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Short communication

Stable production of peptide antigens in transgenic tobacco chloroplasts by fusion to the p53 tetramerisation domain

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Abstract

The production of short peptides as single molecules in recombinant systems is often limited by the low stability of the foreign peptide. In the plant expression system this problem has been solved by translational fusions to recombinant proteins that are highly stable or are able to form complex structures. Previously, we demonstrated that the highly immunogenic 21 amino acid peptide 2L21, which is derived from the canine parvovirus (CPV) VP2 protein, did not accumulate in transgenic tobacco chloroplasts. In this report, we translationally fused the 2L21 peptide to the 42 amino acid tetramerisation domain (TD) from the human transcription factor p53. The chimaeric 2L21-TD protein was expressed in tobacco chloroplasts. Leaves accumulated high levels of the recombinant protein (up to 0.4 mg/g fresh weight of leaf material, equivalent to ~6% of total soluble protein; 2% considering only the 2L21 peptide). The 2L21-TD protein was able to form tetramers in the stroma of the chloroplast. Mice immunised intraperitoneally with partially purified leaf extracts containing the 2L21-TD protein developed specific antibodies with titres similar to those elicited by a previously reported fusion between 2L21 and the B subunit of the cholera toxin. Mouse sera were able to detect both the 2L21 synthetic peptide and the CPV VP2 protein, showing that the antigenicity of the 2L21 epitope was preserved in the chimaeric protein. These results demonstrate that the p53 TD can be used as a carrier molecule for the accumulation of short peptides (such as 2L21) in the chloroplast without altering the immunogenic properties of the peptide.

Introduction

One of the problems related to foreign protein production in nuclear transgenic plants is often the low accumulation of the protein of interest. Yields depend on both the synthesis and stability of xenoproteins. The usual strategy to improve the yield of recombinant proteins is to maximise the efficiency of gene expression and protein stability by targeting the protein to specific subcellular compartments and selecting the appropriate tissue type (Fischer et al. 2004; Streatfield 2007; Twyman et al. 2003).

Plastid transformation is an alternative method to increase foreign protein yield. Recently, a phage lytic protein for antibiotic use was expressed in tobacco chloroplasts at very high levels (more than 70% of the total soluble protein, TSP) (Oey et al. 2009a). This shows the enormous capacity of the chloroplast to produce and accumulate recombinant proteins.

The expression of peptides as independent products in transgenic plants seems to be difficult, probably due to low stability of the foreign peptide (Düring 2001; Florack et al.1995; Molina et al. 2004). This problem has been circumvented by translational fusions to recombinant proteins. Fusion of a peptide antigen to a carrier protein that has been shown to accumulate in plants can stabilise the target peptide. Translational fusions to GUS (Dus Santos et al. 2002; Gil et al. 2001) or to proteins that naturally produce multimeric structures have been proven to be effective for the expression of peptide antigens. For example, the fusion of a tuberculosis antigen to the B subunit of the *Escherichia coli* heat-labile enterotoxin (LTB), which self-assembles as a pentamer (Rigano et al. 2004), and the fusion of amino acids 21-47 of the hepatocyte receptor-binding presurface 1 region with the hepatitis B virus surface antigen, which is able to form virus-like particles (Qian et al. 2008), follow this strategy.

As an alternative to large protein carriers, the 42 amino acid fragment containing the tetramerisation domain (TD) of the human transcription factor p53 (Sakamoto et al. 1994) was fused to a highly immunogenic peptide antigen (2L21) derived from the canine parvovirus (CPV) VP2 protein (Gil et al. 2007). Nuclear transgenic Arabidopsis plants produced stable peptide tetramers that accumulated to more than 1% of TSP and elicited specific humoral responses in mice. In a previous work, we generated transplastomic tobacco plants with a nucleotide sequence encoding the 21 amino acid 2L21 peptide antigen (Molina et al. 2004). Despite the presence of the specific mRNA, the peptide did not accumulate in tobacco chloroplasts. Here, we produced transplastomic tobacco plants carrying a translational fusion between the TD and the 2L21 peptide to analyse the validity of this fusion strategy in the chloroplast. Our results show that the p53 TD mediated tetramerisation of the 2L21 peptide, and that the recombinant protein accumulated to up to 6% of TSP in young leaves. Chimaeric 2L21-TD tetramers elicited the production of specific antibodies able to recognize the VP2 protein of CPV after mouse immunisation.

Results and discussion

The DNA sequence corresponding to the 2L21 epitope (22 amino acids, including the initial methionine), fused in frame to the sequence of the p53 TD (42 amino acids), was inserted in the pAF chloroplast expression vector (Fernandez-San Millan et al. 2008), giving the pAF-2L21-TD vector (Figure 1a). The recombinant protein included a carboxy terminal 6xHis-tag.

Chloroplast transformation of tobacco (*Nicotiana tabacum* var. Petite Havana) was performed by particle bombardment of *in vitro*-grown leaves with gold microparticles coated with plasmid pAF-2L21-TD as described previously (Daniell 1997). Stable integration of the foreign genes into the plastid genome of regenerated plants was confirmed by PCR by using the primer pair 3P/3M (Figure 1a). The expected 1.65 kb PCR product was obtained in all the transplastomic plants analysed but was absent in the wild-type plant (Figure 1b). Confirmed PCR transformants were subjected to Southern blot analysis. The 0.81 kb probe, homologous to the flanking regions *trnI* and *trnA* (Figure 1a), was used to check homoplasmy (all the plastid genomes in a single cell and all the cells of the plant were transformed). Transplastomic plants produced two fragments (5.21 and 1.13 kb), whereas the wild-type plant produced a single fragment of 4.47 kb (Figure 1a). Plants #2, #3 and #6 were homoplasmic, and the rest were heteroplasmic (presence of both transformed and wild-type plastid genomes) (Figure 1c).

The detection of the chimaeric polypeptide in transplastomic plants was performed with the monoclonal antibody (mAb) 3C9 (specific for the 2L21 epitope; Ingenasa, Spain). Total soluble protein was obtained by grinding leaves in phosphate-buffered saline (PBS), pH 7.4 (1:1 w/v). After centrifugation, the supernatant was the soluble fraction and the pellet was resuspended in 3 volumes (w/v) of Laemmli buffer (Laemmli 1970). After boiling and centrifugation, the new supernatant was considered the insoluble fraction. As can be observed in Figure 2a, the soluble and insoluble fractions of the three homoplasmic plants showed a clear signal of the expected size (8 kDa) for the monomeric 2L21-TD. No signal was detected in protein extracts from plants transformed with the nucleotide sequence encoding the 2L21 peptide as an independent sequence (Figure 2a). Despite the denaturing conditions, bands corresponding to oligomeric structures were also detected. The CPV VP2 protein was used as a positive control. There was no cross-reaction between mAb 3C9 and wild-type tobacco proteins. Similar expression levels of 2L21-TD were observed in the different plants. Most of the protein was present in the soluble fraction. Line #2 was selected and self-pollinated to obtain the T_1 generation. Leaf material from these plants was used for further experiments.

The formation of oligomeric structures was tested with unboiled soluble protein extracts (obtained as described above and loaded in the gel with buffer lacking SDS and b-mercaptoethanol) that were resolved by 12% PAGE (including tricine). Western blot analysis showed the presence of a band with an electrophoretic mobility of ~32 kDa, as was expected for the 2L21-TD tetramer (Figure 2b). A boiled sample with loading buffer including SDS and b-mercaptoethanol showed a predominant band of 8 kDa, corresponding to the monomer, and faint bands of the trimer, tetramer and higher oligomeric structures. This result demonstrates the

capacity of the chloroplast to assemble tetrameric forms of the chimaeric 2L21-TD protein. The formation of oligomeric structures in the stroma of tobacco chloroplasts was previously shown for the cholera toxin B subunit (CTB) (Daniell et al. 2001) and the LTB protein (Kang et al. 2003).

Accumulation of foreign proteins levels above 1-2% of TSP can usually be directly visualized on Coomassie blue-stained polyacrilamide gels (Oey et al 2009a). To determine whether the 2L21-TD accumulates to high levels in the stroma of our transplastomic plants, soluble protein extracts were separated by PAGE and stained with Coomassie brilliant blue. An 8 kDa band corresponding to the expected size of the 2L21-TD protein was observed in the samples of the transplastomic plant but was absent in the wild-type plant (Figure 2c). This result confirms the high levels of 2L21-TD protein accumulation in tobacco chloroplasts.

The chloroplast protein biosynthesis is highly active in young leaves but successively declines with leaf age. This feature is frequently used as indicator of foreign protein stability. Relatively unstable proteins show a decline in protein accumulation with leaf age (Birch-Machin et al 2004; McCabe et al 2008). By contrast, stable proteins do not show this decline (Oey et al 2009a,b). To test the stability of the 2L21-TD protein, young, mature and old leaves (12, 8 and 3 leaf number, respectively, counted from the bottom of the plant) from 2L21-TD transplastomic plants grown for 60 days in a phytotron were used for TSP extraction. The TSP content of young, mature and old leaves were 12.9, 4.1 and 1.2 mg/g of fresh weight, respectively. When equal amounts of TSP were separated by SDS-PAGE and Coomassie blue-stained, no age-dependent decline in 2L21-TD was detectable (Figure 2c). This result suggests that the 2L21-TD protein is stable in the stroma of the chloroplast.

For biotechnological applications it is important to quantify the yield of recombinant protein per plant and the relative contributions of young, mature and old leaves. Enzyme-linked immunosorbent assay (ELISA) was performed for quantification of the 2L21-TD protein. The ELISA protocol includes the mAb 3C9 as the primary antibody and has been described elsewhere (Molina et al. 2004). Young leaves showed the highest level of 2L21-TD, 0.4 mg/g fresh weight of leaf material, equivalent to ~6% TSP (2% considering only the 2L21 peptide) (Figure 2d). The amount of 2L21-TD in mature leaves was slightly lower, and the levels in old senescent leaves were 2-fold lower than that in young leaves. According to the average number of leaves per plant and their fresh weight, approximately 30 mg of 2L21-TD can be extracted from a single plant. As expected, accumulation of the 2L21-TD protein in tobacco chloroplasts was much higher than in nuclear transformants of Arabidopsis (Gil et al. 2007). The pattern of lower levels of chimaeric proteins (relative to leaf fresh weight) in chloroplasts of old leaves has been previously described (Farran et al 2008; Molina et al. 2004; Scotti et al 2009). It has been shown that the decrease in recombinant protein accumulation according to the age of the leaf is parallel to the decrease in TSP content (Stevens et al 2000).

These experiments demonstrate that the p53 TD could be expressed (as a fusion molecule to the 2L21 peptide) in tobacco chloroplasts and had the ability to form tetramers in the stroma. In addition, the use of the TD as a carrier molecule allowed the accumulation of the short 2L21

peptide in the chloroplast. As mentioned previously, the expression of short peptides as single molecules in recombinant systems, and specifically in transgenic plants, is difficult (Düring 2001). For example, the antibacterial cecropin peptide was never detected in nuclear transgenic plants in one study (Florack et al. 1995), and transplastomic plants carrying the *2L21* sequence did not accumulate the corresponding peptide despite the presence of its specific mRNA (Molina et al. 2004). An exception is the expression of a 22-amino acid antimicrobial peptide in tobacco chloroplasts (DeGray et al. 2001). Although quantification was not performed, *in vitro* and *in planta* functional assays confirmed that this peptide was correctly expressed. The 2L21 peptide is also 22 amino acids long, but it was not detected in tobacco chloroplasts. Differences in the amino acid sequence and in the estimated charge could explain the different stability of these peptides in the chloroplast. The antimicrobial peptide studied by DeGray et al. (2001) has an estimated charge of +6 at pH 7 (normal stromal pH in the dark), while the estimated charge of the 2L21 peptide is –1.

The immunogenicity of chloroplast-derived 2L21-TD was analysed by intraperitoneal immunisation of female Balb/c mice and subsequent quantification of the induction of 2L21 and VP2-specific antibodies. Soluble protein extracts of leaves from transformed and non-transformed plants were partially purified with 40-80% ammonium sulphate precipitation. Precipitated proteins were dialysed against PBS and antigens quantified by ELISA (Molina et al. 2004). The parenteral immunisation protocol was performed as previously described (Molina et al. 2005). Briefly, antigen-enriched leaf extracts (29% purity; 20 μg 2L21-TD/dose and 70 μg total protein/dose) were administered with complete (first dose) or incomplete (second and third doses) Freund's adjuvant. Blood was collected from the retro-orbital plexus at days 0 and 50. All serum samples were evaluated for the presence of 2L21 and VP2-specific antibodies by ELISA and dot blot. Titration of sera against the 2L21 epitope was carried out by an in-house ELISA test as described previously (Molina et al. 2004). As a positive control, a group of mice was immunised with a plant-derived CTB protein fused to the 2L21 peptide (CTB-2L21; 20 μg/dose) (Molina et al. 2004). All mice immunised with protein extracts from 2L21-TD or CTB-2L21 transplastomic plants showed high antibody titres against the 2L21 peptide (Figure 3a). No specific antibodies (titre <10) were detected in the group immunised with protein extracts from the wild-type plant. In addition, sera from 2L21-TD- and CTB-2L21-immunised mice were able to recognise the recombinant VP2 protein produced in insect cells infected with baculovirus (Lopez de Turiso et al. 1992) (Figure 3b). This showed that the antigenicity of the 2L21 epitope was preserved in the 2L21-TD chimaeric product. Pre-immune sera and sera from control animals immunised with protein extracts from wild-type plants did not show positive reactions in the dot blot (Figure 3b).

These immunisation experiments demonstrated that the 2L21-TD tetramers were at least as immunogenic as the CTB-2L21 pentamers. There are three reasons that favour the use of the p53 TD as an epitope carrier. First, the tertiary structure of the tetramer, as analysed by crystallography (Jeffrey et al. 1995) and nuclear magnetic resonance spectroscopy (Clore et al. 1994), showed that the four N termini are equidistant and projected to the outer core of the

tetramer. This would facilitate proper arrangement of the epitope and favour its presentation to the immune system. Second, the TD is highly stable. It has been reported that TD-derived miniantibodies can remain stable for at least 72 h after inoculation in mice (Willuda et al. 2001). Third, despite the higher accumulation of CTB-2L21 (7.49 mg/g FW) (Molina et al. 2004) as compared to 2L21-TD (0.4 mg/g FW) in tobacco chloroplasts, CTB is a highly immunogenic protein. The recurrent use of CTB as a carrier molecule can induce tolerance and selectively suppress the anti-hapten antibody response (George Chandy et al. 2006; Renjifo et al. 1998; Schutze et al. 1989). As a consequence, CTB would not make a good general carrier protein for different antigens. On the other hand, the TD is a small domain (42 amino acids) derived from the p53 tumour suppressor protein that is highly conserved throughout vertebrate evolution (Walker et al. 1999). Therefore, it is expected to present low antigenicity in humans. These characteristics make the p53 TD a good candidate to be used as a general epitope carrier for applications in multiple vaccination protocols.

In conclusion, the results presented in this paper demonstrate that the 64 amino acid sequence (2L21-TD) comprising the 2L21 peptide fused to the p53 tetramerisation domain can be efficiently expressed in tobacco chloroplasts, and that this chimaeric fusion is able to form tetramers that favour protein accumulation. This approach offers an easy and convenient way to express short peptides within the chloroplast.

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