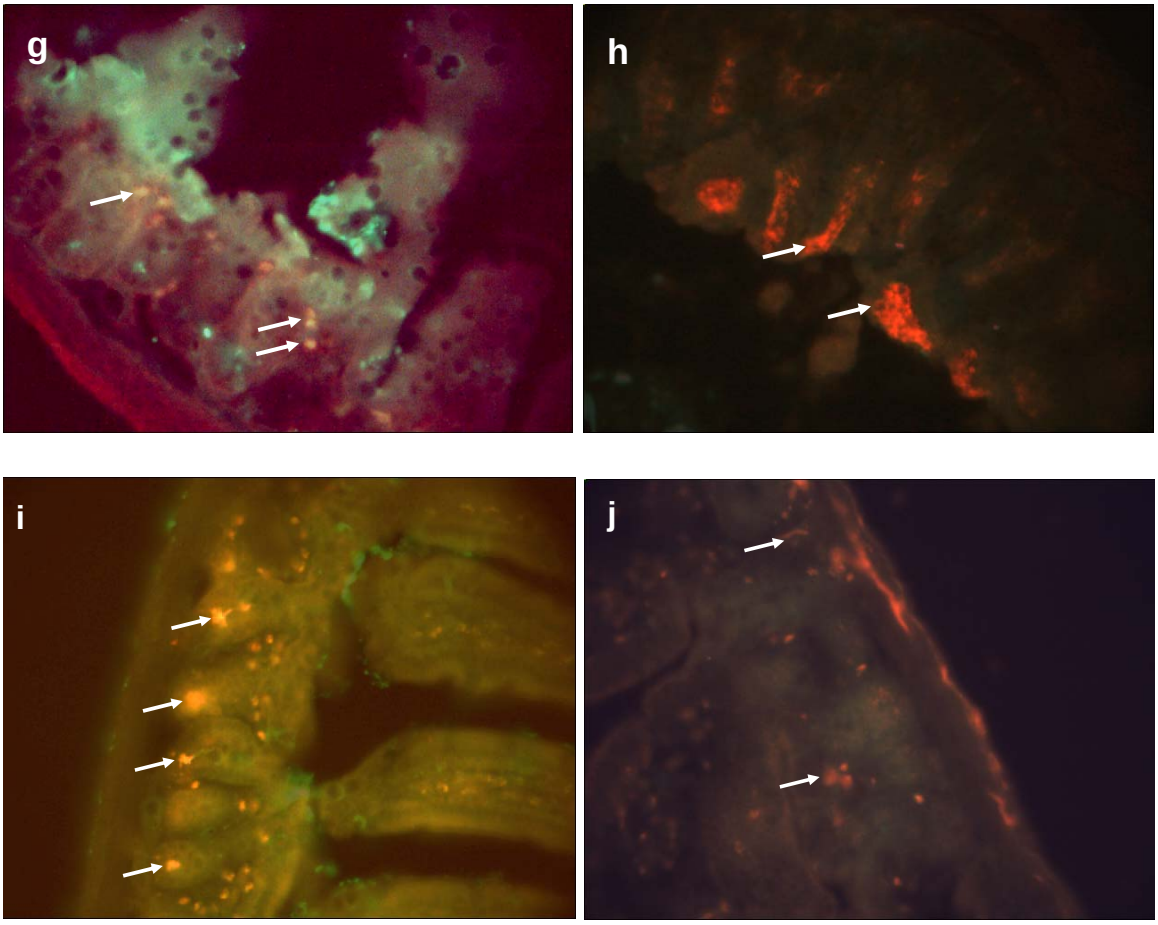


Figure 10: Immunohistochemistry of ileum, liver, spleen tissues of mice fed with wildtype leaves or CTB-GFP or IFN-GFP expressing leaves.

(a) Section of the intestine of a CTB-GFP treated mouse. The arrows indicate CTB in the submucosa of the intestinal villi.

(b) Section of mouse ileum fed with wild type plant, immunostained for CTB.

(c, d, e, f) Double staining for macrophage (red) and CTB (green) in mouse intestine and liver. **(c)** Arrows show macrophages in the submucosa of the intestine containing CTB, in a mouse fed with CTB-GFP expressing plant leaf material. The merged color is yellow. **(d)** Arrows indicate F4/80 positive cells (macrophages, in red) in a merged picture in the

intestine of a mouse fed with wild-type leaf material. **(e)** A merged picture showing double staining for macrophage (Kupffer cells) and CTB in mouse liver. Arrows show macrophages (red) in the liver. There is no sign of CTB (green) in the liver of CTB-GFP fed mouse. **(f)** Liver section of an IFN- GFP fed mouse used as a negative control for CTB. Macrophages are seen in red.

(g) F4/80 antibody was used as a marker of macrophages in the intestine. Arrows indicate macrophages which have entrapped GFP (yellow after merging the red and the green). Many of the macrophages are not associated with GFP.

(h) Many macrophages are seen in the intestine of mouse fed with IFN-GFP expressing plant leaf material, which do not show GFP immunoreactivity.

(i and **j)** CD11c (red) and GFP (green) immunoreactivities. **(i)** Arrows indicate CD11c (red, presumably dendritic cells, due to having a star shape morphology) with internalized GFP (green) which can be seen in yellow color when the red and green channels were merged. **(j)** Arrows indicate CD11c positive cells in intestine of mice fed with IFN-GFP expressing plant leaf material.

Scale bar for A and B = 25 μm

Scale bar for C - J = 50 μm

DISCUSSION

In this study, detection of GFP and CTB in the intestinal mucosa (Fig. 8, 9) suggests that CTB–GFP has been taken up by the enterocytes and the gut-associated lymphoid tissue (GALT). The CTB domain of the CTB-GFP forms the pentameric structure within chloroplasts through disulfide bond formation; pentameric form binds to the GM_1 receptors on enterocytes and is endocytosed into the intestinal cells as endosomes (Lencer 2001). GM_1 functions to concentrate CTB in detergent-insoluble, glycolipid This study shows internalization of CTB-GFP by mouse intestinal mucosal cells as well as the antigen presenting cells in the intestinal mucosa and submucosa. We also showed the prescence of GFP but not CTB in the liver as well as the spleen of mice following oral delivery of CTB-GFP leaf material. Expression of both CTB and GFP in mouse intestinal cells following oral administration of transgenic chloroplast expressed CTB-GFP shows that the recombinant protein will be protected from peptidases and/or acids by bioencapsulation within the plant cells. In the intestine, certain enzymes break the linkages in the cellulose molecules causing pores to form in the wall through which proteins leave the plant cells into the intestinal lumen.

In the current study, detection of GFP and CTB expressions in the intestinal mucosa (fig. 8, 9) suggests that CTB–GFP has been taken up by the enterocytes and the gut-associated lymphoid tissue (GALT). The CTB domain of the CTB-GFP forms the pentamer structure within chloroplasts through which it binds to the GM_1receptors on enterocytes as well as the GALT cells and will be endocytosed into the intestinal cells as endosomes (Lencer, 2001). GM_1functions to concentrate CTB in detergent-insoluble,

glycolipid rich apical membrane microdomains called lipid rafts (Orlandi and Fishman, 1998; Brown and Lendon, 2000). Binding to lipid rafts is required to couple the lipid-anchored toxin with intracellular machinery for protein sorting and vesicular traffic (Lencer 1995; Wolf et al., 1998; Badizadegan et al., 2000). After endocytosis, the CTB-GM_1complex trafficking occurs retrogradely through Golgi cisternae and/ or trans-Golgi network (TGN, Lencer 1995 and 2001; Feng et al., 2004) into the lumen of the endoplasmic reticulum (ER, Fujinaga et al., 2003). The GM_1-CTB-GFP complex in the lipid rafts, targeted to the TGN, loses it's endosomal covering. Within the TGN, ubiquitously-expressed furin cleaves numerous polypeptide precursors as it gets activated. In eukaryotes, many essential secreted proteins and peptide hormones, enzymes and neuropeptides are initially synthesized as proproteins (inactive precursors) and are activated by proteolytic cleavage by member of a calcium-dependent endoproteases, the prohormone-proprotein convertases (PCs, Van den Ouweland et al., 1990; Nakayama, 1997, Seidah and Chretien, 1998, Thomas, 2002). Furin is the best-characterized member of the PC family in the mammalian tissues and is located in trans-Golgi network (TGN, Bosshart et al., 1994; Molloy et al., 1994) as well as in the endosomes and plasma membranes of polarized intestinal and renal epithelial cells and endothelial cells of the capillaries (Thomas 2002; Anderson et al., 2002; Mayer et al., 2004). Crystal structure studies show that furin cleaves protein precursors with narrow specificity following basic Arg-Xaa-Lys/ Arg-Arg-like motifs (Henrich et al., 2003). There is abundant experimental evidence that the CTB-GFP protein with furin cleavage

site in between the fusion protein gets cleaved during retrograde trafficking in the TGN and as a result the CTB and GFP get separated.

The CTB is taken into the ER and from there to the baso-lateral surface of the cell (transcytosis), where it remains membrane bound to GM_1receptor (Lencer, 2001). The GFP molecule getting out of the TGN (presumably membrane bound) is exocytosed through the basolateral membrane and finds its way into extracellular fluid and into the submucosal vessels including the lymphatic system.

Due to the large size fenestrations of the lymphatic vessels, lymphatics return over 3L of fluid and approximately 120g of protein to the blood stream every 24 hours in an adult human (Granger D.N., in Essential medical physiology). Beside, the entry of CTB-GFP through the GM_1 ganglioside receptor, the M cells in intestinal epithelium covering the mucosa associated lymphoid tissue in the digestive tract also serve as the port of entry by pinocytosis (Sansonetti, 1999). M cells internalize macromolecules and microorganisms efficiently and deliver them to the underlying antigen presenting cells. Small amount of GFP associated with macrophages in the intestine of the INF-GFP fed mice is likely to be taken up by the M-cells non-specifically. The IFN- GFP fusion protein also contains a furin cleavage site, but due to limited uptake by the intestinal epithelial cells, there is not a significant GFP transport to the tissues of the IFN-GFP fed mice. The amount of CTB-GFP reaching the enterocytes via GM_1 receptor is very high compared to the entry of IFN-GFP through M-cells. This is quite evident due to the GFP detected in various organs of the CTB-GFP fed mice (fig: 8, 9). Presence of GFP and not CTB in the liver and/or spleen of CTB-GFP treated mice in our current study (fig: 8, 9)

indicates that the cleavage of the CTB-GFP fusion protein in enterocytes and uptake of GFP into the vasculature of the lamina propia and the submucosa. CTB however, might be translocated to the basolateral cell membrane and remain bound to GM_1 (Lencer 2001).

The main focus of our current study using GFP as a model protein (of therapeutic value) conjugated to CTB with a furin cleavage site expressed in plants is to show an efficient oral delivery of protein through GM_1 receptor mediated endocytosis. Moreover, furin cleavage site facilitates the cleavage of the candidate protein in the cell, so that it could be passed into the extracellular space and into the circulation. Internalization of GFP using receptor mediated endocytosis suggests a possible way of protein delivery across the impermeable intestinal mucous membrane. Since there is a rapid turnover of the intestinal epithelial cells (Heath JP, 1996) in human (renewal of the intestinal epithelium occurs in every 3-6 days), repeated feeding of the CTB fused to a therapeutic protein is possible due to the continuous availability of GM_1 receptors in the new epithelium. Moreover Peterson and colleagues suggested a recycling mechanism for GM_1receptor as well (Boonyarattanakalin S, 2004 et al.,)

In addition, it is known that the M cells transport the macromolecules and antigens to the underlying lymphoid tissue (GALT) (Jepson 1998). Therefore, a small amount of CTB-GFP could be untaken by the GALT. This is shown in our current study by CTB and GFP expression in the antigen presenting cells including the macrophages as well as the dendritic cells in the intestinal lamina propia and submucosa.

Ability of cholera-like enterotoxins to act as adjuvants to unrelated antigens to increase the systemic antibody titer was first reported by Northrup and Fauci (1972). Many investigators have used CT as an adjuvant to boost immune responses to unrelated antigens, hence, increasing the mucosal and systemic antibodies through oral vaccines (Elson and Ealding, 1984; Clements et al, 1988; Millar et al, 2001). On the other hand, recent evidences suggest that oral administration of CTB conjugated to insulin (Arakawa et al., 1998) or CTB conjugated to myelin basic protein (Sun et al., 2000) induces tolerance and decreases the autoimmunity in animal model of type1 diabetes (Arakawa et al., 1998) or experimental autoimmune encephalomyelitis (Sun et al., 2000), perhaps through Th2 and Th3 immunomodulation and induction of immunosuppressive cytokines such as IL4 and IL10 and TGF-β.

In either way, involvement of the intestinal antigen presenting cells as it is shown in our current study is the first step towards immunity and/or immunosuppression. Association of macrophages and dendritic cells with CTB and/or GFP in our study shows the efficacy of our plant expressing fusion proteins in involving the intestinal lymphoid tissue as well.

The high cost of therapeutic proteins can be attributed to their production in fermentation based system, purification using expensive methods including chromatography, low temperature storage, transportation and sterile delivery through health professionals. Most of the expenses could be avoided by expressing therapeutic proteins in plant cells and their oral delivery. The ability to express high levels of foreign proteins in edible plant parts (Kumar et. al, 2004a) makes this approach a reality.

CONCLUSIONS

This study demonstrates the use of CTB as an efficient transmucosal carrier system to carry biopharmaceuticals across the intestinal membrane. GFP is used as a visible marker in this proof of concept project. It shows internalization of CTB-GFP by the mouse intestinal mucosal cells as well as the antigen presenting cells in the intestinal mucosa and submucosa. We also showed the presence of GFP but not CTB in the liver of mice following oral delivery of CTB-GFP leaf material. Detection of both CTB and GFP in mouse intestinal cells following oral administration of CTB-GFP expressing leaf material shows that the recombinant protein has been protected from peptidases and/or acids by bioencapsulation within the plant cells. The high cost of therapeutic proteins can be attributed to their production in fermentation based system, purification using expensive methods including chromatography, low temperature storage, transportation and sterile delivery using syringes through health professionals. Most of these expenses could be avoided by expressing therapeutic proteins in plant cells and through their oral delivery. The ability to express high levels of foreign proteins in edible plant parts makes this approach a reality.

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