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Transgenic chloroplasts are efficient sites for high-yield production of the vaccinia virus envelope protein A27L in plant cells†

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Summary

Orthopoxviruses (OPVs) have recently received increasing attention because of their potential use in bioterrorism and the occurrence of zoonotic OPV outbreaks, highlighting the need for the development of safe and cost-effective vaccines against smallpox and related viruses. In this respect, the production of subunit protein-based vaccines in transgenic plants is an attractive approach. For this purpose, the A27L immunogenic protein of vaccinia virus was expressed in tobacco using stable transformation of the nuclear or plastid genome. The vaccinia virus protein was expressed in the stroma of transplastomic plants in soluble form and accumulated to about 18% of total soluble protein (equivalent to approximately 1.7 mg/g fresh weight). This level of A27L accumulation was 500-fold higher than that in nuclear transformed plants, and did not decline during leaf development. Transplastomic plants showed a partial reduction in growth and were chlorotic, but reached maturity and set fertile seeds. Analysis by immunofluorescence microscopy indicated altered chlorophyll distribution. Chloroplast-synthesized A27L formed oligomers, suggesting correct folding and quaternary structure, and was recognized by serum from a patient recently infected by a zoonotic OPV. Taken together, these results demonstrate that chloroplasts are an attractive production vehicle for the expression of OPV subunit vaccines.

Keywords: A27L, chloroplast transformation, plant vaccine, smallpox vaccine, tobacco, transgenic plants.

Introduction

The occurrence of zoonotic orthopoxvirus (OPV) outbreaks in Africa and the USA and the potential use of these viruses in bioterrorism have led to increased attention in the development of new-generation vaccines against smallpox and related viruses (Heraud *et al*., 2006; Wiser *et al*., 2007). The existing smallpox vaccine, i.e. scarification with vaccinia virus (VACV), is extremely effective, but has a high reactogenicity with cardiac adverse events and a wide range of contraindications (Wiser *et al*., 2007). Its stockpiles are also low, as smallpox vaccine production ceased in the 1980s. Because antibodies to VACV appear to be the main mediators of protection, it may be possible to avoid the use of a live, attenuated or not, virus by developing a safe subunit protein-based vaccine that elicits a protective antibody response. Of the 200 genes included in the vaccinia genome, only five encode proteins that are known to elicit a neutralizing antibody response: *H5R, A27L, B5R, D8L* and *L1R* (Hooper *et al*., 2000). The VACV *A27L* gene encodes a 14-kDa polypeptide that assembles into homotrimers and is localized on the virus envelope. A27L is required for the formation of the intracellular enveloped virus (IEV), and is involved in VACV assembly and virus penetration by fusion of the virus

envelope with the cell membrane. The viral A27L protein is also involved in cell–cell fusion, plaque size and virus attachment to cells because of its ability to bind heparin sulphate (Lai *et al*., 1991; Ho *et al*., 2005). These important roles make A27L a good component of a smallpox subunit vaccine. Lai *et al*. (1991) have demonstrated that vaccination with the *Escherichia coli*-expressed A27L elicits neutralizing antibodies and protects mice against a lethal challenge with vaccinia. Demkowicz *et al*. (1992) have confirmed that *E. coli*-expressed A27L completely protects mice, also eliciting cellular proliferative immune responses.

Transgenic plants are an attractive alternative to conventional systems, such as bacteria, yeast, animal and insect cell cultures, for the production of recombinant antigens that can be used as subunit vaccines. Transgenic plants have low raw material cost, enable rapid scale-up capacity and possess minimal risks of contamination with animal pathogens. There have been many reports of biopharmaceutical expression in plants and these proteins have a diverse range of applications (Walmsley and Arntzen, 2003), but the expression levels of these plant-derived proteins need to be relatively high [above 1% of total soluble protein (TSP)] before the full potential of this technology can be reached and commercial production accomplished. To date, the majority of plant-derived pharmaceuticals have been produced by nuclear transformation; however, sites of integration into host DNA vary among transformed lines, expressed sequences can be subjected to gene silencing and foreign proteins are often rapidly degraded in plant tissues (Benchabane *et al*., 2008). Thus, the average protein accumulation levels are unsatisfactory in many cases (0.01–0.4% TSP).

A promising strategy for the plant-based expression of biopharmaceuticals is to integrate transgenes into plastid genomes. Chloroplast transformation offers several advantages over nuclear transformation, including uniform transgene expression rates, no gene silencing and gene/product containment. The chloroplast stroma also allows post-translational modifications, such as oligomerization and disulphide bond formation (Staub *et al*., 2000; Daniell *et al*., 2001; Fernandez-San Millan *et al*., 2003; Bally *et al*., 2008; Benchabane *et al*., 2008). The high copy number (about 10 000 copies) of the chloroplast genome in each plant cell and the increased stability of many proteins in the stroma compared with the cytoplasm constitute further advantages for protein accumulation (Molina *et al*., 2004; Bock, 2007). Since the first vaccine was produced in transgenic plastids (Daniell *et al*., 2001), a number of antigenic proteins have been expressed via chloroplast genetic engineering (Daniell, 2006; Bock, 2007), including vaccine antigens against biosecurity-related organisms, such as the *Bacillus anthracis* protective antigen (Koya *et al*., 2005) and the F1-V *Yersinia pestis* antigen (Arlen *et al*., 2008). It has been calculated that 1 acre of chloroplast transgenic plants can produce up to 360 million doses of a purified anthrax vaccine antigen (Koya *et al*., 2005). Furthermore, immunological studies in animal models have shown that the chloroplast-produced antigenic proteins are active and elicit a protective immune response (Tregoning *et al*., 2003; Koya *et al*., 2005; Ruhlman *et al*., 2007; Arlen *et al*., 2008).

However, not all proteins can be expressed successfully in plastids and, in order to find the best approach to produce foreign proteins in plants, studies comparing different subcellular compartments for protein yield, correct processing and assembly are necessary. Although, in several instances, it has been demonstrated that the level of foreign protein accumulation in transplastomic plants is 100-fold higher than that reached by expression via integration into the nuclear genome (Staub *et al*., 2000; Fernandez-San Millan *et al*., 2003; Leelavathi and Reddy, 2003; Zhou *et al*., 2008), few investigations have directly compared the two approaches using the same target molecule, plant genotype and growth conditions.

In this study, we investigated the feasibility of producing the VACV A27L protein in plant cells. We first performed transient expression of A27L in tobacco protoplasts. Having verified the suitability for plant expression, we stably expressed the VACV protein in tobacco plants transformed by *Agrobacterium*-mediated transformation of the nuclear genome and by biolistic transformation of the plastome. The 14-kDa protein of VACV was produced in large amounts in tobacco chloroplasts. The chloroplast-produced A27L polypeptide was similar, with regard to several criteria, to the native protein found in the virus particle and in VACV-infected cells.

Results

Transient expression

To test the feasibility of producing A27L in plant cells, we first performed transient expression of A27L in protoplasts prepared from tobacco leaves. A sequence encoding the FLAG epitope (DYKDDDDK) was added at the C-terminus of the *A27L* coding sequence for immunodetection. The construct (termed *A27L-FLAG*) was cloned into the vector pDHA for transient expression under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Tabe *et al*., 1995). Transfected protoplasts were subjected to 1 h of pulse labelling with a mixture of ^{35}S-labelled methionine and

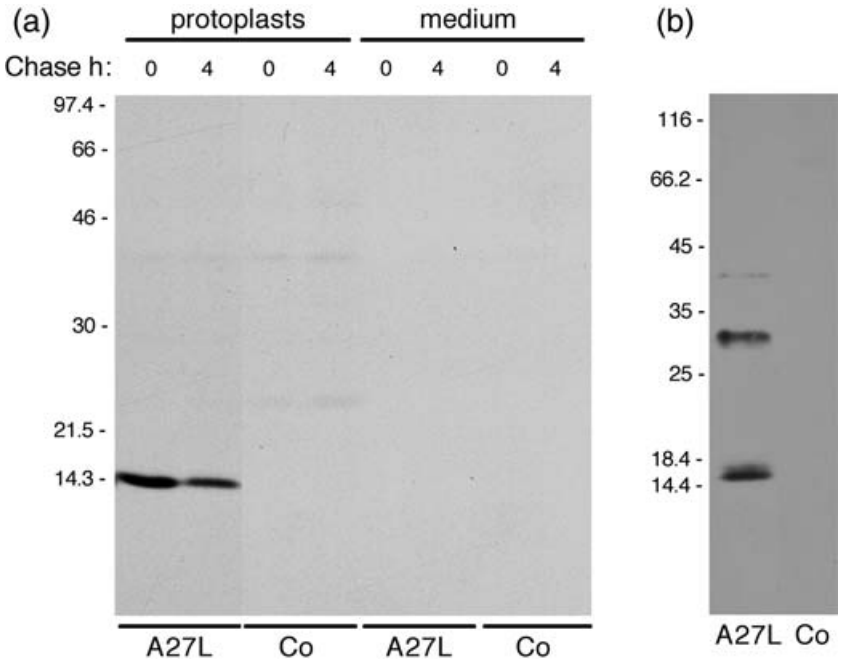

Figure 1 Expression studies by pulse chase (a) and Western blot (b) in transiently transformed protoplasts. These were isolated from young leaves of wild-type tobacco plants and plants transfected with either pDHA-*A27L-FLAG* (A27L) or empty pDHA plasmid (Co). (a) After overnight incubation, protoplasts were pulse labelled with [^{35}S]methionine and [^{35}S]cysteine for 1 h and subjected to chase for 0 or 4 h. Proteins were immunoselected from protoplast homogenates (protoplasts) or incubation media (medium) using anti-FLAG antiserum. Analysis was by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. (b) After transfection and overnight incubation, protoplasts were collected and homogenized. Total proteins were analysed by SDS-PAGE and protein blot using anti-FLAG antiserum. In both panels, the numbers on the left indicate the positions of the molecular mass markers in kilodaltons.

cysteine, followed by a 0- or 4-h chase, to determine the electrophoretic banding pattern and stability of the recombinant protein. After pulse labelling, anti-FLAG antibodies immunoselected, from protoplast homogenates, a ^{35}S-labelled protein with a molecular mass slightly larger than 14 kDa (Figure 1a): this is the expected apparent molecular mass for A27L (Vazquez *et al*., 1998). The 14-kDa polypeptide was not synthesized in protoplasts transfected with the empty plasmid as a control (Figure 1a). Band intensities indicated that the amount of newly synthesized A27L decreased by about 50% between the 0- and 4-h chase. Immunoselection of proteins present in the protoplast incubation medium indicated that A27L was not secreted, an expected result as it is a cytosolic protein (Vazquez *et al*., 1998). The results of pulse chase were confirmed and extended by Western blot analysis of protoplast homogenates. On A27L expression, anti-FLAG antibodies detected a major component around 14 kDa and two additional components around 30 and 40 kDa, absent in control protoplasts transfected with the empty plasmid (Figure 1b). These higher molecular mass forms were observed despite the denaturing conditions during sample preparation and electrophoresis, and may represent nonspecific protein interactions or dimers and trimers of A27L. A small proportion of dimers were detected by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) when A27L was expressed in bacteria, and abundant dimers and trimers were detected by cross-linking (Vazquez *et al*., 1998). Therefore, the 30- and 40-kDa forms observed here probably correspond to dimers and trimers of the 14-kDa polypeptide, strongly suggesting that plant-expressed A27L acquires the correct conformation. A27L has a concentration-dependent tendency to trimerize and its biological function requires self-assembly (Vazquez *et al*., 1998; Ho *et al*., 2005). The minor component with a slightly higher molecular mass than the major 14-kDa form, visible in Figure 1b, was detected in various proportions with respect to the 14-kDa form in different experiments (see, for example, Figure 5), or not detected at all; the molecular basis for this was not investigated further in this study. However, the same component has also been detected in other studies (Vazquez *et al*., 1998).

The results presented in Figure 1 indicate that the recombinant A27L is not rapidly degraded and oligomerizes in plant cells, suggesting correct folding. Having verified the suitability for A27L cytosolic expression, we produced nuclear transgenic plants. In addition, the A27L protein was expressed in tobacco, using stable transformation of the plastid genome, in order to compare the two expression systems.

Production of transgenic and transplastomic plants

For stable nuclear transformation of *Nicotiana tabacum*, the same *A27L-FLAG* sequence as used for transient expression was inserted into the binary vector pK7WG2 (Karimi *et al*., 2002), downstream of the 35S CaMV promoter and the tobacco mosaic virus (TMV) leader and upstream of the 35S CaMV terminator. The resulting plasmid pK7A27L (Figure 2a)

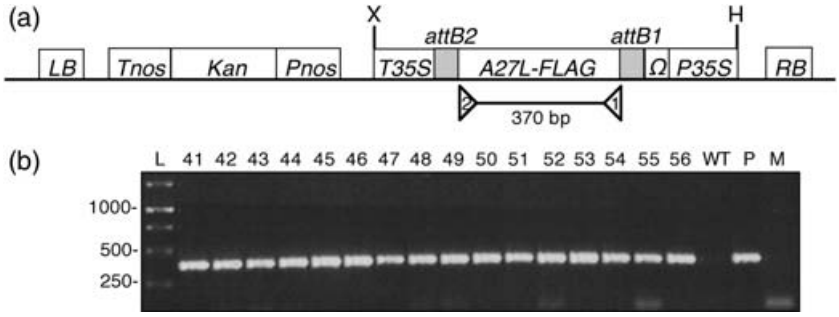

Figure 2 Production of transgenic plants. (a) Schematic map of the T-DNA region of the pK7A27L vector. *LB* and *RB*, left and right border repeats of the T-DNA, respectively; *Kan,* neomycin phosphotransferase II gene; *Pnos* and *Tnos*, nopaline synthase promoter and terminator; X, *Xba*I; *P35S*, promoter of 35S; H, *Hin*dIII; *T35S*, terminator of 35S; *attB2* and *attB1*, recombination sites of the Gateway system; *A27L-FLAG*, *A27L* coding sequence with a FLAG tag at the C-terminus. The expected sizes of the fragments derived by polymerase chain reaction (PCR) analysis are shown. The primers used and their approximate locations are indicated by triangles: 1, A27LF; 2, A27LR (see Experimental procedures). (b) Transgene-specific PCR analysis of total DNA extracts from pK7A27L-transformed tobacco plants. L, molecular mass marker; 41–56, independent transgenic lines; WT, wild-type tobacco genomic DNA as template; P, vector DNA as template; M, PCR mix without DNA.

was used for *Agrobacterium tumefaciens*-mediated transformation of tobacco. Regenerated T_0 plants were analysed by *A27L*-specific polymerase chain reaction (PCR; Figure 2b). Fifty-one of 56 putative transgenic plants that germinated on kanamycin were positive for the insertion. In 18 of the 21 plants analysed, the full-length *A27L* mRNA was detected by reverse transcription (RT)-PCR amplification of total mRNA using *A27L*-specific DNA primers (data not shown).

The *A27L* coding sequence was also introduced into the tobacco plastome. The *A27L-FLAG* sequence was placed under the control of the strong *rrn* operon promoter, the 5′-untransformed region (5′-UTR) of the T7 phage gene 10 and the *rbcL* terminator (Figure 3a). The transgene was cloned into the pPRV vector, which allows the targeting of exogenous DNA to the *trnV-rps12/7* region of the tobacco plastid genome (Zoubenko *et al*., 1994). Transplastomic tobacco plants were produced by the bombardment of about 30 tobacco leaves. This resulted in 32 putative transformation events (primary regenerants), 15 of which were subjected to a second round of selection on spectinomycin. Twenty-one secondary regenerants were analysed for transgene integration by PCR, and a representative set is reported in Figure 3b. Eighteen secondary shoots derived from eight primary regenerants were positive, the others probably being spontaneous mutants. Mutations can occur in the 16S rRNA gene, conferring high levels of spectinomycin resistance (Daniell *et al*., 2001). Southern blot analysis was performed to verify site-specific integration and homoplasmy (Figure 3c). The targeting sequence (P1) probe identified a 3.3-kb fragment in wild-type *N. tabacum* and a 5.3-kb fragment in transplastomic plants, as expected. The absence of the 3.3-kb wild-type fragment indicated that the transplastomic lines were homoplasmic. Homoplasmy was further confirmed by inheritance assays, which demonstrated uniform green progenies, and thus a lack of phenotypic segregation for spectinomycin resistance, in the T_1 generation (data not shown).

Transgene expression in transgenic and transplastomic plants

Recombinant A27L protein accumulation was determined in TSP extracts from leaves of nuclear transgenic and transplastomic plants using anti-A27L polyclonal antiserum. Two nonspecific components of around 20 and 32 kDa were detected in the negative control and in all the transgenic *N. tabacum* lines (Figure 4a). Recombinant A27L was clearly detected in 10 of the 14 independent nuclear transgenic lines tested, with variable signal intensity among the lines (Figure 4a, arrowhead; seven transgenic lines are shown, four of them positive). The approximately 14-kDa polypeptide was also detected in the three independent transplastomic lines analysed (Figure 4b, arrowhead). The additional larger components of approximately 30 and 40 kDa were clearly detected in these samples, most probably representing oligomers, similar to those shown in Figure 1b. The detection of the larger components in transplastomic plants was probably a result of the higher accumulation of the recombinant protein in the latter, when compared with nuclear transgenic plants (see below), and to the consequent incomplete sample denaturation.

The oligomerization state of A27L was verified by velocity centrifugation on a linear sucrose gradient. The different molecular mass forms of A27L, observed in Figure 5a, migrated as a single peak along the gradient, indicating that they

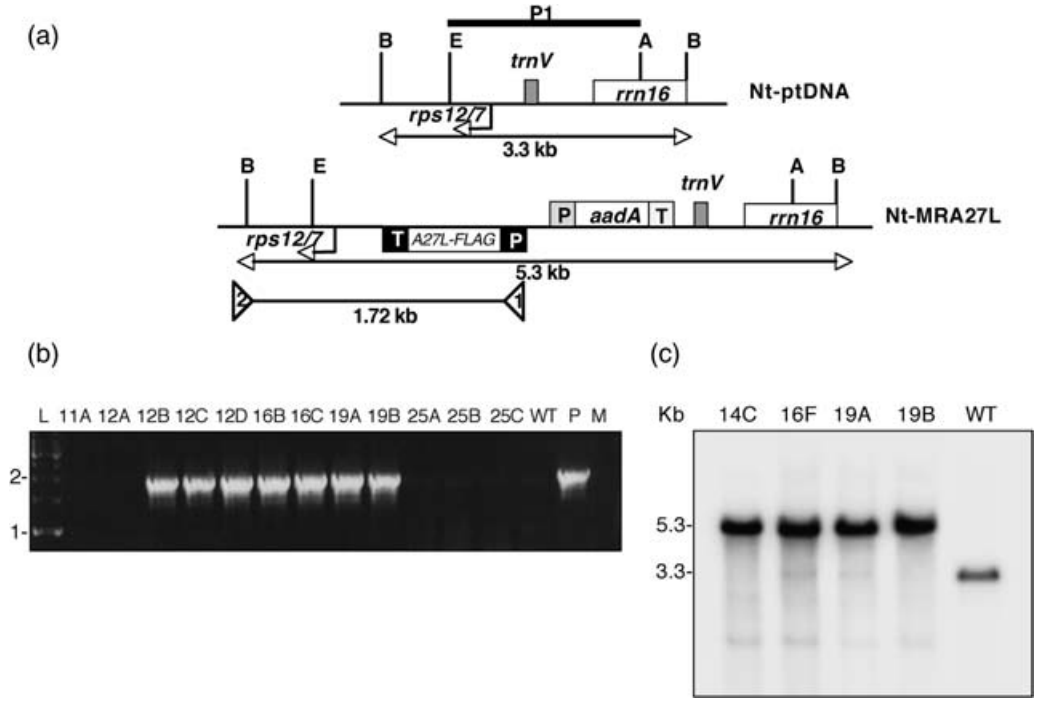

Figure 3 Production of transplastomic plants. (a) Map of wild-type (Nt-ptDNA) and transformed plastid genomes. Genes above and below the horizontal line are transcribed clockwise and counterclockwise, respectively, in inverted repeat B (IR_B) (Wakasugi *et al*., 1998). The expected sizes of the fragments derived by polymerase chain reaction (PCR) or Southern analysis are shown. The spectinomycin resistance (*aad*A), *A27L-FLAG* and plastid genes *rrn16*, *trnV* and *rps12/7* are also shown. The primers used and their approximate locations are indicated by triangles: 1, P1; 2, P2 (see Experimental procedures). A, *Apa*I; B, *Bam*HI; E, *Eco*RI restriction sites. (b) PCR analysis with P1 and P2 primers using total DNA from secondary regenerants as template. L, molecular mass marker; 11A–25C, spectinomycin-resistant secondary regenerants; WT, wild-type tobacco total DNA as template; P, total DNA from one transplastomic plant as positive control; M, PCR mix without DNA. (c) Southern blot analysis of four secondary regenerants. Analysis was carried out with probe 1 after digestion of total cellular DNA with *Bam*HI.

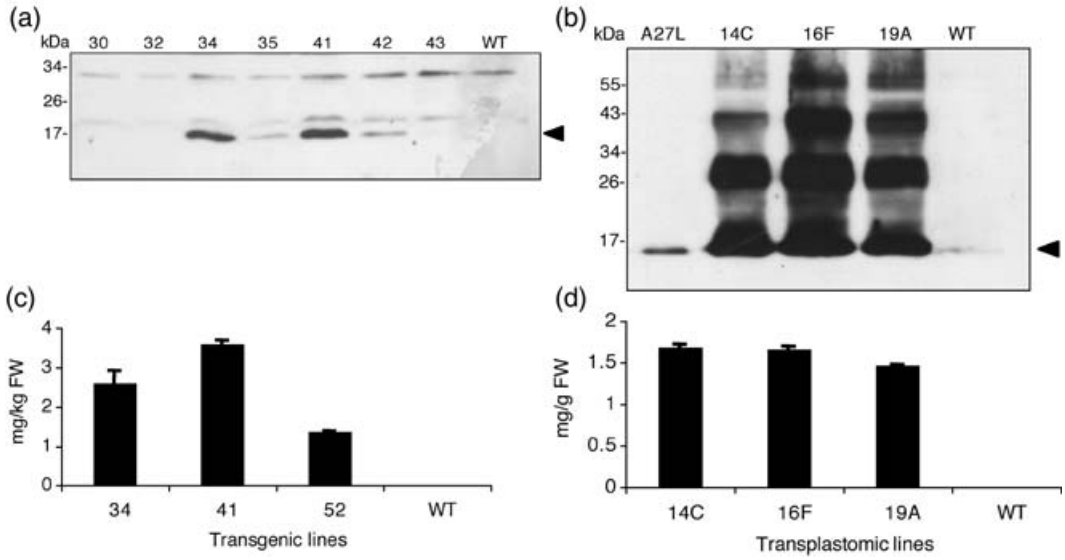

Figure 4 Accumulation of A27L in nuclear transgenic (a, c) and transplastomic (b, d) plants. (a, b) Immunoblot detection using polyclonal anti-A27L antibody: 40 μg (a) and 10 μg (b) of total soluble protein were loaded per lane. A27L, 25 ng of purified baculovirus-derived A27L protein; WT, protein extracts from wild-type plants. Band sizes are given in kilodaltons. (c, d) Anti-A27L enzyme-linked immunosorbent assay (ELISA). Error bars, standard deviation of the mean; FW, fresh weight.

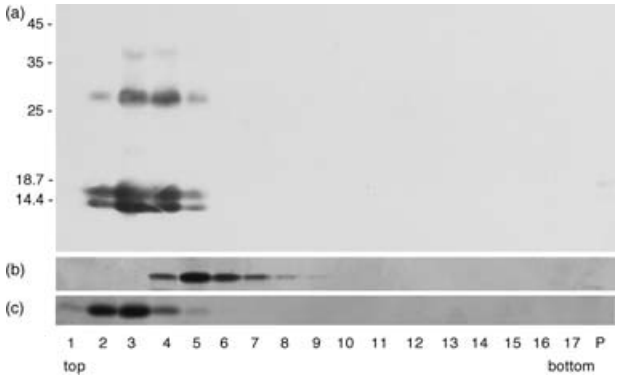

Figure 5 Oligomeric state of A27L accumulated in chloroplasts. (a) Young leaves of transplastomic tobacco expressing A27L were homogenized in the presence of non-ionic detergent. The homogenate was fractionated by sedimentation velocity centrifugation on a continuous 5%–25% (w/v) sucrose gradient. Aliquots of gradient fractions (lanes 1–17) and a proportional amount of the material precipitated at the bottom of the tube (P) were analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein blot, and visualized using anti-FLAG antiserum. The sedimentation markers bovine serum albumin (b, 67 kDa) and cytochrome C (c, 12.4 kDa) were also fractionated on a similar gradient and analysed by SDS-PAGE and Coomassie blue staining. Numbers on the left indicate the positions of molecular mass markers along the SDS-PAGE gel.

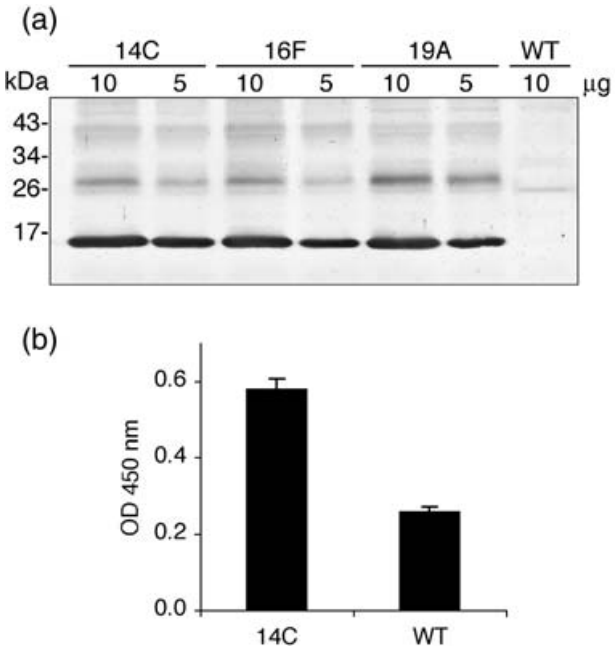

Figure 6 Western blot (a) and enzyme-linked immunosorbent assay (ELISA) (b) of transplastomic lines carried out using serum from a patient recently infected with a zoonotic orthopoxvirus. (a) Immunoblot detection. The amount of total soluble proteins (µg) loaded per lane is indicated. Band sizes are given in kilodaltons. (b) ELISA. Error bars, standard deviation of the mean. WT, protein extracts from wild-type plants; 14C–19A, transplastomic lines.

represent a single oligomer not completely disassembled by SDS-PAGE. The position of the peak along the gradient is intermediate between those of the molecular mass markers cytochrome C (12.4 kDa; Figure 5c) and bovine serum albumin marker (67 kDa; Figure 5b), consistent with A27L being assembled *in vivo* into trimers.

Comparison of the protein blots showed a marked increase in A27L protein accumulation in transplastomic relative to nuclear transgenic lines (Figure 4a,b). These results were confirmed by enzyme-linked immunosorbent assay (ELISA) performed to determine the amount of A27L protein in transgenic and transplastomic plants. The three independent transplastomic lines analysed accumulated similar levels of the recombinant protein, ranging from 1.5 to 1.7 mg/g fresh weight (FW), corresponding to around 18% TSP (Figure 4d). This was at least 500-fold higher than in the best three nuclear transgenic plants, where accumulation ranged from 1.4 to 3.6 mg/kg FW, corresponding to 0.01–0.04% TSP (Figure 4c).

Protein blot was also performed using serum from one patient recently infected with a zoonotic OPV (C. Castilletti *et al*., unpubl. data). Monomeric and oligomeric A27L proteins were recognized by the antiserum in the three transplastomic lines (Figure 6a). Minor, nonspecific components with different molecular masses were also detected in wild-type tobacco and in transplastomic plants (Figure 6a). Only nonspecific polypeptides were detected in protein extracts from nuclear transgenic plants, most probably because of the much lower accumulation of recombinant protein (data not shown). These results were confirmed by ELISA (Figure 6b), demonstrating that the human serum was able to recognize the chloroplast-made A27L protein under both denaturing and native conditions. A low signal was detected in wild-type plant extracts (Figure 6b), probably caused by nonspecific interactions with native tobacco proteins. These results suggest that the plant-synthesized protein retained at least some of the antigenic epitopes present in the native, virus-synthesized A27L protein.

To verify whether the much higher accumulation of A27L in the transplastomic plants could be related to recombinant mRNA levels, RNA blot analysis was performed on total leaf RNA extracts. The lines to be analysed were selected according to A27L protein accumulation (Figure 4c,d). The *A27L* probe detected transcripts of 650 and 450 bp in the transgenic and transplastomic lines, respectively. No transcript was recognized in wild-type tobacco (Figure 7). Probably, the nuclear transcripts were longer because of the addition of a polyA tail. Quantification of the band intensities indicated that transplastomic plants accumulated at least 2000-

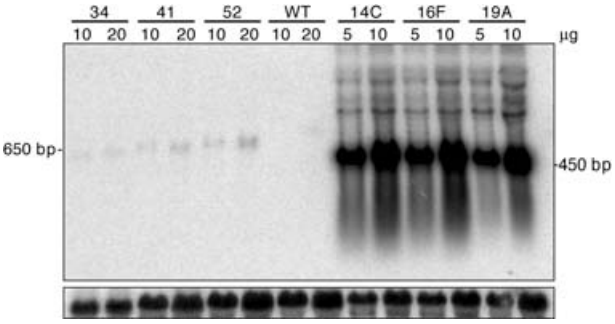

Figure 7 Northern blot analysis of transgenic and transplastomic tobacco lines. The blots were probed for *A27L* (top) and cytoplasmic 18S rRNA (bottom). The amount of RNA (µg) loaded per lane is indicated. Leaf samples were tested from three independent pK7A27L nuclear transgenic lines (34, 41, 52) and from three independent pMRA27L transplastomic lines (14C, 16F, 19A). WT, total RNA extracted from a wild-type tobacco plant.

fold more transcript than the highest expressing nuclear transformants. We conclude that the much higher protein accumulation can be related to the transcript levels.

Subcellular localization in transplastomic plants

We investigated the subcellular localization of A27L in transplastomic plants by immunofluorescence microscopy and subcellular fractionation.

Immunofluorescence microscopy, followed by the collection of optical sections using Zeiss Apotome and Axiovision 4.1 software (Jena, Germany), was performed on protoplasts prepared from pale green leaves of transplastomic plants, or from green leaves of untransformed plants as a control. Protoplasts were fixed, permeabilized and incubated with anti-FLAG antibodies, followed by detection with Alexa.Fluor488 secondary antibody. Chlorophyll autofluorescence was also detected to visualize chloroplasts. Anti-FLAG antibody gave little background labelling in control protoplasts (Figure 8h), but intense and specific labelling of chloroplasts in the transplastomic samples (Figure 8a,e). In all transplastomic protoplasts observed, labelling was consistently present at the chloroplast periphery. Treatment with secondary antibody alone did not give the same pattern (Figure 8i), indicating that the labelling of the chloroplast periphery was a result of recognition of the FLAG epitope in the recombinant protein. Some of the transplastomic chloroplasts showed additional, less intense and more diffuse anti-FLAG labelling occupying part of the organelle (Figure 8a), but uniform, complete labelling was never observed. The accumulation of A27L markedly altered the pattern produced by chlorophyll autofluorescence. Although untransformed chloroplasts showed uniform fluorescence spanning the whole organelle (Figure 8n), in transplastomic plants fluorescence was mainly limited to the chloroplast periphery (Figure 8b,f,j) and almost completely overlapped with the signal given by the anti-FLAG antibodies (Figure 8c,g). These results confirmed the expected subcellular localization of A27L and suggest that this protein alters the organization of chloroplasts. The overall autofluorescence of chlorophyll in the transplastomic chloroplasts was also markedly weaker than in wild-type protoplasts (compare the fluorescence intensity in Figure 8n with that in Figure 8b,f,j).

A27L associates with the cytosolic side of membranes because it interacts with the membrane protein p21, produced by the *A17L* gene (Rodriguez *et al*., 1993). In the absence of p21, A27L is therefore expected to be soluble. The results of immunofluorescence microscopy indicate that either A27L associates with the inner chloroplast envelope or is simply displaced to the chloroplast periphery because of alterations in chloroplast structure. We therefore investigated whether A27L is soluble in the stroma of transgenic chloroplasts (Figure 9). Leaves were homogenized in the absence of detergent and the presence of sucrose, and the homogenate was fractionated by isopycnic sucrose gradient centrifugation. Chloroplasts break during homogenization and therefore soluble proteins of the stroma should remain on top of the gradient, together with cytosolic and soluble vacuolar proteins (vacuoles also break; see Pedrazzini *et al*., 1997). A27L remained almost exclusively on top of the gradient (Figure 9a). Chlorophyll (marker of thylakoids) migrated around density 1.18 (not shown). We therefore conclude that A27L is not strongly associated with the membranes of the chloroplast.

To confirm the A27L localization within chloroplasts, these were purified from transplastomic plants and subjected to further separation into soluble (stromal) and membrane fractions. Isolated fractions were analysed by protein blot using anti-FLAG antibodies. As shown in Figure 9b, A27L was detected in the whole chloroplast extracts and in the chloroplast soluble fraction. Only an extremely minor proportion was present in the chloroplast membrane fraction. No immunoblot signal was detected in any subcellular fraction from wild-type plants (not shown). To verify possible contamination of the chloroplast preparation by other membranes, the subcellular fractions were also probed with antiserum raised against endoplasmin (molecular mass, 97 kDa), a soluble resident of the endoplasmic reticulum (Klein *et al*., 2006). Endoplasmin was detected in the total leaf homogenate, but not in the chloroplast fraction, indicating that the latter is not contaminated by the abundant endoplasmic reticulum membranes (Figure 9c).

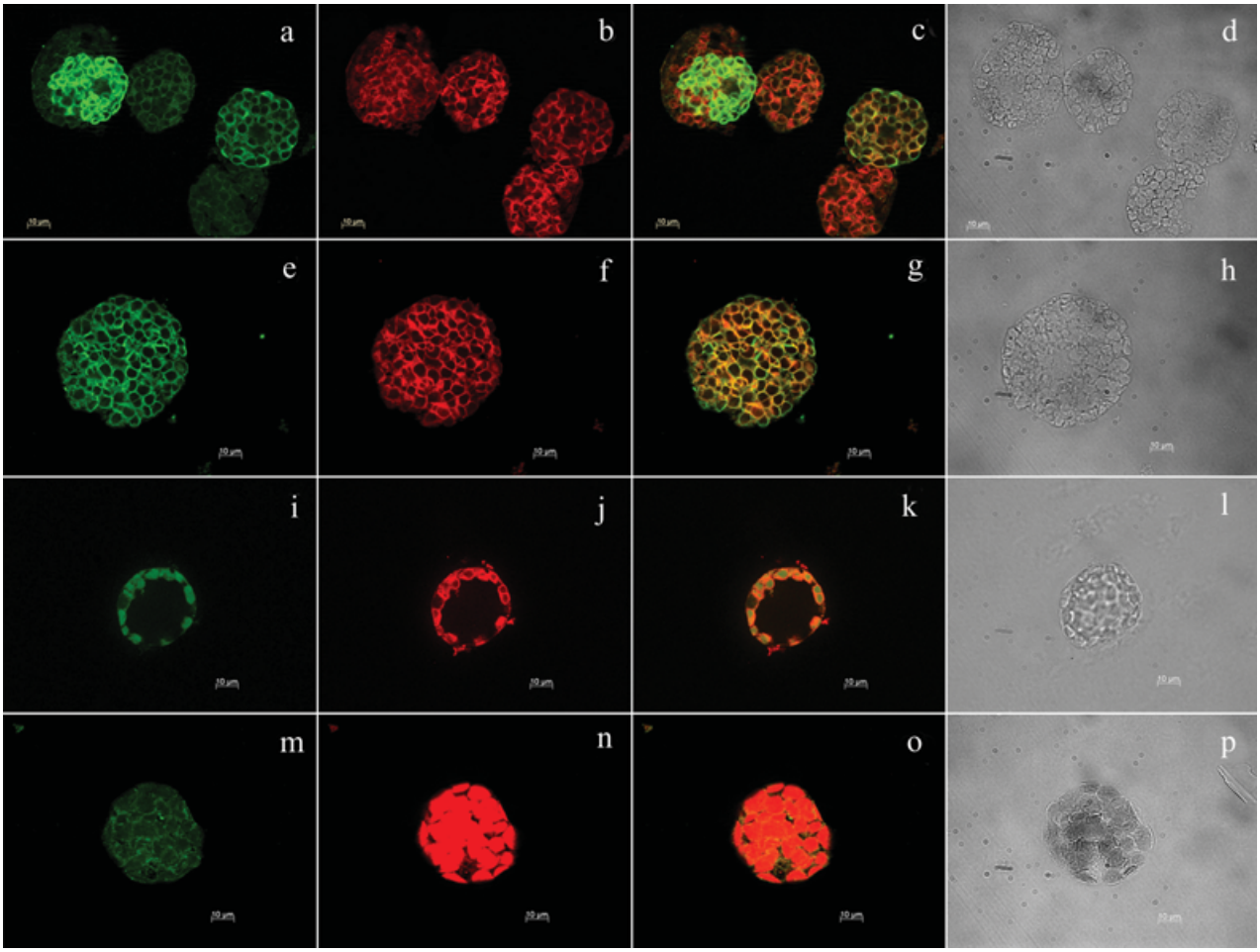

Figure 8 Immunofluorescence localization of A27L in transplastomic tobacco. Protoplasts isolated from young leaves of A27L transplastomic (a–l) or wild-type (m–p) tobacco plants were fixed, permeabilized, treated with antibodies and analysed by epifluorescence microscopy, followed by the collection of optical sections using a Zeiss Apotome system. Each horizontal series of panels refers to the same sample, visualized in different ways. (a, e, m) Anti-FLAG polyclonal antibodies and Alexa Fluor 488 secondary goat anti-rabbit antibody; (i) secondary antibody alone; (b, f, j, n) chlorophyll autofluorescence; (c, g, k, o) merge; (d, h, l, p) bright field. Thickness of optical sections, 0.525 μm. Bars, 10 μm.

Effect of leaf age on transplastomic accumulation

To study the influence of leaf age on the accumulation of A27L in chloroplasts, TSP extracts were prepared from young and mature leaves (first, third and fifth from the top) of transplastomic plants grown in soil in a phytotron, and analysed by ELISA or protein blot. ELISA showed that young and mature leaves accumulated approximately the same amount of A27L (1.4–1.6 mg/g). There was also no significant difference in A27L accumulation between plants grown *in vitro* or in soil (Figure 10a). Protein blot analysis mainly confirmed the ELISA results, but indicated a slight increase in the accumulation of oligomeric forms with age (Figure 10b). In Coomassie blue-stained SDS gel, the approximately 14-kDa band, migrating immediately above the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (SSU), was clearly detectable in transplastomic plants (Figure 10c). The multimeric structures of higher molecular masses were detected by protein blot, but not by Coomassie blue staining, suggesting that they were preferentially recognized by the antibodies, possibly because of epitopes formed by non-primary structures. Staining with Coomassie blue also showed that the accumulation of the Rubisco large and small subunits is reduced in the transplastomic lines. In soil, the transplastomic plants grew more slowly and had a slightly chlorotic phenotype, in agreement with the weaker chlorophyll autofluorescence of chloroplasts, but this did not influence flowering and seed setting. In contrast, nuclear transgenic lines showed a phenotype similar to the wild-type, non-transformed plants (Figure 10d). The mutant phenotype may thus be linked to the A27L expression levels, as demonstrated previously for other recombinant proteins produced in

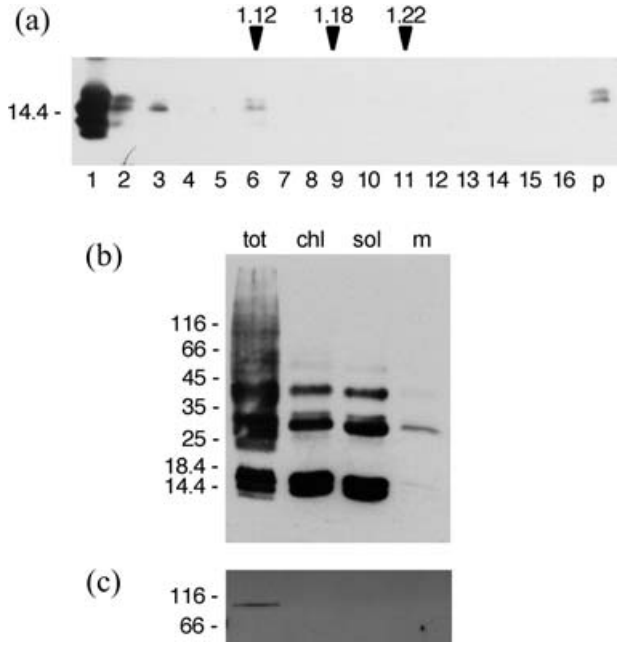

Figure 9 Subcellular fractionation of transplastomic tobacco plants. (a) Young leaves from A27L transplastomic plants were homogenized in the absence of detergent and the presence of sucrose. The homogenate was fractionated by ultracentrifugation on a 16%–55% (w/w) isopycnic sucrose gradient. An equal amount of each gradient fraction (16 fractions in total) was analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein blot, using anti-FLAG polyclonal antibodies. The gradient density is from left (less dense, fraction 1) to right. The pellet precipitated at the bottom of the tubes, probably representing unbroken tissue, was also analysed (p). The numbers at the top indicate the density (g/mL). (b) Chloroplasts (chl) were isolated from a total leaf homogenate (tot) of transplastomic MRA27L-19A plant. Chloroplasts were then further fractionated into a soluble (sol) and a membrane (m) fraction. Proteins were analysed by Western blot using anti-FLAG antibodies. (c) The same fractions analysed in (b) were immunodetected using anti-endoplasmin antiserum. In each panel, the numbers on the left indicate the positions of molecular mass markers in kilodaltons.

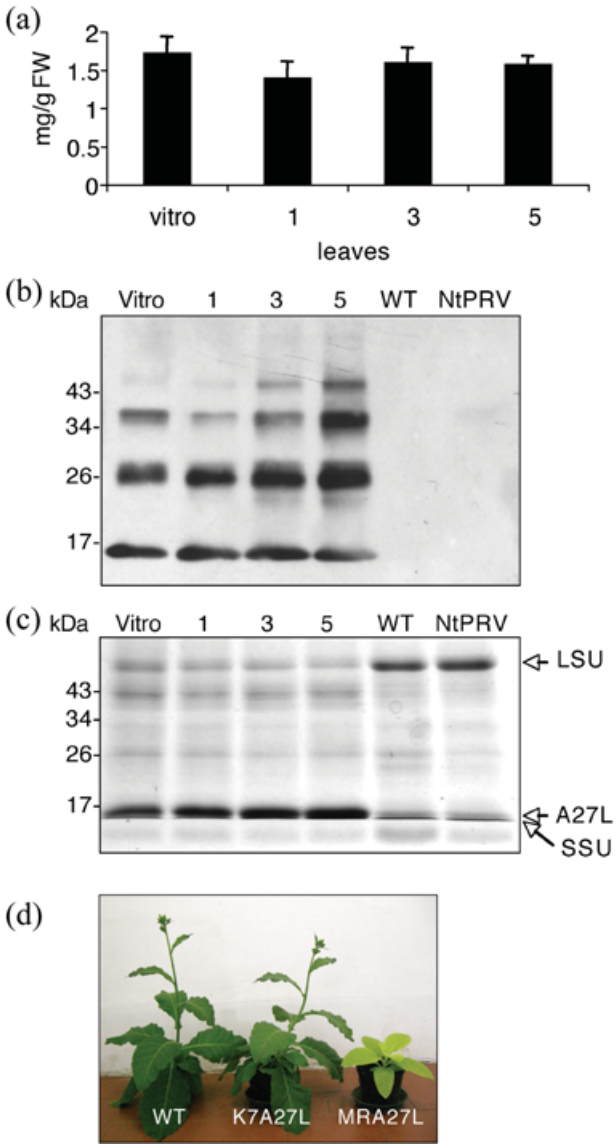

Figure 10 A27L accumulation in young and mature leaves (first, third and fifth from the top) of transplastomic MRA27L-19A plant grown in soil in a phytotron, and in leaves from the same line grown as axenic shoot cultures (vitro). (a) Anti-A27L enzyme-linked immunosorbent assay (ELISA). Error bars, standard deviation of the mean. (b) Immunoblot detection using polyclonal anti-A27L antibody. Five micrograms of total soluble proteins were loaded. (c) Protein gel [50 μg total soluble protein (TSP)/lane] stained with Coomassie blue. The positions of A27L and the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large and small subunits (LSU, SSU) are indicated. Band sizes are given in kilodaltons. WT, protein extracts from wild-type plant; NtPRV, protein extracts from tobacco line transformed with the empty vector pPRV111A. (D) Phenotypes of wild-type (WT), transgenic (K7A27L) and transplastomic (MRA27L) plants.

transgenic chloroplasts (Tregoning *et al*., 2003; Lenzi *et al*., 2008; Zhou *et al*., 2008).

Discussion

The use of transgenic plants as bioreactors for the production of antigenic proteins is a promising technology, but the yield of recombinant protein is often too low for cost-effective production. A major goal in this field of research is therefore to obtain high accumulation of the antigen in a form that can be used for immunological applications. Subcellular localization, tissue specificity and, in recent years, the choice between the nuclear or plastid genome have been proven to have a strong influence on final accumulation. In this study, we compared the expression of the VACV immunogenic protein A27L in transplastomic and nuclear transgenic tobacco. In transplastomic plants, A27L accumulated to about 18% of TSP (equivalent to approximately 1.7 mg/g FW). This is 500-fold higher than the best level obtained in nuclear

transformed plants, and a similar or even greater difference was observed in the respective transcript levels. Therefore, the much greater accumulation in chloroplasts can be explained by the difference in mRNA levels. The latter is most probably a result of the high copy number of the plastid genome per cell compared with the nuclear genome (Bock, 2007; Fernandez-San Millan *et al*., 2008). We do not know whether protein stability also contributes to the different accumulation levels. Our transient expression results showed that the half-life of A27L in the cytosol is around 4 h. Recombinant proteins that are known to be folded incorrectly in plant cells are often almost fully degraded within 2 h (Nuttall *et al*., 2003; de Virgilio *et al*., 2008), whereas correctly folded proteins stabilized by endoplasmic reticulum localization signals have half-lives that can be longer than 24 h (Frigerio *et al*., 2001; Mainieri *et al*., 2004). In the cytosol, A27L therefore seems to have an intermediate stability and no gross misfolding, as also indicated by the fact that it is able to assemble into trimers. Previous studies have reported that A27L maintains its native conformation over a wide pH range, suggesting high stability (Vazquez and Esteban, 1999).

We observed that the ratio between recombinant mRNA accumulation in transplastomic and nuclear transformed plants was higher than the ratio measured for protein accumulation. This suggests that either translational efficiency or A27L protein stability is lower in chloroplasts. Alternatively, the observed discrepancy may be a result of exhaustion of the gene expression capacity of the chloroplast by overuse of the translation machinery, which also results in Rubisco depletion, as reported for other transplastomic plants (Zhou *et al*., 2008).

A27L is highly hydrophilic and requires interaction with the viral p21 membrane protein to associate with the envelope of the intracellular mature virus (Vazquez and Esteban, 1999). We confirmed that A27L is expressed in transplastomic plants in a soluble form. Subcellular fractionation indicated that it was localized in the stroma. The high accumulation of A27L somehow altered the internal organization of chloroplasts. Immunofluorescence microscopy showed that the autofluorescence of chlorophyll in transplastomic plastids was weak and limited to the chloroplast periphery, where most of A27L was also immunodetected. In soil, the transplastomic plants expressing A27L grew more slowly and had a chlorotic phenotype. The observed phenotype correlates with the reduced level of Rubisco large and small subunits. A chlorotic phenotype has also been observed in transplastomic plants expressing other recombinant proteins at high levels, using the same expression cassette as in our study (Tregoning *et al*., 2003; Zhou *et al*., 2008).

Protein turnover during plant development obviously plays an important role in the final accumulation of recombinant proteins, and transplastomic plants are no exception. Interesting examples have been provided for the rotavirus VP6 protein, the fungal xylase and the insecticidal *Bacillus thuringiensis* (Bt) toxin CryAa2, which showed high accumulation in chloroplasts of young tobacco leaves but negligible levels in older leaves (reviewed in Benchabane *et al*., 2008). By contrast, the amount of A27L did not decline with leaf ageing, in spite of the mutant phenotype of chloroplasts and the negative effect on plant growth. Thylakoid protein degradation occurs mainly within chloroplasts, whereas there is growing evidence for an important role of vacuoles in the degradation of stromal soluble proteins (Martínez *et al*., 2008a,b). Whether the high stability of A27L accumulation during leaf ageing is related to its peculiar distribution within the chloroplast remains to be determined. The stability of A27L is important for its total yield in transplastomic plants. Indeed, although the precise amount of recoverable protein remains to be determined, it can be roughly estimated that transplastomic plants, notwithstanding the reduced growth rate, could eventually accumulate more than 35–40 mg of recombinant protein per plant, an amount sufficient for cost-effective downstream applications.

The 14-kDa protein expressed in transplastomic plants was similar to the native protein found in the virus particle and in infected cells, based on size, oligomerization pattern and reactivity against specific antibody. The central domain of A27L is an α-helix with a high tendency to form coiled-coil trimers (Vazquez *et al*., 1998). At high protein concentration, two trimers can form hexamers by the interaction of the leucine zippers in the C-terminal region (Vazquez *et al*., 1998). Recent data have suggested that the oligomeric structure of A27L acts as an indispensable scaffold that stabilizes the overall structure of A27L and is essential for binding to heparin and heparan sulphates at the cell surface (Ho *et al*., 2005). It has also been demonstrated that trimeric forms of the 14-kDa proteins elicit higher titre neutralizing antibodies than do monomers (Lai *et al*., 1990). Oligomerization of A27L does not require cellular factors (Lai *et al*., 1990). In this study, we have demonstrated the ability of the plant-derived A27L protein to form oligomers in the chloroplast environment.

In conclusion, we have demonstrated that VACV protein yields attainable in transplastomic plants are sufficient for the downstream production steps required for the development of a novel smallpox vaccine. The high expression level achieved in this study could allow the use of a limited amount of plant material for a single vaccine dose and facilitate the rapid production of sufficient smallpox vaccine stockpiles.

We have demonstrated that the chloroplast-made A27L protein is recognized by serum from a patient recently infected with a zoonotic OPV, suggesting that the recombinant protein retains at least some of the antigenic epitopes present in the native, virus-synthesized A27L protein. In addition, the ability of the plant-made A27L protein to oligomerize, and the stability of the A27L protein over a wide range of pH values (Vazquez and Esteban, 1999), could be a distinct advantage for the induction of intestinal secretory immunoglobulin A (IgA) following oral immunization. To date, numerous data have been obtained on the immunogenicity and safety of chloroplast-made vaccines against biosecurity-related organisms (Daniell, 2006; Streatfield, 2006; Rigano *et al*., 2009). Future studies addressing the immunogenicity and efficacy of vaccination with chloroplast-synthesized A27L will be important in determining the potential of this antigenic protein as a safe and effective subunit vaccine. Several studies have demonstrated that immunization with *E. coli*-expressed recombinant proteins or DNA vaccines composed of different combinations of VACV antigens (such as A27L + A33R + L1R + B5R) confers an enhanced level of protection relative to vaccination with one or two immunogens (Hooper *et al*., 2003; Heraud *et al*., 2006; Berhanu *et al*., 2008). Multigene expression in a single transformation step is feasible by plastid transformation because of the ability of chloroplasts to process polycistrons. This opens up the way for the production of a multivalent subunit vaccine against OPVs in chloroplast-transformed plants.

Experimental procedures

Plasmid construction

The VACV-1 *A27L* sequence (GenBank, Vaccinia Virus complete genome, Accession no. 001559) was obtained from the Vaccinia Virus – Lancy strain (Berna Biotech Ltd., Bern, Switzerland) by PCR amplification using the following primers: forward, 5′-ATGGACG GAACTCTTTTCCCC-3′; reverse, 5′-TTACTCATATGGRCGCCGTC CA-3′. The amplified *A27L* coding sequence has a G + C content of 39% and encodes for 110 amino acids. The resulting PCR product was ligated into pCR4-TOPO (Invitrogen Life Technologies, Carlsbad, CA, USA).

To construct pDHA-*A27L-FLAG*, the *A27L* coding sequence was PCR amplified, adding a *Xba*I site at the 5′ end and a *Sph*I site and the coding sequence for the FLAG epitope (eight amino acids, DYKDDDDK) at the 3′ end, using the following primers: forward, 5′-GCTCTAGAATGGACGGAACTCTTTTCC-3′; reverse, 5′-ACATG CATGCCTATCATTACTTGTCGTCGTCGTCCTTGTAGTCCTCATATG GCGCCGTCCA-3′. The resulting PCR product was ligated into the *Xba*I/*Sph*I restricted pDHA plasmid (Tabe *et al*., 1995). The A27LpCR8GWTOPO construct, containing the *A27L* gene and the coding sequence for the FLAG epitope at the 3′ end, was provided courtesy of Drs A. Porceddu and F. Alagna (CNR-IGV, Perugia, Italy). The DNA fragment containing the sequence coding for *A27L-FLAG*, flanked by *att*L recombination sites, was recombined into the Gateway vector pK7WG2 (Karimi *et al*., 2002) using LR clonase (Invitrogen Life Technologies). Recombined plasmid pK7A27L was transformed into *E. coli* DH5-α cells using heat shock. Recombinant plasmid was then transformed into *A. tumefaciens* strain LBA4404 for subsequent plant transformation. In order to construct the vector pMRA27L, the gene *A27L* fused to the FLAG tag was PCR amplified from A27LpCR8GW using the primers A27LR (5′-CTA*GCTAGC*GACG-GAACTCTTTTCCCC-3′, restriction sites are in italic) and A27LF (5′-CCG*GATTCTCTAGA*CTATCATTACTTGTCGTC-3′, restriction sites are in italic), adding a *Nhe*I site at the 5′ end and a *Xba*I site at the 3′ end. Plasmid pMRA27L is a pHK40 (Kuroda and Maliga, 2001) vector derivative in which the *Nhe*I-*Xba*I fragment encoding *neo* was replaced with a *Nhe*I-*Xba*I fragment encoding the A27L-FLAG fusion protein. The vector targets the insertion of the foreign gene into the inverted repeat region of the tobacco plastome. Details about the cloning strategy are available on request.

Transient expression in tobacco protoplasts and pulse-chase labelling

For transient expression of A27L, protoplasts were isolated from small (length, 4–7 cm) leaves of wild-type tobacco SR1 plants grown in axenic conditions and subjected to polyethylene glycol-mediated transfection, as described previously (Pedrazzini *et al*., 1997), using 40 μg of pDHA-*A27L-FLAG*, or an equivalent amount of empty pDHA, for one million protoplasts. After overnight recovery, pulse-chase labelling of protoplasts was performed using Pro-Mix (a mixture of [^{35}S]methionine and [^{35}S]cysteine; GE Healthcare), as described previously (Pompa and Vitale, 2006). The homogenization of protoplasts or their incubation medium was performed by adding to frozen samples 2 vol of ice-cold 1.5 × protoplast homogenization buffer [150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 1.5% Triton X-100 and Complete protease inhibitor cocktail (Roche, Basel, Switzerland)]. Immunoselection was performed as described previously (Pedrazzini *et al*., 1997) using anti-FLAG rabbit polyclonal antibodies (Sigma-Aldrich, St Louis, MO, USA). Samples were analysed by SDS-PAGE and fluorography as described previously (Pompa and Vitale, 2006). Rainbow ^{14}C-methylated proteins (Sigma-Aldrich) were used as molecular mass markers. For protein blot, aliquots of protoplast homogenates were separated by SDS-PAGE, electrotransferred onto Hybond-P membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and probed with anti-FLAG rabbit polyclonal antibodies (Sigma-Aldrich; 1 : 400 dilution). Detection was performed using Super-Signal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL, USA), following the manufacturer's instructions. Protein molecular mass markers (Fermentas, Vilnius, Lithuania) were used.

Plant transformation

For nuclear and plastid transformation, plants were grown on Murashige and Skoog (MS) medium with B5 vitamins (Duchefa, Haarlem, the Netherlands) containing 3% (w/v) sucrose and 0.8% (w/v) agar in sterile culture conditions (24 °C, 16 h/8 h light/dark, 50–70 μE/m^2/s daylight fluorescent tubes).

Agrobacterium tumefaciens-mediated transformation of *N. tabacum* with the vector pK7A27L was accomplished using the leaf disc method (http://www.plants.leeds.ac.uk/jd/pdf/Tobacco%20transgenic.pdf). Briefly, leaf discs from *N. tabacum* cv. Petite Havana were transformed by co-cultivation with *A. tumefaciens* harbouring pK7A27L. After co-cultivation, the leaf discs were grown on regeneration medium containing 500 mg/L cefotaxime and 100 mg/L kanamycin. After 5 weeks, regenerated shoots were plated on MS medium plus 100 mg/L kanamycin until the new plants developed.

For plastid transformation, plants of *N. tabacum* cv. Petite Havana were grown in sterile conditions on MS medium. Leaves from 3–8-week-old plants were used for bombardment. DNA for plastid transformation was prepared using the Qiagen Plasmid Maxi Kit (Qiagen, GmbH, Hilden, Germany). DNA was introduced into leaf chloroplasts on the surface of gold particles (0.6 μm) using the Bio-rad (Hercules, CA, USA) PDS1000He Biolistic Gun. Transplastomic shoots were produced with the vectors reported in Figure 3a and with the empty vector pPRV111A (Zoubenko *et al*., 1994), after selection on RMOP medium (Svabetal, 1990) containing 500 mg/L spectinomycin dihydrochloride. Resistant shoots were subjected to a second round of selection under the same conditions. Transgenic and transplastomic plants were maintained as axenic shoot cultures, as described previously. Established tobacco lines generated from nuclear and chloroplast transformation were transferred to soil and grown in a phytotron (25/20 °C, 14 h/10 h day/night, 200 $\mu E/m^2/s$ light), where they were allowed to self-fertilize, for further experiments.

PCR and Southern blot analysis

Total DNA was isolated from the leaves of putative transgenic and transplastomic plants, and from control plants, using the cetyltrimethylammonium bromide extraction method (Doyle and Doyle, 1990). The total DNA of putative tobacco transgenic plants was used as template for the detection of the *A27L* gene in a transgene-specific PCR employing A27LR and A27LF primers. Using these primers, an amplicon of 370 bp was expected. To confirm transgene cassette integration into the chloroplast genome, PCR was performed using the primer pairs P1 (5′-TATTTCTGGGAGGGAGACC-3′) and P2 (5′-CCCCGAAGAGTAACTAGGACCAAT-3′), product size = 1720 bp, landing on the *Prrn* promoter and on the chloroplast genome downstream of the *rps12/7* gene, respectively (Figure 3a).

Homoplasmy of the transplastomic plants was confirmed by DNA gel-blot analysis. Total plant DNA (5 μg) was digested with *Bam*HI, separated on a 0.8% (w/v) agarose gel and transferred to a Hybond-N membrane (GE Healthcare). A double-strand DNA probe was prepared by random priming ^{32}P labelling using the NEBlot Kit (New England Biolabs, Ipswich, MA, USA). The template for probing was a PCR fragment amplified from total tobacco cellular DNA with the primers (forward, 5′-GGATATCTATCTAATCCGATCG-3′; reverse, 5′-GGGCCCCCGTCAATTCCTTT-3′) prepared from the targeting *rrn16-rps12* flanking regions.

RNA gel-blot analysis and RT-PCR

Total cellular RNA was isolated by phenol–chloroform extraction from the leaves of plants grown in sterile conditions (http://www.tigr.org/tdb/potato/images/SGED_SOP_3.2.1.pdf). Total RNA was electrophoresed on 1% agarose–formaldehyde gel and transferred to a Hybond-N membrane (GE Healthcare). The membrane was hybridized with a random-primed ^{32}P-labelled probe using templates containing the *A27L* coding sequence. The template for probing the tobacco cytoplasmic 18S rRNA was a PCR fragment amplified from total tobacco cellular DNA with primers 18S1 (5′-TAGATAAAAGGTCGACGCGG-3′) and 18S2 (5′-CCCAAAGTCCAACTACGAGC-3′). Signals from blots were quantified using the Typhoon Variable Mode Imager 9200 (GE Healthcare) and normalized to the 18S rRNA signal. RT-PCR was performed to detect the presence of *A27L* mRNA in transgenic tobacco plants. Traces of DNA contamination in the total RNA samples from transgenic and control plants were enzymatically removed using the RQ1 RNase-Free DNase Kit (Promega, Madison, WI, USA). cDNA was prepared from 1 μg of total RNA samples using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). Targets were amplified using the primer sets A27LR and A27LR, and 18S1 and 18S2.

SDS-PAGE and immunoblot analysis

Leaves for protein extraction were collected from nuclear transgenic and transplastomic plants grown in either sterile conditions or in soil, as described above. Analyses with plants grown in soil were carried out using plants micropropagated as shoot cultures for 4 weeks and then grown in a phytotron for a further 8 weeks.

To extract soluble proteins, 200 mg of leaves were homogenized in 600 μL of buffer containing 100 mM Tris-HCl (pH 7.8), 200 mM NaCl, 1 mM EDTA, 2% β-mercaptoethanol, 0.2% Triton X-100 and 1 mM phenylmethylsulphonylfluoride (PMSF). Protein concentration was determined using the Bradford Protein Assay Reagent Kit (Bio-Rad, Hercules, CA, USA). The extracts were heated for 10 min at 95 °C. Proteins were separated by SDS-PAGE (12.5% acrylamide gel) and transferred onto nitrocellulose membranes. Blocked membranes were incubated with 1 : 1000 diluted polyclonal anti-VACV (WR) A27L antiserum. Anti-rabbit horseradish peroxidase conjugate (GE Healthcare), diluted 1 : 50 000, was used as the secondary antibody. Detection was performed using the ECL Plus Western Blotting Detection system (GE Healthcare). The VACV (WR) A27L recombinant protein from baculovirus (NR-2622) and the polyclonal anti-A27L (NR-627) antiserum were obtained through the Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Manassas, VA, USA.

Western blot analysis was also performed using a 1 : 100 diluted human serum from a veterinary physician scratched by a diseased cat with feline-poxvirus infection, collected 2 weeks after the onset of symptoms (C. Castilletti *et al*., unpubl. data). A secondary horseradish peroxidase-conjugated antihuman IgG, diluted 1 : 10 000 (Sigma-Aldrich), was used to bind the primary antibodies. The signal was developed using 3,3′,5,5′-tetramethylbenzidine (TMB).

Capture ELISA

Crude soluble extracts were obtained by the homogenization of leaves from nuclear transgenic and transplastomic plants in high salt buffer [1 × phosphate-buffered saline (PBS), 0.5 M NaCl, 10 mM EDTA, 1 mM PMSF]. The extracts were centrifuged at 18 900 ***g*** for 5 min and the supernatants were collected. Microtitre plates (high

bind polystyrene EIA/RIA 96-well microplate, Corning Costar, Corning, NY, USA) were coated overnight with a 1 : 500 dilution of the mouse monoclonal antibody anti-A27L protein (BEI Resources, Manassas, VA, USA), and then washed and blocked for 1 h at 37 °C with 5% milk in PBS-T (PBS + 0.05% Tween-20). Three samples of varying dilutions of each plant extract were added, starting from 6.25 and 3.12 μg of total proteins from transgenic and transplastomic lines, respectively (1 h at 37 °C). The plates were washed three times with PBS-T and incubated with 1 : 2000 diluted polyclonal anti-VACV (WR) A27L antiserum (BEI Resources; 1 h at 37 °C). The plates were washed again and incubated with a 1 : 5000 dilution in 1% milk of anti-rabbit peroxidase-conjugated antibody (GE Healthcare) for 1 h at 37 °C. Alternatively, plates were incubated with a 1 : 30 diluted human serum (C. Castilletti *et al*., unpubl. data), followed by an antihuman IgG (Fab specific)-peroxidase antibody diluted 1 : 10 000 (Sigma-Aldrich). Plates were developed with the TMB Peroxidase EIA Substrate Kit (Bio-Rad) for 5 min at room temperature. The absorbance at 450 nm was measured spectrophotometrically in a Victor Microplate reader (Perkin-Elmer, Waltham, MA, USA). ELISA data obtained by anti-A27L ELISA were converted to milligram/kilogram or milligram/gram of FW by reference to a standard curve constructed using baculovirus-derived VACV (WR) A27L protein (BEI Resources).

Analysis of oligomeric state by velocity gradient centrifugation

For velocity centrifugation on sucrose gradients, young leaves were homogenized in ice-cold 0.2% Triton X-100, 200 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, pH 7.8, supplemented with Complete protease inhibitor cocktail, using a 2 : 1 volume to FW ratio. The homogenate (about 700 μL) was loaded onto a linear 5%–25% (w/v) sucrose gradient prepared in 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 50 mM Tris-HCl, pH 7.5. After centrifugation at 154 700 ***g*** for 17 h at 4 °C in a Beckman SW40 rotor (Beckman Instruments, Fullerton, CA, USA) fractions of about 650 μL were collected. An equal aliquot of each fraction was analysed by SDS-PAGE and protein blot using anti-FLAG rabbit polyclonal antibodies. The sedimentation markers cytochrome C (12.4 kDa) and bovine serum albumin (67 kDa) were fractionated on a similar gradient and visualized by SDS-PAGE and Coomassie blue staining.

Subcellular fractionation and chloroplast isolation

For subcellular fractionation, leaves were homogenized in an ice-cold mortar with 10 mM KCl, 100 mM Tris-HCl, pH 7.8, containing 12% (w/w) sucrose and 2 mM $MgCl_2$, using 4 mL of buffer per gram of fresh leaf tissue. Eight hundred microlitres were loaded onto a 11.5-μL linear 16%–55% (w/w) sucrose gradient prepared in the same buffer. After centrifugation at 154 400 ***g*** for 2 h at 4 °C in a Beckman SW40 rotor, fractions were collected and an equal aliquot of each fraction was analysed by SDS-PAGE and protein blot. Protein blot and immunodetection used the anti-FLAG rabbit polyclonal antibodies, as described above.

Chloroplasts were isolated from transplastomic and wild-type tobacco plants as follows: leaves (1 g) were cut into small pieces and homogenized with 7 mL of ice-cold SRM buffer [50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES)-KOH, pH 8.0, 0.33 M sorbitol and Complete protease inhibitor cocktail (Roche)]. The homogenates were filtered through four layers of Miracloth and the filtrates were centrifuged for 5 min at 650 ***g***. The supernatants were discarded and the pellets were resuspended in 1.5 mL SRM buffer and layered onto the top of a two-step 40%–80% Percoll step gradient. The gradients were centrifuged for 20 min at 7000 ***g***. Intact chloroplasts were carefully removed from the interface with a Pasteur pipette, diluted in 10 mL of SRM buffer and centrifuged for 1 min at 8000 ***g***. The integrity of the isolated chloroplasts was checked using light microscopy. Chloroplast pellets were resuspended in 600 μL of lysis buffer (5 mM dithiothreitol, 1 mM EDTA, 10 mM Tris-HCl, pH 8) and placed on ice for 10 min. After centrifugation at 14 000 ***g*** for 5 min, the resulting supernatants (stroma) were removed and the membrane pellets were resuspended in the same buffer. Subcellular fractions were subjected to Western blot analysis using anti-FLAG polyclonal antibodies (1 : 1000 dilution) or rabbit polyclonal antiserum raised against a C-terminal portion of *Arabidopsis* endoplasmin (Klein *et al*., 2006; 1 : 1000 dilution).

Immunofluorescence microscopy

One million protoplasts isolated from the young leaves of A27L transplastomic plants or tobacco wild-type plants were washed with 4 vol of W5 medium (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2.2H_2O$, 5 mM glucose), pelleted for 10 min at 60 ***g*** and resuspended in MaCa buffer [0.5 mM mannitol, 20 mM $CaCl_2$, 0.1% (w/v) 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.7] containing 4% (w/v) paraformaldehyde for 2 h at room temperature. After fixation, protoplasts were washed with 4 vol of W5 medium and permeabilized with TSW buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.02% SDS, 0.1% Triton X-100) for 30 min. Protoplasts were then incubated with the primary antibody (anti-FLAG 1 : 100) diluted in TSW for 1 h at room temperature. After three washes in TSW, the protoplasts were incubated for 1 h at room temperature with Alexa.Fluor488 goat anti-rabbit secondary antibodies (1 : 1000 dilution; Invitrogen-Molecular Probes, Eugene, OR, USA). After three final washes in TSW, protoplasts were resuspended in PBS–glycerol supplemented with 0.1% phenylenediamine, an antifade agent. Fluorescence was visualized using a Zeiss Axiovert 200 microscope equipped for epifluorescence, followed by the collection of optical sections using Zeiss Apotome and Axiovision 4.1 software.

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