High-yield expression of a viral peptide vaccine in transgenic plants

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Abstract A high-yield production of a peptide vaccine in transgenic plants is described here. A 21-mer peptide, which confers protection to dogs against challenge with virulent canine parvovirus, has been expressed in transgenic plants as an amino-terminal translational fusion with the *GUS* gene. Transformants were selected on the basis of their GUS activities, showing expression levels of the recombinant protein up to 3% of the total leaf soluble protein, a production yield comparable to that obtained with the same epitope expressed by chimeric plant viruses. The immunogenicity of the plant-derived peptide was demonstrated in mice immunized either intraperitoneally or orally with transgenic plant extracts, providing the suitability of the GUS fusions approach for low-cost production of peptide vaccines.

Key words: Peptide vaccine; Canine parvovirus; Transgenic plant; Fusion protein

1. Introduction

Subunit vaccines have improved conventional attenuated or killed vaccines in many aspects including safety and production systems. However, production of recombinant vaccine proteins in expression systems is expensive, requiring large scale fermenters and stringent purification protocols. These fermentation processes for vaccine production may be a prohibitively expensive technology for developing countries or for application in animal health. The expression of foreign proteins in plants has become an attractive alternative to conventional production systems as they allow to obtain recombinant proteins in large quantities at low cost. Plant-made vaccines or antibodies are specially attractive as plants are free of human or animal diseases, reducing screening costs for viruses and bacterial toxins. Proteins produced in transgenic plants are capable of invoking protective immune responses against important pathogens as it has already been demonstrated [1–9]. In addition, plants enable oral delivery of vaccine antigens eliminating the injection-related manipulations of animals or hazards in humans. At present, vaccines for human and animal disease prevention comprise the most competitive area for plant-based production of xenogenic proteins [10].

One of the major disadvantages of using transgenic plants as bioreactors to produce vaccines is the expression levels and accumulation in plant tissues of the recombinant vaccine proteins. In contrast, chimeric plant viruses expressing peptides or complete proteins from pathogens are very efficiently accumulating the xenogenic proteins in plants [10]. Nevertheless, the production of vaccine antigens by genetically manipulated viruses requires an extra step of inoculation of the host plant with the chimeric virus and could present additional risks derived from the spread of recombinant viruses to the environment.

In the present work, we have assayed an alternative to produce high levels of a vaccine peptide fused to β-glucuronidase (GUS) protein, which shows a reduced turnover and additionally enables the rapid screening of high level expression transformants. We selected a linear antigenic peptide derived from the VP2 capsid protein of canine parvovirus (CPV). This 21-mer peptide has been shown to be effective in protection of dogs and minks against parvovirus infection [11,12]. In this report, we demonstrate that this peptide, fused to the GUS protein, was efficiently expressed and accumulated in transgenic plants, at similar levels obtained with recombinant plant viruses [13,14].

2. Materials and methods

2.1. Construction of plasmid, plant transformation and analysis of GUS activity

The DNA sequence corresponding to 2L21 epitope was obtained by a PCR-based gene assembly by overlap extension using the following oligonucleotides: (A) 5′-GCGCGGATCCATGTCTGATGGAGCTGTTCAACCAGATGGTGGTCAACCTG-3′ and (B) 5′-GCGCGGATCCTCCAGTAGCTCTCTGTTTCTAACAGCAGGTTGACCACCA-3′. Sequence of primers was adapted to the most frequent codon usage in *Arabidopsis* [15] as it is shown in Fig. 1A. An extra ATG was added to the 5′ end of 2L21 DNA to introduce an initiation codon. The annealed oligomers were cloned into the *Bam*HI site of the plasmid pUC19 and then digested with *Xba*I and *Sma*I for subcloning the 2L21 encoding DNA fragment into the binary plasmid pBI121 (Clontech, Palo Alto, CA, USA). The resulting plasmid, named pBI-2L21, contained the 2L21 encoding region fused in-frame to the 5′ end of the *GUS* gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter.

Arabidopsis plant transformation was carried out as described elsewhere [16] with slight modifications [7]. T1 plants were analyzed for the presence of the foreign DNA sequence by PCR using primers for amplification of a fragment of 1 kb from 2L21-GUS fusion gene as described [7]. Selected T1 plants were self-pollinated to obtain T2 and T3 generations. GUS activities were determined in plant tissues according to the method of Jefferson et al. [17].

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2.2. Analysis of expression of 2L21-GUS fusion protein in transformed plants

Plant soluble proteins were obtained by homogenization of leaves in a blender with liquid nitrogen, and the resulting powder was resuspended in buffer (0.3 g of fresh weight/ml) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6, 10 mM NaCl, 5 mM EDTA, 0.6% Triton X-100, 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The extract was filtered and centrifuged 10 min at $12\,000 \times g$. Plant extracts were resolved in 15% sodium dodecyl sulfate (SDS)–PAGE and transferred to nitrocellulose filters. The presence of 2L21-GUS protein was detected in Western blot using monoclonal antibody (MAb) 3C9 (Ingenasa, Madrid, Spain; [18]) at 0.1 μg/ml dilution and an anti-mouse IgG conjugated with alkaline phosphatase (Roche Diagnostics, Germany). Reactions were developed with nitro-blue tetrazolium chloride (Gibco-BRL Life Technology, Rockville, MD, USA) and bromochloroindoyl phosphate (Pierce Chemical Company, Rockford, USA). Intensity of MAb 3C9-reacting bands in Western blots of known concentrations of baculovirus-expressed CPV VP2 was compared in multiple reactions to that of 2L21-GUS protein contained in different concentrations of total soluble protein from leaf extracts. Quantification of reacting signals was carried out by using Howteck Scanmaster 3+ scanner and Bioimage 3.3 software (Millipore, USA). The average integrated densitometry values were calculated for every transgenic plant in at least three independent analyses.

2.3. Immunization of animals

Adult BALB/c mice divided in two different groups of 10 mice each were immunized intraperitoneally on days 0, 15 and 30 with leaf extracts from the highest 2L21-GUS expressing plant in complete Freund's adjuvant for the first inoculation and in incomplete adjuvant for the others. One group received 100 μg (low-dose experiment) and the other 1.2 mg (high-dose experiment) of leaf soluble proteins, containing 3.3 and 39.6 μg of recombinant 2L21-GUS fusion protein, respectively. A third group of 10 mice was immunized by oral gavage with leaf proteins extracted in 5 mM sodium phosphate buffer (pH 7.2). Doses of 200 μg of total soluble protein from leaf extracts were administered to mice three times per week and during 4 alternative weeks. Control mice were immunized by using the same protocols with extracts from transgenic plants transformed with plasmid pBI121. 15 days after the last immunization dose, mice were bled and the antibody reactivity against 2L21 peptide was analyzed.

2.4. Determination of specific antibodies in immunized mice

Baculovirus-expressed CPV VP2 forming virus-like particles (VLPs, [19]) was used as antigen source for determination of 2L21-specific antibodies. Different concentrations (0.2–4 μg) of VLPs purified as described previously [20] were resolved in a 9% SDS–PAGE and transferred to a Hybond-C membrane (Amersham Pharmacia Biotech Europe, Germany) for Western blot analyses of sera from the respective 2L21-GUS-immunized mice diluted 1/100.

Titration of specific antibodies from immunized mice by enzyme-linked immunosorbent assay (ELISA) was carried out by two indirect ELISAs. Peptide 2L21 coupled to keyhole limpet hemocyanin (KLH) or purified VLPs were used as coating antigens. Polystyrene microtiter plates (Labsystem, Finland) were coated overnight at 4°C with the peptide or VLPs at 5 μg/ml diluted in 50 mM carbonate buffer, pH 9.6 and then serial 2-fold dilutions of the respective mouse antiserum were added. After a 1 h incubation at 37°C, peroxidase-labeled anti-mouse IgG (Pierce Chemical Company, Rockford, USA) was added to each well and was incubated 1 h at room temperature. Bound enzyme was detected by adding ABTS (2,2′-azino-di[ethyl-benzothiazoline-sulfonic acid]) (Sigma-Aldrich Quimica, Madrid, Spain) as substrate and stopped after 10 min with 2% SDS. The optical density of samples was determined at 405 nm using an ELISA reader (Bio-Tek Instruments). ELISA titers were determined as $\log_2$ reciprocal serum dilution where absorbance amounted to three times the blank (preimmunization serum).

The induction of antibodies to GUS protein in immunized mice was determined by Western blot using 2.5 μg per strip of GUS from *Escherichia coli* (Boehringer Mannheim, Germany). Sera belonging to every group of immunized mice were pooled and reacted in Western blot at 1/100 dilution. For positive control of reaction an anti-GUS rabbit IgG (2 mg/ml) was used (Molecular Probes, Leiden, The Netherlands). A preimmune serum was used as negative control.

3. Results

3.1. Construction of 2L21-GUS fusion protein expression vector

To examine the GUS fusion approach in plants, we used a DNA encoding 21 amino acids corresponding to peptide 2L21, obtained by a PCR-based gene assembly, to construct the pBI-2L21 expression vector. The peptide encoding sequence was mutagenized for optimizing the codon usage in *Arabidopsis* and cloned in-frame to 5′ end of the *GUS* reporter gene in the plasmid pBI121, a diagram of which appears in Fig. 1. The recombinant pBI-2L21 plasmid enables selection of transformants on media containing kanamycin and stable integration into nuclear chromosomal plant DNA. This plasmid uses the CaMV 35S promoter to drive nominally constitutive transcription of the cloned gene.

3.2. Expression of 2L21-GUS fusion protein in transgenic plants

Transformation of *Arabidopsis* plants with pBI-2L21 was carried out by *Agrobacterium tumefaciens* infiltration. Twenty five independent kanamycin-resistant transgenic lines were obtained appearing similar in morphology to wild-type plants. All lines were positive when screened for the presence of the recombinant genes by PCR analysis (not shown). The different levels of GUS activity showed by independent transformants are illustrated in Fig. 2A. As expected, when fully expanded upper leaves from those plants were analyzed in Western blot with MAb 3C9 against 2L21 peptide, a 73 kDa immunoreactive band was detected in most plants, corresponding to the 2L21-GUS fusion protein (Fig. 2B). No immunoreactive bands could be detected in leaf extracts from plants transformed with pBI121 plasmid or in plants showing low levels of GUS activity. A good correlation between GUS

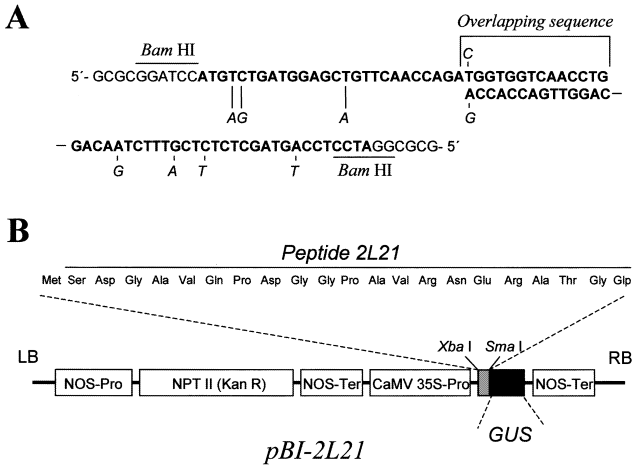

Fig. 1. Schematic structure of the binary plasmid pBI-2L21 used for *Agrobacterium*-mediated plant transformation. A represents the sequence of two overlapping oligonucleotides used for the synthesis of the DNA fragment encoding the peptide 2L21 by recombinant PCR. Nucleotides in bold represent the initiation codon and the encoding 2L21 region. Modifications introduced in the sequence of these oligonucleotides to optimize the most common codon usage in *Arabidopsis* are represented in italics. B represents the resulting plasmid pBI-2L21 after cloning of 2L21 encoding sequence downstream of the CaMV 355 promoter in plasmid pBI121. The amino acid sequence of 2L21 peptide derived from the nucleotide sequence represented in A is also shown.

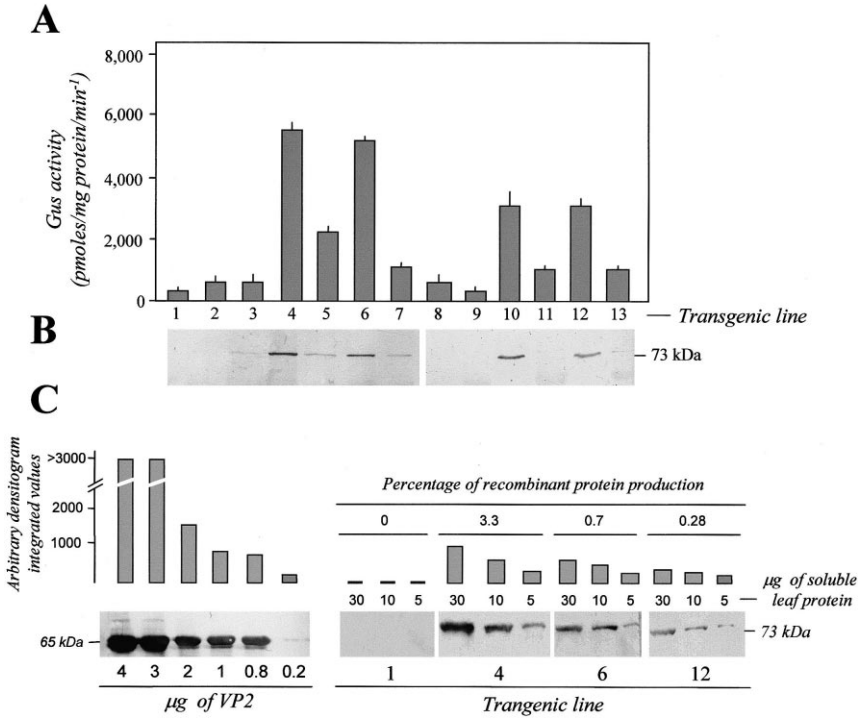

Fig. 2. Characterization of transgenic plants obtained with plasmid pBI-2L21. A represents the levels of GUS activity in transgenic T2 lines transformed with plasmid pBI121 (1) or pBI-2L21 (2–13). GUS activity of leaves in individual transgenic lines was determined and shown as produced amount of 4-methylumbelliferone per min per mg soluble leaf protein at 37°C. Bars represent the mean of GUS activity and standard deviation of different leaves from the same plant. B shows the expression of 2L21-GUS protein in leaves from the same plants, detected by Western blot using 30 μg of soluble leaf proteins and MAb 3C9. C represents the quantification of 2L21 peptide production by comparative integrated densitometry values obtained from Western blot reactions using MAb 3C9. This panel shows reactivity with the MAb of different known amounts of recombinant CPV VP2 (0.2–4 μg) and the recombinant 2L21 peptide contained in different amounts of soluble leaf proteins (5–30 μg) from several homozygous transgenic plants (1, 4, 6 and 12). The calculated percentage of recombinant fusion protein production in plants is also indicated.

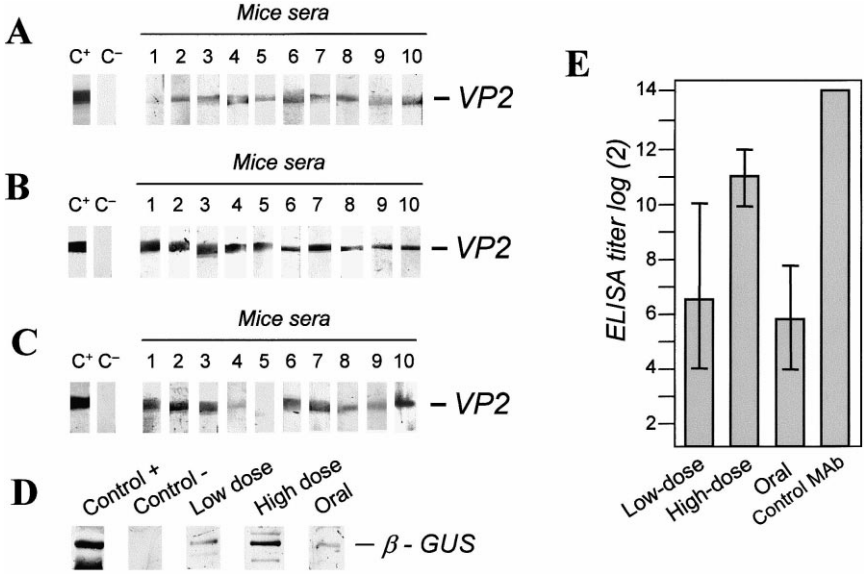

Fig. 3. Antibody responses to the plant-derived 2L21 peptide and GUS protein in immunized mice. Reactivity in Western blot against recombinant CPV VP2 of sera from mice immunized intraperitoneally with low (A) or high (B) antigen doses and orally (C). MAb 3C9 was used as positive control of reactions (C^+) and a pool of sera from mice immunized with plant extracts transformed with plasmid pBI121 was used as negative control (C^-). D shows the reactivity in Western blot against GUS protein of pools of sera from mice immunized intraperitoneally (low and high doses) and orally. A specific serum against GUS (2 mg/ml) was used as positive control and a preimmune serum was used as negative control. E represents the mean ELISA titers and standard deviations of sera from the different groups of immunized mice compared to those obtained with MAb 3C9. The MAb was used in ELISA and Western blot at a 0.1 μg/ml concentration.

activity and reactivity of fusion protein in Western blot was observed. Homozygous plants from lines showing the highest GUS activity were isolated and used in further experiments. They showed an increase of about 20% of GUS activity with respect to their parental T2 segregating transgenic lines.

Quantification of 2L21-GUS fusion protein in leaves from transgenic plants using MAb 3C9 rendered percentages of 2L21-GUS protein accumulation in the highest expressing plants between 0.15 and 3.3% of total soluble leaf protein. This represents yields of 2L21-GUS protein production of about 75 μg/g of fresh weight of transgenic plant leaves. Fig. 2C shows a representative reactivity of MAb 3C9 in Western blot of different concentrations of recombinant CPV VP2 compared to that obtained from different leaf extracts. These results were confirmed analyzing the extinction signal of reacting bands with MAb 3C9 in Western blot of serial dilutions of known amounts of CPV VP2, a synthetic 2L21 peptide coupled to KLH protein and leaf extracts (not shown). As expected from using the CaMV 35S promoter, recombinant protein was histochemically detected by GUS expression in all plant tissues (not shown).

3.3. Antibody response to plant-derived 2L21 peptide

To test the ability to stimulate production of CPV-specific antibodies, several amounts of transgenic plant extracts expressing 2L21-GUS fusion protein were used intraperitoneally or orally to immunize mice. All mice belonging to the parenterally immunized groups developed specific antibodies against 2L21 peptide that reacted in Western blot with the baculovirus-expressed CPV VP2 (Fig. 3A,B). The antibody response was quantified by ELISA using recombinant CPV VLPs (Fig. 3D) or a synthetic 2L21 peptide coupled to KLH protein (not shown), showing similar results. The specific antibody titers were proportional to the amount of recombinant fusion protein used in the immunization protocol, reaching ELISA titers of 12 for the peptide or CPV VLPs when mice were immunized with 1.2 mg of plant extract. Interestingly, most mice (nine out of 10) immunized orally with leaf soluble proteins from transgenic plants developed specific antibodies against 2L21 peptide detectable in Western blot and ELISA (Fig. 3C,D). Specific antibodies against GUS protein were also detected in immunized mice by Western blot (Fig. 3E), indicating that, as expected, the immune response was not exclusively restricted to the 2L21 peptide.

4. Discussion

Two major prerequisites for application of transgenic plant-derived vaccines to human or animal health have to be addressed. First is to determine the dosage, best delivery method and immune response type obtained with the plant-derived vaccine. Information about these aspects has been acquired with several infectious models such as *Vibrio cholerae* [6], HIV [21], *Pseudomonas aeruginosa* [22], murine hepatitis virus [23], foot and mouth disease virus [4,5], transmissible gastroenteritis virus [7,8] or *E. coli* heat labile enterotoxin [24,25] among others. Nevertheless, current information is still insufficient to generalize procedures for every vaccine antigen obtained in transgenic plants. Second is the ever-present mission of increasing the level of transgene expression within transgenic plants. While several opportunities exist to increase expression, which ones are likely to produce the best effect need to be determined on a case by case basis. For vaccine production in transgenic plants, a reduced number of alternatives to increase recombinant protein accumulation have been assayed. Good results were obtained by the use of endoplasmic reticulum retention signals [6,26], increasing the translational efficiency of RNAs with 5′ untranslated region from tobacco etch virus [2] or using a plant-optimizing synthetic gene [24]. At present, expression levels of vaccine proteins higher than 0.4% of soluble plant protein have not been reported [2] and in most cases expression levels of vaccine proteins in transgenic plants were lower than 0.1%.

Additionally to the above mentioned low expression levels of vaccine proteins found in plants, instability of short peptides in plant cells could also make difficult the production of peptide vaccines in transgenic plants. In the present work an approach has been assayed to increase a vaccine peptide expression, the 2L21 peptide from CPV, as a fusion protein with GUS, a reporter protein commonly used in plant molecular biology because of its optimal expression and accumulation. The selection of 2L21 peptide to be expressed by this strategy is because it constitutes an example of an efficient peptide vaccine [11]. Previously, this peptide was successfully expressed in two plant viruses, the cowpea mosaic virus [13] and the plum pox potyvirus [14]. This feature allows to compare the expression levels obtained by using transgenic plants versus those obtained by recombinant plant viruses. Here, we have demonstrated that expression of 2L21 peptide as a translational fusion protein with GUS compares favorably with expression levels of the same peptide obtained by recombinant plant viruses. To our knowledge, this is the first description of stable expression of a viral peptide in transgenic plants, opening the possibility of a low-cost alternative for producing peptide vaccines. Additionally, GUS fusion proteins provide a simple system for screening a high number of transgenic plants to select those with higher levels of peptide accumulation.

In general, peptides themselves evoke poor immune responses in immunized animals. In previous experiments [11,26], humoral or cellular immune responses against 2L21 peptide were detectable only when animals were immunized with the peptide coupled to KLH protein but not with the free peptide. Protein KLH not only serves as a carrier for the correct presentation of the peptide to the immune system, but also because it contains T-cell epitopes which potentiate the immune response against to the coupled peptide. In fact, all protective results obtained with peptide 2L21 in target animals were only obtained when animals received high doses of the peptide coupled to KLH protein. GUS protein seems not negatively affecting the immune response obtained in immunized mice against the 2L21 peptide, which was immunogenic by the intraperitoneal and oral route. However, the antibody immune response we obtained with the fusion protein was lower than using the same peptide coupled to KLH protein, suggesting that GUS protein does not act as a similarly efficient immunological carrier.

How the fusion of peptides to GUS protein may facilitate the recombinant protein accumulation in transgenic plants requires further investigation. Apparently, in plants transformed for expressing the GUS fusion, the specific mRNA levels, examined by Northern blot analysis using a 2L21 encoding DNA fragment as the hybridization probe, showed a good correlation with the GUS activity and protein accumu-

lation detected in the different plants (not shown). GUS fusions also could eliminate inefficient processing or premature degradation of the peptide, allowing accumulation of the fusion protein at levels up to 3% of total soluble protein. We have successfully expressed other short amino acid sequences from foot and mouth disease virus and bovine rotavirus in plants fused to GUS protein (unpublished results), suggesting that this methodology is applicable to any peptide of interest. The possibility to increase large vaccine proteins accumulation by translational fusions to GUS is currently under investigation.

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