1-1-2004

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Plant Biotechnology Journal (2004) **2**, pp. 141–153 Doi: 10.1111/j.1467-7652.2004.00057.x

High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts

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Received 29 July 2003;
revised 22 October 2003;
accepted 25 October 2003.
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Keywords: canine parvovirus, CTB, fusion protein, GFP, peptide vaccine, plastid transformation.

Summary

The 2L21 peptide, which confers protection to dogs against challenge with virulent canine parvovirus (CPV), was expressed in tobacco chloroplasts as a C-terminal translational fusion with the cholera toxin B subunit (CTB) or the green fluorescent protein (GFP). Expression of recombinant proteins was dependent on plant age. A very high-yield production was achieved in mature plants at the time of full flowering (310 mg CTB-2L21 protein per plant). Both young and senescent plants accumulated lower amounts of recombinant proteins than mature plants. This shows the importance of the time of harvest when scaling up the process. The maximum level of CTB-2L21 was 7.49 mg/g fresh weight (equivalent to 31.1% of total soluble protein, TSP) and that of GFP-2L21 was 5.96 mg/g fresh weight (equivalent to 22.6% of TSP). The 2L21 inserted epitope could be detected with a CPV-neutralizing monoclonal antibody, indicating that the epitope is correctly presented at the C-terminus of the fusion proteins. The resulting chimera CTB-2L21 protein retained pentamerization and G_{M1}-ganglioside binding characteristics of the native CTB and induced antibodies able to recognize VP2 protein from CPV. To our knowledge, this is the first report of an animal vaccine epitope expression in transgenic chloroplasts. The high expression of antigens in chloroplasts would reduce the amount of plant material required for vaccination (~100 mg for a dose of 500 μg antigen) and would permit encapsulation of freeze-dried material or pill formation.

Introduction

Traditional vaccines use either killed or attenuated whole disease-causing organisms. The advances in molecular biology have enabled the identification of antigens capable of eliciting a protective immune response. Antigen production in a heterologous expression system allows the development of subunit vaccines, improving aspects such as safety and processing (Walmsley and Arntzen, 2000). The production of recombinant vaccine proteins in microorganisms is expensive and requires stringent purification protocols. The alternative production in plants could circumvent these problems for a number of reasons: (i) the culture of plants in the field is straightforward, fairly cheap and can be scaled up with a low cost in relation to fermentation processes (10–50 times cheaper) (Kusnadi *et al*., 1997); (ii) the health risks arising from contamination with human pathogens or toxins are minimized; (iii) the plant material can be used directly as food or feed, avoiding purification and generating an oral vaccine; (iv) plant-based vaccines for oral delivery may be particularly useful for inducing immunity to disease organisms that colonize mucosal surfaces. Other advantages of plant-produced vaccines include heat stability, no need for injection-related manipulations of animals or hazards in humans, and the production of multicomponent combined vaccines.

It has been demonstrated that proteins produced in plants are able to elicit immune responses against important human or animal pathogens (Thanavala *et al*., 1995; Arakawa *et al*., 1998a; Modelska *et al*., 1998; Tacket *et al*., 1998, 2000; Castanon *et al*., 1999; Wigdorovitz *et al*., 1999; Streatfield

et al., 2001). Indeed, the first plant oral vaccine against the Norwalk virus is pending approval in the USA. Nevertheless, one of the main problems related to vaccine production in nuclear transgenic plants is the low expression level of foreign antigens. The average expression levels of recombinant antigens in stably transformed plants are generally on the level of 0.01–0.4% of the total soluble protein (TSP) (Daniell *et al*., 2001c), making the immunization protocols very long and economically non-viable. However, some recombinant proteins reach exceptionally high expression levels by nuclear transformation; for example, the *Aspergillus niger* phytase accumulates to 14% TSP in tobacco leaves (Verwoerd *et al*., 1995) and the heat-labile toxin of *Escherichia coli* has been reported to reach 12% in maize seeds (Streatfield *et al*., 2003). The quantity of plant tissue for an oral vaccine dose must be of practical size for consumption, enabling pill processing. Increasing the expression levels of recombinant proteins is therefore a key step.

An alternative to improve foreign protein expression is chloroplast transformation. Foreign genes integrated in the tobacco chloroplast genome result in the accumulation of exceptionally large quantities of recombinant proteins, up to 46% of TSP (De Cosa *et al*., 2001). Hyperexpression of the transgenes in the chloroplast is attributed to the high copy number of the foreign gene (up to 10 000 copies/cell) and to the higher stability of the protein in the stroma of the chloroplast in relation to the cytoplasm. Biopharmaceutical compounds, such as human somatotropin (Staub *et al*., 2000), human serum albumin (Fernández-San Millán *et al*., 2003), antimicrobial peptides (DeGray *et al*., 2001), the B-subunit of the cholera toxin (CTB) (Daniell *et al*., 2001a) and the tetanus toxin (Tregoning *et al*., 2003), have been expressed at high levels (4–25% of TSP), suggesting that high expression is gene independent. Thus, chloroplast technology could be a feasible and forthcoming strategy to improve oral vaccine expression.

Additionally, chloroplast transformation has other advantages over nuclear transformation (Bogorad, 2000; Bock, 2001; Daniell *et al*., 2002). So far, gene silencing has not been described in transgenic chloroplasts achieving high expression levels of recombinant proteins (De Cosa *et al*., 2001; Tregoning *et al*., 2003). The environmental concern of transgene escape through pollen in nuclear transgenic plants is overcome by chloroplast transformation due to the maternal inheritance of plastids in most of the cultivated plants (Daniell, 2002). Recently, Huang *et al*. (2003) have described the transfer of DNA from the chloroplast to the nuclear genome at a frequency of 0.006%. Daniell and Parkinson (2003) have pointed out some technical aspects of this article, deducing that the simultaneous integration of transgenes into the nuclear and chloroplast genomes occurred in the transgenic lines analysed. Notwithstanding, both articles conclude that, even if transgenes with plastid regulatory sequences are transposed from chloroplast to nuclear genomes, they will not be functional, eliminating concerns about such transfer. Multigene engineering is feasible by chloroplast transformation in a single step because of the ability of chloroplasts to process polycistrons (De Cosa *et al*., 2001; Daniell and Dhingra, 2002; Ruiz *et al*., 2003). This will be very useful to produce multicomponent vaccines in transgenic chloroplasts.

In the present work, we have obtained high levels of a peptide-based vaccine by chloroplast transformation of tobacco plants. We selected a linear antigenic peptide (named 2L21) from the VP2 capsid protein (amino acids 2–23) of the canine parvovirus (CPV). This parvovirus infects young animals producing haemorrhagic gastroenteritis and myocarditis. The 2L21 synthetic peptide chemically coupled to KLH carrier protein has been extensively studied and has been shown to effectively protect dogs and minks against parvovirus infection (Langeveld *et al*., 1994b, 1995). Previously, this peptide has been successfully expressed in nuclear transgenic plants as an N-terminal translational fusion of GUS protein (Gil *et al*., 2001). Also, shorter peptides sharing sequence with the 2L21 epitope have been correctly expressed in plant viruses eliciting CPV-neutralizing antibodies in mice (Fernandez-Fernandez *et al*., 1998; Langeveld *et al*., 2001). We have expressed the 2L21 peptide fused either to CTB or the green fluorescent protein (GFP). In this report, we demonstrate that the resulting fusion proteins are efficiently expressed and accumulated in chloroplast transgenic plants at higher levels (up to 10-fold) than those previously reported in nuclear transformation (Gil *et al*., 2001).

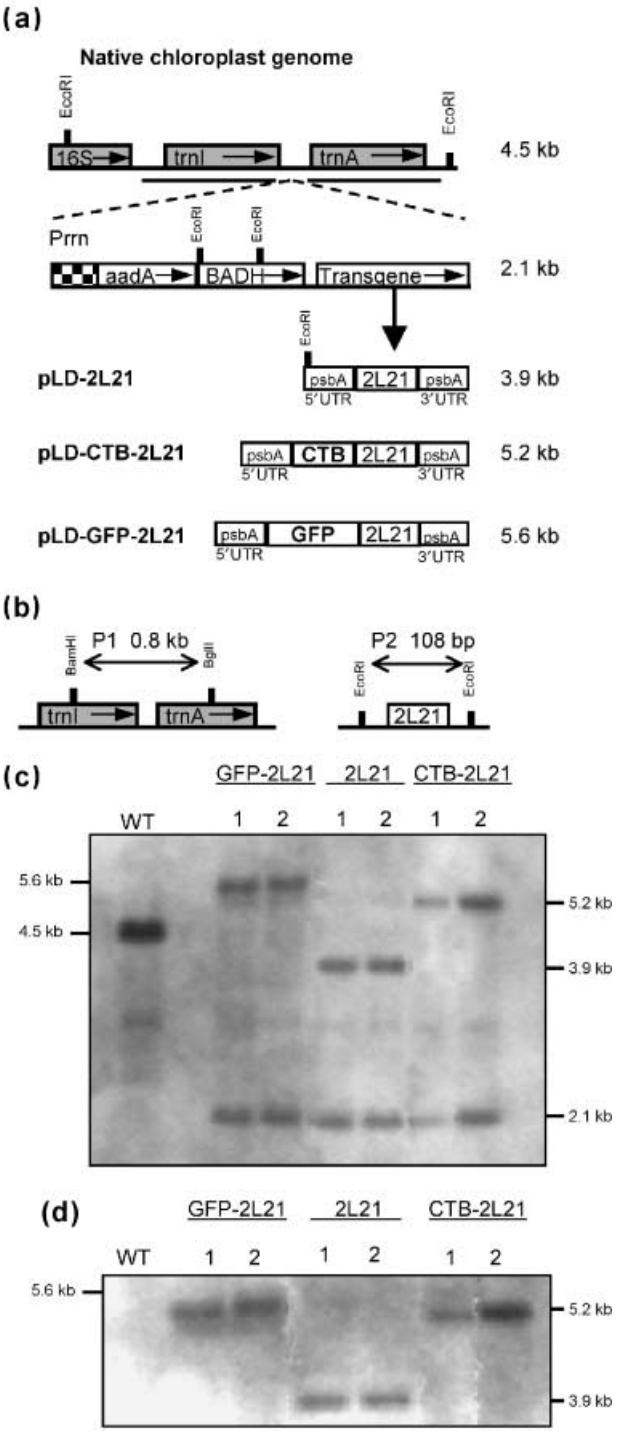
Results and discussion

Vector construction

Three chloroplast transformation vectors were designed (Figure 1a). All included: (i) the *trnI* and *trnA* border sequences for homologous recombination in the inverted repeat regions of the chloroplast genome; (ii) the chimeric aminoglycoside 3′-adenylyltransferase (*aadA*) gene, conferring resistance to spectinomycin and streptomycin; and (iii) the betaine aldehyde dehydrogenase (*BADH*) gene, conferring resistance to betaine aldehyde. Both genes are driven by the constitutive promoter of the rRNA operon (*Prrn*), each with individual ribosome binding sites (GGAGG). The foreign gene was inserted immediately downstream of the promoter

and the 5′-untranslated region (UTR) of the *psbA* gene (Figure 1a). In the pLD-2L21 vector, the foreign gene consisted of a 66 bp long DNA sequence encoding the 2L21 epitope of the CPV VP2 protein. The pLD-CTB-2L21 vector included a gene fusion of the *2L21* sequence to the C-terminus of the *CTB*, with the sequence of a flexible hinge tetrapeptide (GPGP) in between. This tetrapeptide may reduce steric hindrance between the CTB and the 2L21 peptide, facilitating CTB subunit assembly. It has been successfully used to express CTB fused to insulin or NSP4 rotavirus in potato plants (Arakawa *et al*., 1998b, 2001). The pLD-GFP-2L21 vector included the gene encoding the soluble modified GFP protein (Davis and Vierstra, 1998) instead of the *CTB* gene. We chose CTB for the fusion protein because it is a potent mucosal immunogen as a result of CTB binding to the intestinal epithelial cells via G_{M1}-ganglioside receptors, enhancing the immune response when coupled to other antigens (Holmgren *et al*., 1993; Mor *et al*., 1998). GFP was selected for the fusion protein because the 14-amino acid N-terminal GFP fusion to 5-enolpyruvylshikimate-3-phosphate synthase increased the protein accumulation in transgenic chloroplasts of tobacco (Ye *et al*., 2001). In addition, GFP can be used as a reporter gene and therefore facilitates the selection of transgenic plants.

After bombardment, leaf fragments were cultured under the selection of spectinomycin. Five weeks later, 4–5 shoots appeared from each bombarded leaf. Previously described protocols (Daniell, 1993, 1997) were followed to obtain homoplasmic plants.



Determination of chloroplast integration and homoplasmy

Integration of the foreign genes into the chloroplast genome was confirmed by polymerase chain reaction (PCR) in the shoots developed on spectinomycin-selective medium. The strategy was based on one primer, landed on the chloroplast genome upstream of the *trnI* gene used for homologous recombination, and a second primer, landed on the *aadA* gene (Figure 1a). The PCR product cannot be obtained in nuclear transgenic plants, spontaneous mutants or non-transformed shoots. It was found that 100% of the shoots analysed were PCR positive, whereas wild-type shoots did

Figure 1 Vector constructs and integration of transgenes into the chloroplast genome. (a) Regions for homologous recombination are underlined in the native chloroplast genome. The 2L21 sequence or fusion sequences, GFP-2L21 and CTB-2L21, are driven by the psbA promoter. Arrows within boxes show the direction of the transcription. Numbers to the right indicate the predicted hybridizing fragments when total DNA digested with *Eco*RI was probed with probe P1. (b) The 0.8 kb fragment (P1) of the targeting region for homologous recombination and the 108 bp 2L21 sequence (P2) were used as probes for the Southern blot analysis. (c, d) Southern blot analysis of two independent lines (1, 2) for each transformation cassette. Blots were probed with P1 (c) and P2 (d). 2L21, sequence included in the VP2 gene of the canine parvovirus; aadA, aminoglycoside 3′-adenylyltransferase; BADH, betaine aldehyde dehydrogenase; CTB, cholera toxin B; GFP, green fluorescent protein; Prrn, 16S rRNA promoter; UTR, untranslated region; WT, wild-type Petit Havana plant.

not amplify any fragment (data not shown). In order to obtain homoplasmic plants, confirmed PCR transformants were subjected to a second round of selection in a medium of the same composition. Southern blot analysis was performed on shoots developed under these conditions. The 0.8 kb probe, homologous to the flanking regions *trnI* and *trnA* (Figure 1b), was used to verify site-specific integration and to check homoplasmy. Total plant DNA was digested with *Eco*RI. DNA from non-transformed plants produced a 4.5 kb fragment (Figures 1a,c). Transformed plants produced two fragments: (i) a 2.1 kb fragment common to all of them; and (ii) 3.9 (pLD-2L21), 5.2 (pLD-CTB-2L21) or 5.6 kb (pLD-GFP-2L21) fragments. To confirm that the 3.9, 5.2 and 5.6 kb fragments contained the *2L21* sequence, the same blot was hybridized with P2 probe (Figure 1b) homologous to the *2L21* sequence. As expected, hybridization was observed only in the upper bands of the chloroplast transgenic lines (Figure 1d). Southern blot analysis of T_1 generation confirmed that all six transgenic lines analysed maintained homoplasmy.

Plants transformed with pLD-GFP-2L21 vector showed uniform green fluorescence after illumination with ultraviolet light (data not shown). No chimeric plants were observed.

Immunoblot analysis of chloroplast-synthesized 2L21 epitope and fusion proteins

The detection of the recombinant proteins in transformed chloroplasts was performed with the monoclonal antibody (mAb) 3C9, specific for the 2L21 epitope. This antibody has been used previously in the detection of the 2L21 epitope expressed in different systems, such as baculovirus or *Arabidopsis* (Lopez de Turiso *et al*., 1992; Gil *et al*., 2001). There was no cross-reaction between mAb 3C9 and wild-type tobacco plant proteins (Figure 2a,b). Plants transformed with the plasmid pLD-2L21 showed no detectable signal in the Western blot (Figure 2a). To discard a putative problem of detection limit or peptide diffusion in polyacrylamide gel electrophoresis (PAGE), a dot-blot was performed on three plants, loading 50 μg per dot. No signal was detected, although a clear spot appeared when synthetic 2L21 peptide was used as positive control (data not shown). A clear band of the expected GFP-2L21 protein size (29 kDa) was observed in pLD-GFP-2L21-transformed plants (Figure 2a).

Chloroplast-synthesized CTB-2L21 protein developed with mAb 3C9 (Figure 2b) or with anti-CTB serum (Figure 2c) as the primary antibody appeared as aggregates in unboiled samples from pLD-CTB-2L21-transformed plants. Unboiled purified bacterial CTB developed with anti-CTB serum (Figure 2c) also appeared as aggregates although, as expected,

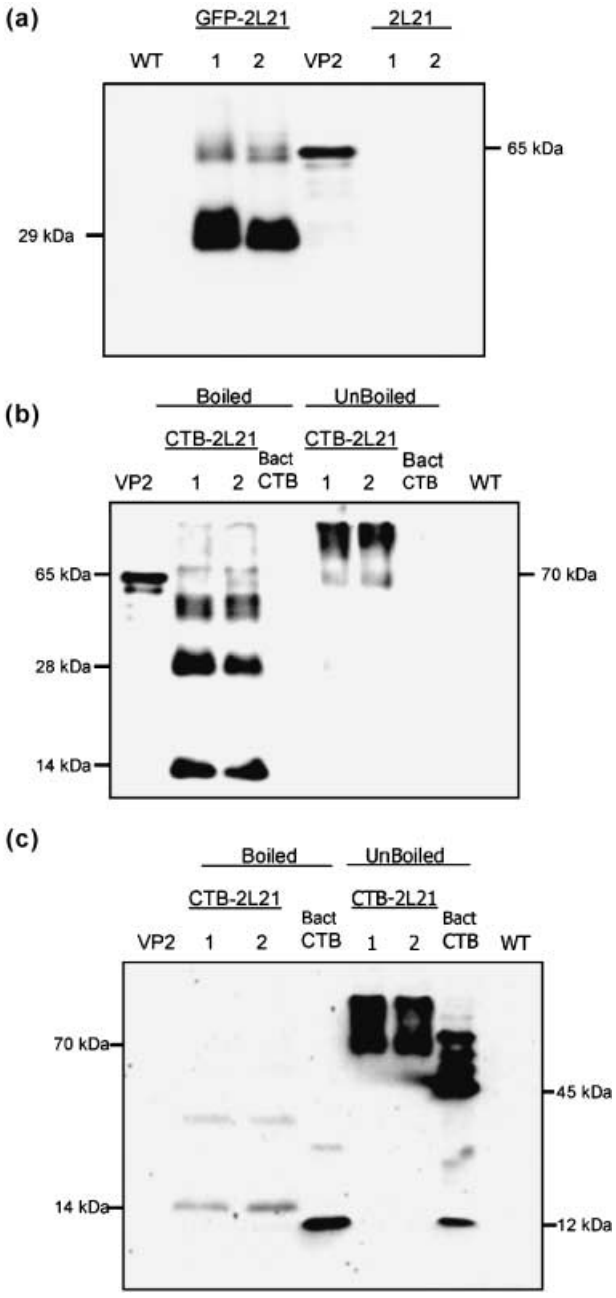


Figure 2 Western blot analysis of 2L21 expression in transgenic chloroplasts. Blots were detected using mouse anti-2L21 (a, b) or rabbit anti-CTB (c) as primary antibody. (a) Two independent lines (1, 2) of 2L21 and GFP-2L21 chloroplast transgenic plants. (b, c) Two independent lines (1, 2) of CTB-2L21 chloroplast transgenic plants. Five micrograms of plant total protein were loaded per well, except for boiled samples in blot (c) where 10 μg of protein were loaded. 2L21, epitope from the VP2 protein of the canine parvovirus; Bact CTB, bacterial CTB; CTB, cholera toxin B; GFP, green fluorescent protein; VP2, protein of the canine parvovirus that includes the 2L21 epitope; WT, wild-type Petit Havana plant.

the molecular weights of these aggregates were lower than those observed in chloroplast-derived CTB-2L21 protein. Bacterial CTB antigen showed no cross-reaction with mAb 3C9 (Figure 2b). To clarify the nature of these aggregates,

chloroplast-derived CTB-2L21 and bacterial CTB samples were boiled for 5 min. After heat treatment, a prominent band corresponding to the CTB monomer form (12 kDa) was observed in bacterial CTB samples hybridized with anti-CTB serum (Figure 2c). In the case of heat-treated chloroplast-derived CTB-2L21 protein, differences were observed depending on the primary antibody used for hybridization. Prominent bands corresponding to the expected size of the CTB-2L21 monomer (14 kDa) and dimer (28 kDa) were detected when the blot was hybridized with mAb 3C9, although higher oligomers were also visualized (Figure 2b). However, in the blot developed with anti-CTB serum, the CTB-2L21 monomer was the most abundant form, although some dimers could also be seen (Figure 2c). It must be noted that native CTB is assembled in a pentameric structure by disulphide bridge formation, with a molecular weight of 45 kDa. Bands above 45 kDa in the case of bacterial CTB, or 70 kDa in the case of CTB-2L21, must reflect aggregates of CTB-2L21 or CTB pentamers labile to heat treatment. Arakawa *et al*. (1998b) expressed the CTB fused to insulin in potato tubers and also observed the CTB-insulin oligomeric form and the corresponding monomeric form after heat treatment. We observed a different sensitivity of the anti-CTB antibody against boiled or unboiled plant samples. Very faint bands were observed in boiled samples in relation to the unboiled ones, even though a double amount of total protein was loaded (Figure 2c). This fact was not observed with mAb 3C9. Unboiled purified bacterial CTB appeared partially dissociated into dimers and monomers on storage at 4 °C for several months. The same behaviour was observed by Daniell *et al*. (2001a).

G_{M1}-ganglioside enzyme-linked immunosorbent binding assay

Chloroplast-synthesized CTB-2L21 protein showed a strong affinity for G_{M1}-ganglioside similar to that shown by purified bacterial CTB (Figure 3). This result indicates that CTB-2L21 fusion protein retained the pentameric structure characteristic of bacterial CTB and conserved the antigenic sites for specific binding to the pentasaccharide G_{M1}-ganglioside receptors on the intestinal epithelial cells. No signal was detected when the plate was coated with bovine serum albumin (BSA) instead of G_{M1}-ganglioside (Figure 3). Daniell *et al*. (2001a) expressed CTB, without any coupled foreign peptide, in tobacco chloroplast and also observed a specific affinity for G_{M1}-ganglioside. The expression of chimera CTB proteins, including foreign sequences, was also performed by nuclear transformation of potato. Fusion proteins were constructed by linking insulin or a 22-amino acid long epitope from the rotavirus enterotoxin non-structural protein to the C-terminus of the CTB. G_{M1}-ganglioside binding assays demonstrated that both fusion proteins were assembled into biologically active pentamers and retained the affinity to G_{M1}-ganglioside (Arakawa *et al*., 1998b, 2001; Kim and Langridge, 2003). Therefore, CTB is a good candidate for C-terminal fusion of foreign sequences in the chloroplast.

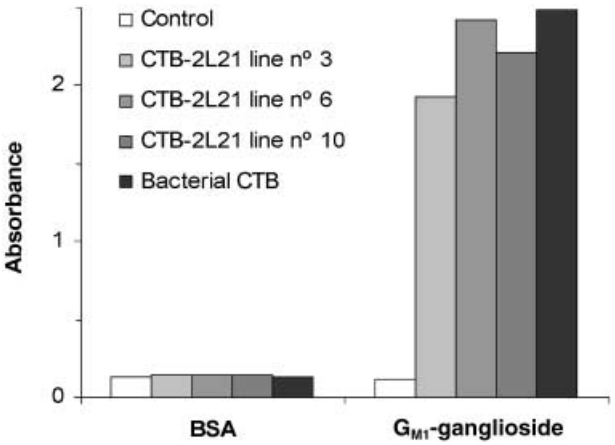


Figure 3 CTB-G_{M1}-ganglioside binding enzyme-linked immunosorbent assay. Plates, coated with bovine serum albumin (BSA) or G_{M1}-ganglioside, were incubated with plant total soluble protein from three independent chloroplast transgenic lines (3, 6, 10) of CTB-2L21, untransformed plant (WT) or 25 ng of bacterial CTB. The absorbance of the G_{M1}-ganglioside-CTB antibody complex in each case was measured. 2L21, epitope from the VP2 protein of the canine parvovirus; CTB, cholera toxin B; G_{M1}-ganglioside, pentasaccharide present in the membrane of the epidermal cells in the intestine of mammals.

Enzyme-linked immunosorbent assay (ELISA) quantification of the 2L21 epitope

Large differences in the accumulation of recombinant proteins were observed between plants transformed with the three different constructs. Three lines transformed with the pLD-2L21 vector were analysed and none showed expression of the 2L21 peptide in the ELISA, despite using a dilution of 1 : 5 (detection limit of 2L21, 6 ng). However, plants transformed with pLD-CTB-2L21 or pLD-GFP-2L21 plasmids expressed the respective fusion protein at high levels (Figure 4a). To study the expression level of the recombinant protein during the life cycle of the plant, we analysed samples of chloroplast transgenic plants grown in a phytotron from transplanting to senescence. In general, the expression of CTB-2L21 was higher than that of GFP-2L21 (Figure 4a,b). The expression of the fusion proteins depended on plant age. Small plants, 30 cm tall (24 days after transplanting), synthesized only 0.84 mg/g fresh weight (FW) of CTB-2L21 or 0.37 mg/g

FW of GFP-2L21 (Figure 4a). There was an arrest or even a decrease in the synthesis 48 days after transplanting, coinciding with the start of flowering. The maximum levels of recombinant protein (7.49 mg/g FW of CTB-2L21, equivalent to 31.1% TSP; 5.96 mg/g FW of GFP-2L21, equivalent to 22.6% TSP) were reached after 60 days, matching with full flowering and the appearance of the first green fruits. There was a decrease in the levels of recombinant proteins when mature fruits appeared, 69 days after transplanting. The large amounts of recombinant proteins were confirmed when total proteins from chloroplast transgenic plants were separated by sodium dodecylsulphate (SDS)-PAGE and stained with Coomassie brilliant blue. Predominant bands of the expected size were observed for CTB-2L21 and GFP-2L21 (Figure 4c). High levels of CTB-2L21 or GFP-2L21 did not affect the phenotype of transgenic plants. Transformed and wild-type control plants grown in the phytotron were indistinguishable. The high expression levels of these chimera proteins could be due to a very efficient translation conducted by the *psbA* 5′-UTR (Eibl *et al*., 1999). Recently, Fernández-San Millán *et al*. (2003) observed that the presence of the *psbA* 5′-UTR enhanced the translation and favoured the accumulation of human serum albumin in tobacco chloroplasts. Daniell *et al*. (2001a) expressed the CTB subunit in transgenic chloroplasts with a maximum accumulation of 4.1% of TSP. This result contrasts with the high expression level obtained for the fusion protein CTB-2L21 (31.1% of TSP). In addition to the difference in the amino acid sequence itself, the *CTB* gene was driven by the promoter of the rRNA operon (*Prrn*), including a ribosome binding site (GGAGG), whereas the *CTB-2L21* gene was driven by the promoter and 5′-UTR of the *psbA* gene. By using mutants generated by sequence deletion and base alteration, Zou *et al*. (2003) have recently demonstrated that the correct primary sequence and secondary structure of the *psbA* 5′-UTR stem-loop are required for mRNA stabilization and translation. Both the different promoter used and the inclusion of the translation enhancer 5′-UTR sequence could therefore explain the higher protein levels found in this work.

To find out whether the leaf age affected the actual level of recombinant proteins, samples from chloroplast transgenic plants were grown in a phytotron for 65 days and analysed. Young leaves showed the highest levels of GFP-2L21 or

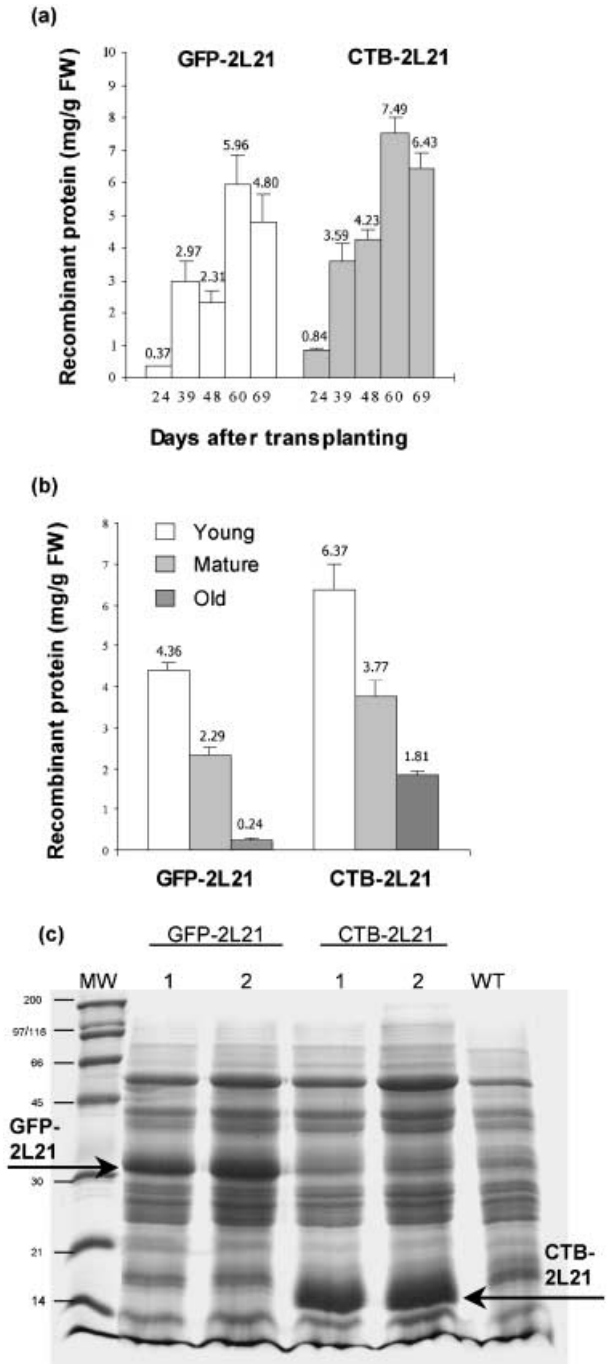


Figure 4 Analysis of recombinant protein accumulation in transgenic chloroplasts of GFP-2L21 and CTB-2L21 plants. (a) Enzyme-linked immunosorbent assay of recombinant protein accumulation in plants at different developmental stages. Total soluble protein was extracted from young leaves of potted plants in the phytotron. The data are presented as the means ± SE of measurements on five individual plants per construction. (b) Recombinant protein accumulation in young, mature and old leaves of plants growing in the phytotron, 65 days after transplanting. Data are presented as the means ± SE of measurements on seven individual plants per construction. (c) Coomassie blue-stained sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel of plant samples, 60 days after transplanting, equivalent to those shown in (a). Two independent lines (1, 2) for each construction were analysed. Recombinant proteins GFP-2L21 and CTB-2L21 are marked. Fifty micrograms of plant total protein were loaded per well. 2L21, epitope from the VP2 protein of the canine parvovirus; CTB, cholera toxin B; GFP, green fluorescent protein; MW, molecular weight marker; WT, wild-type Petit Havana plant.

Table 1 Yield of the CTB-2L21 recombinant protein expressed in tobacco chloroplast relative to leaf age

Leaf age	Average number of leaves per plant	Fresh weight/leaf (g)	Average amount of CTB-2L21 (mg)	Recombinant protein percentage in relation to whole plant
Young	3.2	2.5	51	16.5
Mature	7.8	8.0	235	75.8
Old	2.6	5.0	24	7.7
Total recombinant protein (mg) per plant			310	100

Young, small dark-green leaves from the upper part of the plant; mature, large, well-developed leaves from the middle of the plant; old, bleached leaves or those undergoing senescence from the bottom of the plant; CTB-2L21, cholera toxin B protein including 2L21 epitope from the VP2 protein of the canine parvovirus.

CTB-2L21 (Figure 4b). Old senescent leaves showed much lower amounts of recombinant protein in both types of plant, 4-fold (CTB-2L21) or 18-fold (GFP-2L21) lower than that in young leaves (Figure 4b). Usually, the content of TSP also decreased from young to old leaves, and the profiles of recombinant proteins matched those of TSP. Thus, the amount of recombinant protein expressed as the percentage of TSP remained basically unchanged (Stevens *et al*., 2000) despite the fact that old leaves accumulated very low amounts of recombinant protein in comparison with mature and young leaves.

In order to quantify the yield of recombinant protein per plant and the relative contributions of young, mature and old leaves, CTB-2L21 protein levels were estimated according to the number and weight of leaves per plant (Table 1). A total of 310 mg of CTB-2L21 could be produced per plant, 75.8% of this coming from mature leaves and 16.5% from young leaves. Only 7.7% was produced in old leaves. We believe that the expression of recombinant protein levels as mg/g FW is more reliable than as a percentage of TSP, as TSP values may be highly variable depending on the physiological and environmental conditions (Stevens *et al*., 2000). This fact and the recombinant protein content according to the age of the plant must be taken into consideration when scaling up the processes.

Why did the plants transformed with the pLD-2L21 vector not accumulate the 2L21 peptide? Analysis at the transcriptional level in the three different types of chloroplast transgenic plants revealed transcripts of the expected size in all of them (Figure 5). This precludes deficient transcription or mRNA stability. Our hypothesis is that the messenger may be correctly translated, but the 22-amino acid long peptide is recognized as a foreign molecule in the stroma of the chloroplast and is rapidly degraded. In bacteria, with an equivalent transcriptional and translational machinery to that of the chloroplast, the expression of peptides shorter than 60 amino acids is often ineffective because of degradation (Dobeli *et al*., 1998). Thus peptides are generally expressed in bacteria as

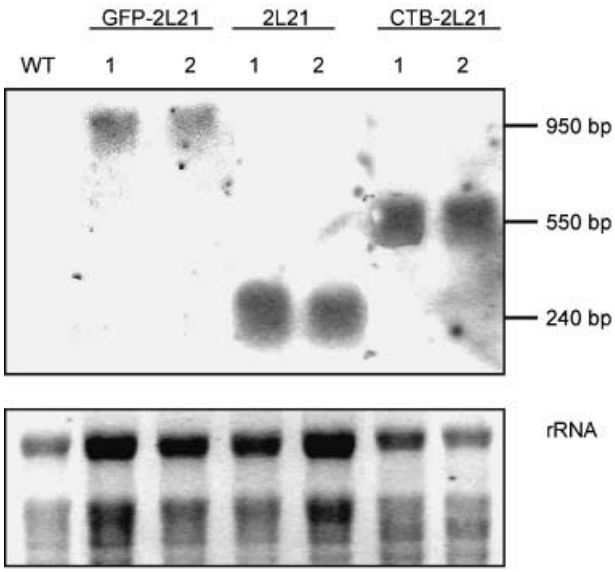


Figure 5 Northern blot analysis of chloroplast transgenic plants. Total RNA was extracted from leaves of potted plants in the phytotron. Two independent lines (1, 2) for each construction (2L21, GFP-2L21, CTB-2L21) were analysed. The 108 bp 2L21 sequence was used as a probe. Ten micrograms of total RNA were loaded per well. Ethidium bromide-stained rRNA was used to assess loading. 2L21, epitope from the VP2 protein of the canine parvovirus; CTB, cholera toxin B; GFP, green fluorescent protein; rRNA, ribosomal RNA; WT, wild-type Petit Havana plant.

fusion proteins. Many proteins located in the stroma of the chloroplast or in the lumen of the thylakoids are synthesized in the nucleus and require a single or double transit peptide. These transit peptides, cleaved by specialized endopeptidases, do not accumulate in chloroplasts, suggesting that they are probably further degraded by proteases (Adam, 2000). Small polypeptides are usually unstable in the stroma of the chloroplast. Leelavathi and Reddy (2003) showed that interferon gamma had a short half-life in transgenic chloroplasts, but this could be extended by GUS fusion. A similar pattern was observed with the fusion between ubiquitin and human somatotropin (Staub *et al*., 2000). Therefore, the 2L21 peptide might be expressed but may be degraded at a

faster rate. DeGray *et al*. (2001) expressed an antimicrobial peptide in transgenic chloroplasts, showing the plant extract's antibacterial activity. Although both peptides are composed of 22 amino acids, their sequences are different and may be degraded accordingly by sequence-specific peptidases and proteases (Adam, 2000). A primary structure more sensitive to chloroplast peptidases could explain the instability of the 2L21 peptide in the stroma of the chloroplast.

Antibody response to plant-derived 2L21 immunogens

In order to study the immunogenicity of plant-derived 2L21 constructions, Balb/c mice were immunized intraperitoneally (i.p.) with leaf extracts from CTB-2L21-, GFP-2L21- and 2L21-transformed plants or non-transformed plants (control) as described in 'Experimental procedures'. All mice immunized with CTB-2L21 elicited anti-2L21 antibodies (titres ranged from 200 to 25 000) (Figure 6). None of the sera from groups immunized with leaf extracts from 2L21-transformed plants and non-transformed plants recognized 2L21 peptide (titre, < 10) and only one of six mice from the GFP-2L21 group showed a marginal response against 2L21 peptide (titre, 20) (data not shown). To determine whether the humoral response elicited recognized viral native protein, sera were titrated against VP2 protein. Only mice immunized with leaf extracts containing CTB-2L21 recognized VP2 in ELISA (Figure 6). No antibody titres (titre, < 10) were found against the control peptide (Figure 6).

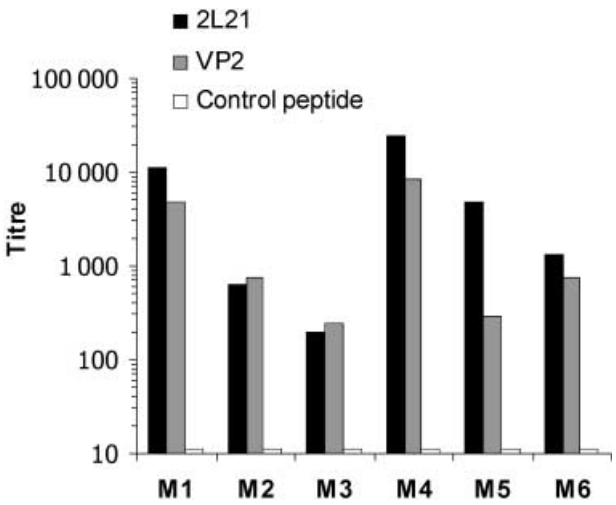


Figure 6 Titres of antibodies at day 50 induced by plant-derived CTB-2L21 recombinant protein. Balb/c mice were intraperitoneally immunized with leaf extract from CTB-2L21 transgenic plants. Animals were boosted at days 21 and 35. Each mouse received 20 μg of CTB-2L21 recombinant protein. Individual mice sera were titrated against 2L21 synthetic peptide, VP2 protein and control peptide (amino acids 122–135 of hepatitis B virus surface antigen). Titres were expressed as the highest serum dilution to yield twice the absorbance mean of pre-immune sera. M1–M6, mouse 1–6; 2L21, epitope from the VP2 protein of the canine parvovirus; CTB, cholera toxin B; VP2, protein of the canine parvovirus that includes the 2L21 epitope.

These results show that plant-derived CTB-2L21 recombinant protein is immunogenic by the i.p. route, being able to induce a humoral response that cross-reacts with native VP2 protein. CTB protein is a well-known carrier protein able to potentiate the immune response against fused peptides (McKenzie and Halsey, 1984). This immunological property would explain the anti-2L21 response detected in the CTB-2L21 group. The conserved pentameric conformation (Figure 2b,c) and G_{M1}-ganglioside binding property (Figure 3) of CTB-2L21 chimera protein augur that this C-terminal CTB fusion protein must also be a good immunogen for mucosal delivery. The fact that no antibody immune response was elicited with extracts from GFP-2L21-transformed plants suggests that GFP protein does not act as an efficient immunological carrier. The lack of humoral response in the 2L21 group could be explained by the poor immunogenicity of the 2L21 peptide and the instability of short peptides in plant cells. Previous reports have shown that free 2L21 peptide is a poor immunogen that requires coupling to KLH protein to induce antibodies (Langeveld *et al*., 1994a,b).

Oral vaccination is able to induce both mucosal and systemic immune response. Indeed, a mucosal immune response is more effectively achieved by oral, rather than parenteral, antigen delivery. Therefore, oral vaccination is of special interest because many infectious agents colonize or invade epithelial membranes. One of the major advantages of the high expression of recombinant proteins in chloroplast transgenic plants is the low dose required for oral vaccination. Clinical trials in phase I have been reported for the Norwalk virus (Tacket *et al*., 2000), bacterial diarrhoea (Tacket *et al*., 1998) and hepatitis B virus (Kapusta *et al*., 2001). Volunteers ingested raw material from potato tubers or lettuce leaves (ranging from 50 to 200 g per dose, equivalent to 0.3–750 μg of antigen). Most of the individuals developed IgG- and IgA-specific antibodies against the plant-derived antigen, although some subjects who ate these large amounts of raw potato tubers showed side-effects, such as vomiting, diarrhoea and fever (Tacket *et al*., 2000). A standard oral delivery vaccination programme consisting of three doses is not practical if a large amount of plant material must be eaten per dose. By using chloroplast transgenic plants with expression levels equivalent to those reported in this paper, very small amounts of plant material would be required per dose (80–100 mg for a quantity of 500 μg recombinant protein), allowing the encapsulation of freeze-dried material or pill

formation. Nevertheless, it must be demonstrated case-by-case that dried leaf material can induce immune responses when ingested, and that the antigen integrity can be maintained for long periods at room temperature. In the present work, we chose tobacco as the host plant because the chloroplast transformation method is very efficient. However, the presence of toxic alkaloids in the tobacco plant make this system incompatible with oral delivery in humans (Mason *et al*., 2002). Thus, a great effort must be made to transfer the plastid transformation strategy to less toxic and more palatable species, such as spinach, lettuce or cereal species that do not need to be cooked, e.g. maize meal.

In conclusion, we have expressed the fusion proteins CTB-2L21 and GFP-2L21 at very high levels in tobacco chloroplasts. This will facilitate the use of raw plant material for the delivery of an oral vaccine against CPV. Recognition of the 2L21-inserted epitope by the anti-2L21 mAb 3C9 in Western blot (Figure 2) and ELISA (Figure 4) indicates that this epitope is correctly presented at the C-terminus of CTB and GFP proteins. Recognition of CTB-2L21 protein by CPV-neutralizing mAb 3C9 (Lopez de Turiso *et al*., 1991) and induction of an anti-VP2 cross-reactive immune response after i.p. injection of plant-derived CTB-2L21 protein suggest that this chimera protein might be able to induce a protective immune response against CPV. Additional experiments are underway to check the ability of this fusion protein to develop specific immune responses after mucosal delivery. So far, mainly human or bacterial proteins have been expressed in chloroplast transgenic plants (Daniell *et al*., 2002). To our knowledge, this is the first time an animal vaccine epitope has been expressed in transgenic chloroplasts.

Experimental procedures

Construction of the chloroplast expression vectors

The tobacco chloroplast expression vector pLD-BADH was constructed by inserting the *BADH* 1.5 kb *Sma*I fragment into the *EcoR*V site of the pLD vector (Daniell *et al*., 1998, 2001a,b; Kota *et al*., 1999; Guda *et al*., 2000), including the new sites *Sac*II, *Mlu*I, *Sal*I, *Dra*II, *Kpn*I and *Eco*RI in its 3′ end. This fragment contains the *BADH* coding sequence preceded by a Shine-Dalgarno (GGAGG) and has an ATG as the initiation codon. These sequences were introduced by using the primers 5′-CACCCCGGGGGAGGCAACCATGGCGTTC-CCAATTCC-3′ and 5′-CACCCCGGGGAATTCGGTACCAG-GCCCCGTCGACACGC GTCCGCGGTCAAGGAGACTTGTACC-3′, and amplified by PCR using the pLD-St-BADH vector as template.

The DNA sequence corresponding to the parvovirus 2L21 epitope was obtained by a PCR-based gene assembly by using the following overlapping primers: 5′-GGGACATGTCT-GACGGTGCTGTACAACCT*GACGGTGGTCAACCTGCTGTA*-3′ and 5′-GGGGCGGCCGCTTAACCAGTAGCACGTTCGT-TACG*TACAGCAGGTTGACCACCGTC*-3′ (italic overlapping sequences). The codon usage was referred to the chloroplast *psbA* gene. The PCR product was cloned into the pGEM-T plasmid (Promega). The *Afl*III/*Not*I pGEM-T fragment was fused to the promoter and 5′-UTR of the *psbA* gene, giving the p2L21 vector. The *Kpn*I/*Not*I fragment from p2L21, including the promoter and 5′-UTR of the *psbA* gene, and the *2L21* sequence, was inserted into the pLD-BADH vector, giving the pLD-2L21 vector.

For the fusion proteins CTB-2L21 and GFP-2L21, the flexible hinge tetrapeptide GPGP (Arakawa *et al*., 1998b) was fused by PCR to the 5′ end of the *2L21* sequence by using the primers 5′-CCCGGGCCCATGTCTGACGGTGCTG-3′ and 5′-GGGGCGGCCGCTTAACCAGTAGCACGTTCGTTACGTA-CAGCAGGTTGACCACCGTC-3′. The 75 bp *Sma*I/*Not*I fragment of the *2L21*, including the hinge tetrapeptide, was inserted at the 3′ end of the *CTB* gene. The *CTB* gene included the promoter and 5′-UTR of the *psbA* gene. Finally, the *Sal*I/*Not*I fusion sequence was inserted into the pLD-BADH vector, giving the pLD-CTB-2L21 vector.

The *GFP* gene was amplified by PCR using the primers 5′-GGGTCATGAGTAAAGGAGAAG-3′ and 5′-GCGGCCGCTC-CCGGGCCCTTTGTATAGTTCATCCATG-3′ and, as template, the soluble modified version of the *GFP* gene (GenBank accession no. U70495, obtained under no. CD3-326 from the Arabidopsis Biological Resource Center, The Ohio State University). The 75 bp *Sma*I/*Not*I fragment of the *2L21*, including the hinge tetrapeptide, was inserted at the 3′ end of the *GFP* gene. The 775 bp *Bsp*HI/*Not*I *GFP-2L21* fragment was fused to the promoter and 5′-UTR of the *psbA* gene. Finally, the *Sal*I/*Not*I fusion sequence was inserted into the pLD-BADH, giving the pLD-GFP-2L21 vector.

Bombardment and regeneration of chloroplast transgenic plants

Gold microprojectiles coated with plasmid DNA (pLD-2L21, pLD-CTB-2L21 or pLD-GFP-2L21) were bombarded into tobacco (*Nicotiana tabacum* var. Petit Havana) *in vitro*-grown leaves using the biolistic device PDS1000/He (Bio-Rad) as described previously (Daniell, 1997; DeGray *et al*., 2001). After bombardment, leaves were incubated in the dark for 2 days at 28 °C. Leaves were then cut into small pieces (~5 mm × 5 mm) and placed abaxial side up on selection medium

(RMOP) (Daniell, 1993) in Magenta vessels (Sigma) containing 500 mg/L spectinomycin dihydrochloride as selecting agent. The growth conditions of the culture chamber were 28 °C, 120 μmol/m^2/s and 16 h photoperiod. Resistant shoots obtained after 6–7 weeks were cut into small pieces (~2 mm × 2 mm) and subjected to a second round of selection in the same selection medium. Regenerated plants were transplanted and grown in a phytotron with the following conditions: 28 °C, 250 μmol/m^2/s, 70% relative humidity and 16 h photoperiod.

Southern and Northern blot analysis

Total plant DNA (10 μg) was digested with *Eco*RI, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. The chloroplast vector DNA digested with *Bgl*II and *Bam*HI generated a 0.8 kb probe (P1) homologous to the flanking sequences. Hybridization was performed using the chemiluminescent AlkPhos direct labelling-detection system (Amersham). After Southern blot confirmation, plants were transferred to soil. Seeds from the T_0 generation were *in vitro* germinated on spectinomycin selection medium. The T_1 seedlings were isolated and cultured for 2 weeks in Magenta vessels. Finally, plants were transferred to pots. Plants of T_0 and T_1 generations were analysed for homoplasmy.

Total RNA was extracted from leaf tissue as described by Logemann *et al*. (1987). RNA (10 μg) was separated on 2% agarose/formaldehyde gels and then transferred to a nylon membrane. The pGEM-T-2L21 vector DNA digested with *Eco*RI generated a 108 bp probe (P2) homologous to the *2L21* sequence. Hybridization was performed using the chemiluminescent detection system mentioned above.

Western blot analysis

Transformed and untransformed leaves (100 mg) from plants grown in a phytotron were ground in liquid nitrogen and resuspended in 200 μL of protein extraction buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Leaf extracts (5–10 μg as determined by Bradford assay), unboiled or boiled for 5 min with β-mercaptoethanol, were electrophoresed in a 13% polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane for immunoblotting. VP2 protein from CPV (Ingenasa, Madrid, Spain) and CTB protein (Sigma, C9903) were used as positive controls. The primary antibody to detect the 2L21 epitope (mouse anti-VP2 mAb 3C9; Ingenasa, Madrid, Spain) was used at 0.5 μg/mL, and the secondary antibody (peroxidase-conjugated rabbit anti-mouse IgG, Sigma A9044) was used at 1 : 30 000 dilution. The primary antibody to detect CTB (rabbit anti-CTB, Sigma C3062) was used at 1 : 5000 dilution, and the secondary antibody (peroxidase-conjugated goat anti-rabbit IgG, Sigma A9169) was used at 1 : 10 000. Detection was performed using the ECL Western blotting system (Amersham).

G_{M1}-ganglioside binding assay

Microtitre plates (Costar Corning) were coated with G_{M1}-ganglioside (Sigma G7641) by incubating the plate (overnight at 4 °C) with a solution of G_{M1}-ganglioside (3.0 μg/mL) in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Alternatively, some wells were coated with BSA as controls. Wells were blocked with 1% BSA in phosphate-buffered saline pH 7.4 (PBS), washed three times with PBS containing 0.1% Tween-20 (PBS-T) and incubated (overnight at 4 °C) with soluble protein from pLD-CTB-2L21-transformed plants, untransformed controls or bacterial CTB as positive control. Later, plates were incubated (2 h at 37 °C) with a 1 : 8000 dilution in 0.5% BSA of rabbit anti-CTB antibody (Sigma C3062) (100 μL/well), washed three times with PBS-T and then incubated (2 h at 37 °C) with a 1 : 50 000 dilution in 0.5% BSA of peroxidase-conjugated goat anti-rabbit IgG (Sigma A9169) (100 μL/well). The plate was incubated for 2 h at 37 °C and washed three times with PBS-T. After washing, the plates were finally incubated with ABTS (Roche 102 946) for 1 h at room temperature (RT) in the dark and read at 405 nm in a Multiskan microtitre reader plate (Labsystems).

ELISA quantification of recombinant proteins

Transformed and untransformed leaves (100 mg) from plants grown in a phytotron were ground in liquid nitrogen and resuspended in 500 μL of bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Samples were bound to a 96-well polyvinyl chloride microtitre plate (Costar Corning) overnight at 4 °C. Background was blocked with 1% (w/v) skimmed milk in PBS-T (PBS-TM) for 1 h at RT, washed three times with PBS-T and incubated with anti-VP2 mAb 3C9 (Ingenasa, Madrid, Spain) at 0.5 μg/mL in PBS-TM (1 h at RT). The wells were washed three times with PBS-T and incubated with 1 : 50 000 dilution of rabbit anti-mouse IgG-peroxidase conjugate in PBS-TM (1 h at RT). Plates were developed with ABTS (Roche 102 946). The reaction was stopped after 1 h with 1% SDS and read at 405 nm in a Multiskan microtitre reader plate (Labsystems). A standard curve to calculate the amount of recombinant protein was made by plating a synthetic 2L21 peptide (Bioworld, Ohio, USA) in the range 6–56 ng per well in 100 mM Na_2CO_3 pH 10.5. Transgenic leaf

extracts were diluted to fit in the linear range of the 2L21 standard.

Immunogenicity measurements

Female Balb/c mice (Harlan Ibérica, Barcelona, Spain) were used in this experiment. Groups of 4-week-old mice (n = 6) were immunized by i.p. injection (0.2 mL) with crude leaf extract from CTB-2L21-, GFP-2L21- and 2L21-transformed plants in Complete Freund's Adjuvant (CFA). CTB-2L21 and GFP-2L21 groups received 20 μg/mouse of 2L21 recombinant protein. The amount of TSP varied between 140 and 200 μg. The 2L21 group received 200 μg of TSP/mouse. As a control, a group of mice (n = 6) was immunized i.p. with leaf extracts from non-transformed plants (200 μg/mouse of TSP). Animals were boosted at days 21 and 35, using Incomplete Freund's Adjuvant (IFA) instead of CFA. Blood was collected from the retro-orbital plexus at days 0 and 50. Individual mice sera were titrated against 2L21 synthetic peptide (1 μg/well), VP2 protein (0.1 μg/well) and control peptide WNSTAFHQTLQDPR (amino acids 122–135 of hepatitis B virus surface antigen) (Bioworld, Ohio, USA) (1 μg/well) by an in-house ELISA described in Hervas-Stubbs *et al*. (1994). Titres were expressed as the highest serum dilution to yield twice the absorbance mean of pre-immune sera.

Acknowledgements

The authors are grateful to Ms Alicia Fernández-San Millán for collaboration at the beginning of this project and for critical reading of the manuscript. This work was supported by Grant BIO2002-02851 from the Ministerio de Ciencia y Tecnología (Spain). A.M. was a recipient of a fellowship from Departamento de Educación y Cultura (Gobierno de Navarra).

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