



## Short communication

# Multimerization of peptide antigens for production of stable immunogens in transgenic plants

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## Abstract

Previous literature addressing the production of recombinant proteins in heterologous systems has consistently shown that proteins capable of forming complex structures tend to accumulate within host cells at relatively higher levels than monomeric forms. In this report, we translationally fused a 21-aminoacids long highly immunogenic peptide (2L21), derived from canine parvovirus (CPV) VP2 protein to a 41-aminoacid long tetramerization domain (TD) from the transcriptional factor p53. The chimerical DNA construction 2L21-TD was cloned in a binary plant transformation vector and used to transform *Arabidopsis thaliana* plants. Fifteen of the 25 transgenic lines obtained in the experiment showed detectable 2L21-TD RNA accumulation and from these we chose 4 to study 2L21-TD protein accumulation. Non-denaturing immunoblotting assays revealed that 2L21-TD chimeras effectively formed tetrameric complexes with yields reaching up to 12 µg/mg of soluble protein. Mice immunized by oral or intraperitoneal routes with crude protein extracts containing 2L21-TD protein were able to detect both 2L21-synthetic peptide and CPV VP2 proteins, with titers similar to those elicited by a previously reported fusion between 2L21 and the β-glucuronidase protein. These results demonstrate that multimerization directed by the small TD domain contributed to the stabilization and consequently to the accumulation of the 2L21 peptide in transgenic plants, without altering its native antigenicity and immunogenicity.

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## 1. Introduction

One of the main concerns about the production of recombinant proteins in heterologous systems regards

to the accumulation of the protein of interest. This is particularly important in proteins expressed in transgenic plants, in which problems associated with low protein yields are usually due to a combination of factors including poor protein folding and stability reduction (Ma et al., 2003; Fischer et al., 2004).

Different strategies have been described to allow the correct folding of recombinant proteins and to make them less susceptible to degradation by cellu-

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lar proteases. Among others, targeting xenoproteins to different cellular compartments (Conrad and Fiedler, 1998; Richter et al., 2000) and fusing them to other proteins that remain stable in the cellular environment (Gil et al., 2001; Dus Santos et al., 2002; Skosyrev et al., 2003) have been reported to improve yields of target proteins. Likewise, proteins which naturally tend to produce multimeric structures, such as virus-like particles formed by viral capsid proteins (Mason et al., 1996; Pérez-Filgueira et al., 2004; Gil et al., 2006; Varsani et al., 2006) or oligomers as is the case for cholera toxin and heat-labile enterotoxin from *E. coli* (Streatfield et al., 2003; Tacket, 2005), tend to accumulate to relatively high levels in plant cells.

Peptide antigens have usually been expressed in plants translationally fused to larger protein carriers (Gil et al., 2001; Dus Santos et al., 2002; Wigdorovitz et al., 2004) or to capsid proteins from plant virus-based vectors (Canizares et al., 2005). However, their expression as fusions to short polypeptides or as independent products, proved to be more difficult as is reflected by the practically total absence of publications or experimental data on this subject (Krebbers and Vandekerckhove, 1990; Sciutto et al., 2002). Here, we present a strategy to enhance peptide antigen expression through its fusion to a 41 aminoacid fragment (TD) containing the tetramerization domain from the human transcription factor p53 (Sakamoto et al., 1994). Although early works utilized this self-assembling sequence in *E. coli* for oligomerization of single-chain antibodies (scFv) designed for tumor targeting (Rheinacker et al., 1996; Willuda et al., 2001), refer to the use of this domain for peptide oligomerization has not been reported yet. Similarly, other peptide sequences have been reported to enhance accumulation of proteins within plant cells, although these studies did not include fusions to other peptides (Kogan et al., 2001; Fernández-Carneado et al., 2004).

Here, we produced transgenic *Arabidopsis thaliana* plants carrying a translational fusion between the TD and a highly immunogenic 21-mer peptide (2L21) derived from the N-terminal domain of the VP2 capsid protein from the canine parvovirus (CPV) (Casal et al., 1995). Our results demonstrated that the p53 tetramerization domain mediated oligomerization of 2L21 peptide, producing stable peptide tetramers that accumulated to more than 1% of the total protein extracted from transgenic leaves. Recombinant 2L21-

TD tetramers elicited CPV-specific humoral responses when administered in mice by intraperitoneal (i.p.) or oral routes. All together these results indicate that the native antigenicity of 2L21 was preserved in the tetramers and that recombinant 2L21 oligomers were clearly immunogenic, eliciting CPV-specific humoral responses similar to those previously reported for a fusion between 2L21 and the  $\beta$ -glucuronidase ( $\beta$ -GUS) protein (Gil et al., 2001).

## 2. Results

CPV-derived 2L21 peptide and p53TD fragment coding sequences were obtained by PCR amplification using the pBI-121 2L21-GUS (Gil et al., 2001) and pCMV-p53 (Clontech, USA) plasmids as templates, respectively. A set of four primers were designed to include appropriate restriction sites at the 5' and 3' ends of the 2L21 and p53TD sequences, allowing in frame fusion of the two fragments and further cloning into an intermediate plasmid (pGemT-easy, Promega, USA). In frame start and stop codons were also added upstream and downstream the 2L21 and p53TD sequences, respectively, and the fusion gene was cloned into the pBI-121p35S/TEV binary vector, under control of the cauliflower mosaic virus 35S promoter (CaMV35S) and tobacco etch virus enhancer leader sequence (TEVe) (Fig. 1). The resulting pBI-2L21-TD and control pBI121 (Clontech, USA) vectors were used to transform *Arabidopsis thaliana* plants (Columbia ecotype) by floral infiltration with *Agrobacterium tumefaciens* (strain C58C0) as described previously (Gomez et al., 1998). Seeds obtained after the infiltration (T0) were screened using Murashige-Skoog (MS) cell culture medium (Sigma, St. Louis, MO) containing 50 mg/l of kanamycin, and those antibiotic-resistant plants (T1) were transferred to soil two weeks after germination for further analysis. Stable integration of the 2L21-TD gene was confirmed at this stage by PCR amplification performed using primers *xho*-2L21-*F* and *kpn*-TDR and genomic DNA from transformed lines as templates (data not shown).

Twenty-five independent kanamycin-resistant transgenic lines were finally obtained appearing similar in morphology to wild-type plants. T1 plants were selected based on their 2L21-TD expression levels and sequentially self-pollinated to obtain a T3 generation

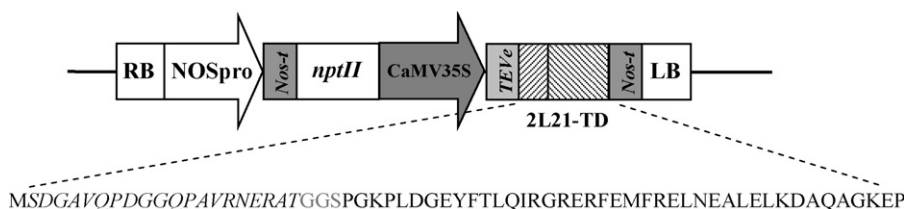


Fig. 1. Fusion of the 2L21 peptide with the p53 TD. Schematic representation of the transgene region of the pBI-2L21-TD binary vector. Fusion gene 2L21-TD was obtained by PCR as described in the text using primers: *xho-2L21-F* (5' ctgagatgtctgatggagca 3'), *sma-2L21-R* (5' cccgggtcatcctgtagctcttc 3'), *sma-TD-F* (5' cccgggcaaacactggatggag 3') and *kpn-TD-R* (5' cctaggtcacctggctctt 3'); RB and LB, right and left borders of the T-DNA; NOSpro and NOS-t, nopaline synthase gene promoter and polyadenylation signals; *nptII*, neomycin phosphotransferase gene; CaMV35S, 35S promoter from cauliflower mosaic virus and TEVe, tobacco etch virus enhancer sequence. Amino acid sequence of the 2L21-TD is depicted below, residues for 2L21 peptide are shown in italics, fragment in grey represents a linker sequence and the minimum TD sequence within the p53-derived fragment is underlined.

that was finally used to estimate recombinant protein yields and carry out immunization experiments.

Transcription the 2L21-TD gene was analyzed by Northern Blot assays performed using total RNA extracted from leaf samples from the T1 of each transgenic line ( $n = 25$ ) and pBI121-transformed plants ( $n = 2$ ). Samples were resolved in 1.2% formaldehyde-agarose gels, transferred to Hybond-N membranes (Amersham, USA) and hybridized overnight with a P32 labelled ssDNA probe complementary to the whole 2L21-TD sequence, following standard procedures (Sambrook et al., 1989). Fifteen lines presented bands of the expected size with variable levels of intensity, probably indicating different levels of transcriptional activity that might correspond to differences in transgene copy numbers and integration sites among independent transgenic lines.

A representative Northern blot experiment is shown in Fig. 2a. Four transgenic lines presenting the highest transcriptional levels for the 2L21-TD gene were further analysed for protein expression.

Expression of the 2L21-TD fusion in transgenic plants was analysed in both denaturing and native PAGE and Western blot assays. For denaturing assays, total soluble proteins were obtained by grinding fresh leaves in extraction buffer containing 50 mM Tris-HCl pH 7.4, 10 M EDTA pH 8.0, 0.1% Triton X-100, 1%  $\beta$ -mercaptoethanol and 2 mM phenylmethylsulphonylfluoride. Plant saps were centrifuged at  $12,000 \times g$  at  $4^\circ\text{C}$  during 15 min and total soluble protein (TSP) concentrations of the resulting supernatants were measured by Bradford assay (BioRad, USA). Samples containing 40  $\mu\text{g}$  of TSP were resolved in a 15%

tricine-SDS PAGE, as previously described (Schagger and von Jagow, 1987). Gels were transferred to nitrocellulose membranes and probed using the 3C9 mAb (Ingenasa, Spain) that recognizes an epitope within the 2L21 sequence (Langeveld et al., 1994). A single band with the expected electrophoretic mobility ( $\sim 7\text{--}8\text{ kDa}$ ) was readily detected in all tested plants indicating effective expression of the transgene which seemed to roughly correlate with the corresponding transcriptional levels previously registered. To test the ability of the recombinant chimeras to form oligomeric structures, native protein extracts obtained as described above but using an extraction buffer lacking EDTA and  $\beta$ -mercaptoethanol, were resolved by native 15% tricine PAGE. Immunoblotting analyses using the 3C9 mAb showed that all plant lines presented a predominant band with an electrophoretic mobility of  $\sim 28\text{--}30\text{ kDa}$  and a second band with lower intensity around 16 kDa as expected for 2L21-TD tetramers and dimers, respectively (Fig. 2c). These results indicated that most of the recombinant 2L21-TD expressed in plants was accumulated in stable tetrameric forms, with expression levels that resembled those registered in the corresponding denaturing Western blot assays. Dimeric species probably represent intermediate forms since it has been described that the native tetramers are actually constituted by dimers of two primary dimers (Sakamoto et al., 1994). In both assays, no immunoreactive bands could be detected in protein extracts from plants transformed with pBI121 plasmid. Based on these results, transgenic line #6 was selected for quantification and immunization experiments.

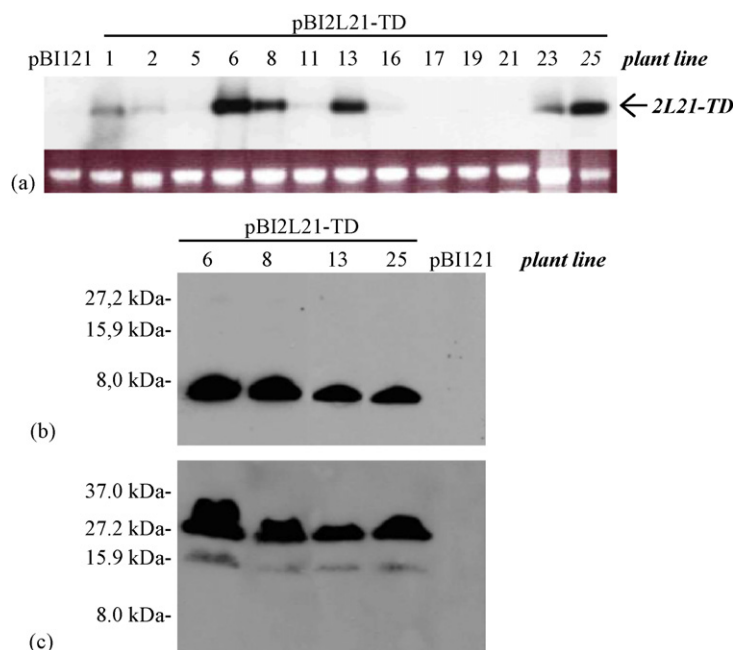


Fig. 2. Expression of the 2L21-TD polypeptide in transgenic plants. (a) Northern blot analyses for pBI-2L21-TD and pBI-121 transgenic lines. Total RNA samples (5  $\mu$ g) were resolved by agarose electrophoresis, transferred to nitrocellulose membranes and tested with a P32 labeled ssDNA probe corresponding to the whole 2L21-TD sequence. Arrow indicates the expected position for 2L21-TD transcripts and corresponding loading controls for each sample are shown below. Protein extracts from four plant lines were studied by: (b) denaturing PAGE and Western blot assays using the 3C9 mAb as detector and (c) non-denaturing PAGE and analyzed by immunoblotting using the same mAb as probe.

Western blot results presented above showed that tetrameric and monomeric forms of the fusion were immunodetected by the 3C9 mAb with approximately similar efficiency. Based on these observations, the amount of recombinant antigen produced in 2L21-TD transgenic plants was roughly estimated by coating ELISA plates with serial dilutions of a 2L21 synthetic peptide and native protein extracts from 2L21-TD transgenic line #6, and using the 3C9 mAb to detect the 2L21 peptide (data not shown). Nanograms of 2L21 synthetic peptide and micrograms of TSP from 2L21-TD transgenic leaves that produced the same ODs within a linear range (between 0.25 and 0.65 absorbance units) were determined and a correlation between them was established (correlation  $R^2 = 0.9977$ ). Using this approach we estimated that line #6 of 2L21-TD transgenic plants contained  $\sim 12$  ng of 2L21 peptide per microgram of TSP (1.2%).

The immunogenicity of the plant-produced 2L21-TD was analyzed by intraperitoneal (i.p.) and oral

immunization experiments performed in mice and quantified in terms of the induction of 2L21 and VP2-specific antibodies. For i.p. immunizations, plant saps from wild-type *Arabidopsis thaliana* and plants transgenic for pBI121, 2L21-GUS (line #4, Gil et al., 2001) or 2L21-TD (line #6) were obtained by grinding fresh leaves in phosphate buffered saline pH7.4. Saps were clarified by centrifugation ( $15,000 \times g$  for 10 m) and vaccines were formulated by emulsifying clarified saps (1 mg of TSP per dose) with complete (first dose) or incomplete (subsequent boosters) Freund's adjuvant. Six weeks old female Swiss mice were immunized at days 0, 15 and 30 and serum samples were obtained 15 days after the last booster. Orally immunized mice were placed in individual cages, deprived of food 4 h before treatment and then offered 2 g of fresh leaves sprayed with 10  $\mu$ g of the subunit B of cholera toxin (CTB) (Sigma, USA) dissolved in a 10% sucrose solution. This treatment was repeated five times during 3 weeks and serum samples were taken 10 days after the last immunization.

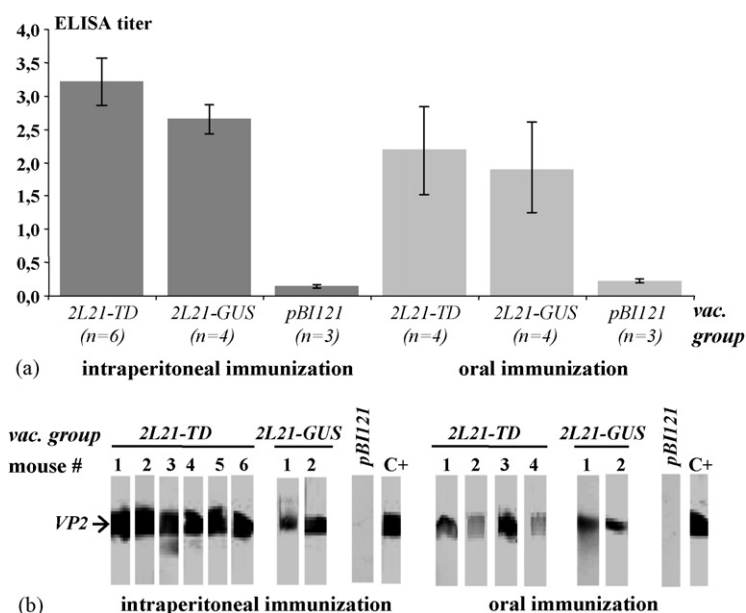


Fig. 3. Immunogenicity of the 2L21-TD antigen produced in plants. (a) Mean antibody titers obtained for each vaccination group against the 2L21 peptide; number of individuals is shown in parenthesis and standard deviations are represented as vertical lines on each bar. ELISA titers were expressed as log 10 of the reciprocal of the highest serum dilution which gave OD readings of at least twice a pool of sera from three animals immunized with extracts from wild-type plants at the same dilution. (b) Western blot assay performed on membranes carrying a recombinant VP2 from CPV expressed in baculovirus and using sera (diluted 1/100 in PBST 4% skim milk) from animals immunized with the antigens described in the text as primary antibody; (C+) positive control lanes detected using the 3C9 mAb as probe.

All serum samples were analyzed and the presence of 2L21 and VP2-specific antibodies was evaluated by ELISA and Western blot. ELISA plates were coated overnight with a synthetic 2L21 peptide (0.8 µg/well), incubated with serial dilutions of serum samples and detected essentially as described before (Pérez-Filgueira et al., 2000). As shown in Fig. 3a, all mice immunized with protein extracts from 2L21-TD transgenic plants presented significant titers of antibodies against the 2L21 peptide, regardless of the immunization route. For both immunization protocols, 2L21-specific antibody titers were similar in the 2L21-TD- and 2L21-GUS immunized groups. Animals immunized by the oral route presented lower and more variable titers than those vaccinated by the i.p. route (Fig. 3a), probably reflecting differences in the antigen doses delivered to animals due to the immunization protocol utilized. Sera from 2L21-GUS and 2L21-TD immunized animals were also able to recognize the 2L21 epitope within a recombinant VP2 protein produced in baculovirus (Lopez de Turiso et al., 1992), while control animals immunized with trans-

genic plants for pBI121 control vector did not show positive reactions in any of the immunoassays (Fig. 3a and b). All together these results suggested that the immunogenic and antigenic features of the 2L21 were preserved within the 2L21-TD chimera.

### 3. Discussion

Expression of peptide sequences in plants has been predominantly obtained by their fusion to larger protein carriers; their production as short peptide fragments is practically absent in literature. The experiments presented in this report demonstrated that a 65 aminoacid sequence (2L21-TD) comprising the 2L21 peptide fused to the p53 tetramerization domain can be efficiently expressed in transgenic plants. This fusion was able to produce stable tetramers of the target peptide and to reach expression levels above the usual yields for non-targeted proteins obtained in plants by nuclear transformation, reported to range between 0.01 and 0.4% of the TSP (Giddings, 2001).

Immunization experiments performed in mice using crude leaf extracts from 2L21-TD transgenic plants, demonstrated that the 2L21-TD tetramers were clearly immunogenic irrespectively of the immunization route. Interestingly, TD-derived tetramers were reported to remain stable up to 72 h after inoculation in mice (Willuda et al., 2001), a factor that may also play a role in achieving an efficient presentation of the antigen to the immune system. Importantly as well, the reported tertiary structure of the tetramer, with the four N termini spatially distant and projecting in different directions in the tetramers (Jeffrey et al., 1995), would provide proper spatial arrangement for epitope. In accordance with this, detection of the 2L21 and CPV VP2 using sera from 2L21-TD-immunized mice have shown that the antigenicity of the native 2L21 peptide was preserved in the recombinant product.

Recent reports presented fusions of 2L21 peptide to CTB in transplantic tobaccos specifically aimed to the development of edible vaccines (Molina et al., 2004, 2005). Despite the very high levels of expression reached with this strategy, the inclusion of highly immunogenic carrier molecules as CTB may prevent the recurring use of such immunogens since immune responses generated against the carrier would eventually affect those to the target hapten (George Chandy et al., 2006; Renjifo et al., 1998). In this regard it is important to stress that the TD is a small domain (<4.9 kDa) that derives from a cellular factor that is highly conserved in vertebrates (Walker et al., 1999) and therefore presents low antigenicity. This would make the approach introduced in this paper completely general since it might be applied to different vaccination protocols and expression systems. It would even be possible to produce constructs carrying fusions between the TD and different protein sequences within a single vector, allowing expression of hetero-tetramers that would combine different peptides or polypeptides in a single molecule.

In summary, we show that a peptide product may be multimerized by a small self-associating protein domain in the transgenic plants, having the potential to induce specific humoral responses against the target sequence through different immunization routes and without altering the native characteristics of the antigen. This approach offers particular advantages for the vaccinology field, although the structural features associated with the possibility of producing stable

recombinant tetramers may have applications in other sanitary or industrial disciplines as well.

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## References

- Canizares, M.C., Nicholson, L., Lomonosoff, G.P., 2005. Use of viral vectors for vaccine production in plants. *Immunol. Cell Biol.* 83, 263–270.
- Casal, J.I., Langeveld, J.P., Cortes, E., Schaaper, W.W., van Dijk, E., Vela, C., Kamstrup, S., Meloen, R.H., 1995. Peptide vaccine against canine parvovirus: identification of two neutralization subsites in the N terminus of VP2 and optimization of the amino acid sequence. *J. Virol.* 69, 7274–7277.
- Conrad, U., Fiedler, U., 1998. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. *Plant Mol. Biol.* 38, 101–109.
- Dus Santos, M.J., Wigdorovitz, A., Trono, K., Ríos, R.D., Franzone, P.M., Gil, F., Moreno, J., Carrillo, C., Escribano, J.M., Borca, M.V., 2002. A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. *Vaccine* 20, 1141–1147.
- Fernández-Carneado, J., Kogan, M.J., Castel, S., Giral, E., 2004. Potential peptide carriers: amphipathic proline-rich peptides derived from the N-terminal domain of gamma-zein. *Angew. Chem. Int. Ed. Engl.* 43, 1811–1814.
- Fischer, R., Stoger, E., Schillberg, S., Christou, P., Twyman, R.M., 2004. Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* 7, 152–158.
- George Chandy, A., Hultkrantz, S., Raghavan, S., Czerkinsky, C., Lebens, M., Teleme, E., Holmgren, J., 2006. Oral tolerance induction by mucosal administration of cholera toxin B-coupled antigen involves T-cell proliferation in vivo and is not affected by depletion of CD25+ T cells. *Immunology* 118, 311–320.
- Giddings, G., 2001. Transgenic plants as protein factories. *Curr. Opin. Biotechnol.* 12, 450–454.
- Gil, F., Brun, A., Wigdorovitz, A., Catala, R., Martínez-Torrecuadrada, J.L., Casal, I., Salinas, J., Borca, M.V., Escribano, J.M., 2001. High-yield expression of a viral peptide vaccine in transgenic plants. *FEBS Lett.* 488, 13–17.
- Gil, F., Titarenko, E., Terrada, E., Arcalis, E., Escribano, J.M., 2006. Successful oral prime-immunization with VP60 from rabbit haemorrhagic disease virus produced in transgenic plants using different fusion strategies. *Plant Biotechnol. J.* 4, 135–143.
- Gomez, N., Carrillo, C., Salinas, J., Parra, F., Borca, M.V., Escribano, J.M., 1998. Expression of immunogenic glycoprotein S polypep-

- tides from transmissible gastroenteritis coronavirus in transgenic plants. *Virology* 249, 352–358.
- Jeffrey, P.D., Gorina, S., Pavletich, N.P., 1995. Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. *Science* 267, 1498–1502.
- Krebbes, E., Vandekerckhove, J., 1990. Production of peptides in plant seeds. *Trends Biotechnol.* 8, 1–3.
- Kogan, M.J., Dalcol, I., Gorostiza, P., Lopez-Iglesias, C., Pons, M., Sanz, F., Ludevid, D., Giralt, E., 2001. Self-assembly of the amphipathic helix (VHLPPP)8. A mechanism for zein protein body formation. *J. Mol. Biol.* 312, 907–913.
- Langeveld, J.P., Casal, J.I., Osterhaus, A.D., Cortes, E., de Swart, R., Vela, C., Dalsgaard, K., Puijk, W.C., Schaaper, W.M., Melen, R.H., 1994. First peptide vaccine providing protection against viral infection in the target animal: studies of canine parvovirus in dogs. *J. Virol.* 68, 4506–4513.
- Lopez de Turiso, J.A., Cortes, E., Martinez, C., Ruiz de Ybanez, R., Simarro, I., Vela, C., Casal, I., 1992. Recombinant vaccine for canine parvovirus in dogs. *J. Virol.* 66, 2748–2753.
- Ma, J.K., Drake, P.M., Christou, P., 2003. The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* 4, 794–805.
- Mason, H.S., Ball, J.M., Shi, J.J., Jiang, X., Estes, M.K., Arntzen, C.J., 1996. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci. U.S.A.* 93, 5335–5340.
- Molina, A., Hervas-Stubbs, S., Daniell, H., Mingo-Castel, A., Veramendi, J., 2004. High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. *Plant Biotechnol. J.* 2, 141–153.
- Molina, A., Veramendi, J., Hervas-Stubbs, S., 2005. Induction of neutralizing antibodies by a tobacco chloroplast-derived vaccine based on a B cell epitope from canine parvovirus. *Virology* 342, 266–275.
- Pérez-Filgueira, M., Wigdorovitz, A., Romera, A., Zamorano, P., Borca, M.V., Sadr, A.M., 2000. Detection and characterization of functional T-cell epitopes on the structural proteins VP2, VP3, and VP4 of foot and mouth disease virus O1 Campos. *Virology* 271, 234–239.
- Pérez-Filgueira, D.M., Brayfield, B.P., Phiri, S., Borca, M.V., Wood, C., Morris, T.J., 2004. Preserved antigenicity of HIV-1 p24 produced and purified in high yields from plants inoculated with a tobacco mosaic virus (TMV)-derived vector. *J. Virol. Methods* 121, 201–208.
- Renjifo, X., Wolf, S., Pastoret, P.P., Bazin, H., Urbain, J., Leo, O., Moser, M., 1998. Carrier-induced, hapten-specific suppression: a problem of antigen presentation? *J. Immunol.* 161, 702–706.
- Rheinhecker, M., Hardt, C., Ilag, L.L., Kufer, P., Gruber, R., Hoess, A., Lupas, A., Rottenberger, C., Pluckthun, A., Pack, P., 1996. Multivalent antibody fragments with high functional affinity for a tumor-associated carbohydrate antigen. *J. Immunol.* 157, 2989–2997.
- Richter, L.J., Thanavala, Y., Arntzen, C.J., Mason, H.S., 2000. Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat. Biotechnol.* 18, 1167–1171.
- Sakamoto, H., Lewis, M.S., Kodama, H., Appella, E., Sakaguchi, K., 1994. Specific sequences from the carboxyl terminus of human p53 gene product form anti-parallel tetramers in solution. *Proc. Natl. Acad. Sci. U.S.A.* 91, 8974–8978.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schagger, H., von Jagow, G., 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368–379.
- Sciutto, E., Fragoso, G., Manoutcharian, K., Gevorgian, G., Rosas-Salgado, G., Hernández-González, M., Herrera-Estrella, L., Cabrera-Ponce, J., López-Casillas, F., González-Bonilla, C., Santiago-Machuca, A., Ruiz-Pérez, F., Sánchez, J., Goldbaum, F., Aluja, A., Larralde, C., 2002. New approaches to improve a peptide vaccine against porcine *Taenia solium* cysticercosis. *Arch. Med. Res.* 33, 371–378.
- Skosyrev, V.S., Rudenko, N.V., Yakhnin, A.V., Zagranichny, V.E., Popova, L.I., Zakharov, M.V., Gorokhovatsky, A.Y., Vinokurov, L.M., 2003. EGFP as a fusion partner for the expression and organic extraction of small polypeptides. *Protein Expr. Purif.* 27, 55–62.
- Streitfield, S.J., Lane, J.R., Brooks, C.A., Barker, D.K., Poage, M.L., Mayor, J.M., Lamphear, B.J., Drees, C.F., Jilka, J.M., Hood, E.E., Howard, J.A., 2003. Corn as a production system for human and animal vaccines. *Vaccine* 21, 812–815.
- Tacket, C.O., 2005. Plant-derived vaccines against diarrheal diseases. *Vaccine* 23, 1866–1869.
- Varsani, A., Williamson, A.L., Stewart, D., Rybicki, E.P., 2006. Transient expression of Human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector. *Virus Res.* 120, 91–96.
- Walker, D.R., Bond, J.P., Tarone, R.E., Harris, C.C., Makalowski, W., Boguski, M.S., Greenblatt, M.S., 1999. Evolutionary conservation and somatic mutation hotspot maps of p53: correlation with p53 protein structural and functional features. *Oncogene* 18, 211–218.
- Wigdorovitz, A., Mozgova, M., Santos, M.J., Parreño, V., Gomez, C., Pérez-Filgueira, D.M., Trono, K.G., Rios, R.D., Franzoni, P.M., Fernandez, F., Carrillo, C., Babiuk, L.A., Escibano, J.M., Borca, M.V., 2004. Protective lactogenic immunity conferred by an edible peptide vaccine to bovine rotavirus produced in transgenic plants. *J. Gen. Virol.* 85, 1825–1832.
- Willuda, J., Kubetzko, S., Waibel, R., Schubiger, P.A., Zangemeister-Wittke, U., Pluckthun, A., 2001. Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) miniantibodies multimerized by self-associating peptides. *J. Biol. Chem.* 276, 14385–14392.