Plant Mol Biol (2007) 63:393–404
DOI 10.1007/s11103-006-9096-9

Amplicon-plus targeting technology (APTT) for rapid production of a highly unstable vaccine protein in tobacco plants

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Received: 27 May 2006 / Accepted: 3 October 2006 / Published online: 14 January 2007

Abstract High-level expression of transgenes is essential for cost-effective production of valuable pharmaceutical proteins in plants. However, transgenic proteins often accumulate in plants at low levels. Low levels of protein accumulation can be caused by many factors including post-transcriptional gene silencing (PTGS) and/or rapid turnover of the transgenic proteins. We have developed an Amplicon-plus Targeting Technology (APTT), by using novel combination of known techniques that appears to overcome both of these factors. By using this technology, we have successfully expressed the highly-labile L1 protein of canine oral papillomavirus (COPV L1) by infecting transgenic tobacco plants expressing a suppressor of post-transcriptional gene silencing (PTGS) with a PVX amplicon carrying a gene encoding L1, and targeting the vaccine protein into the chloroplasts. Further, a scalable ''wound-and-agrospray'' inoculation method has been developed that will permit high-throughput *Agrobacterium* inoculation of *Nicotiana tabacum*, and a spray-only method (named ''agrospray'') for use with *N. benthamiana* to allow large-scale application of this technology. The good yield and short interval from inoculation to harvest characteristic of APTT, combined with the potential for high-throughput achieved by use of the agrospray inoculation protocol, make this system a very promising technology for producing high value recombinant proteins, especially those known to be highly labile, in plants for a wide range of applications including producing vaccines against rapidly evolving pathogens and for the rapid response needed to meet bio-defense emergencies.

Keywords Amplicon-plus Targeting Technology · Labile protein · Vaccine · Transient expression · Tobacco · Agrospray

Abbreviations

APTT	Amplicon-plus Targeting Technology
CTP	chloroplast targeting peptide
P1/HC-Pro	a mutated version of component-proteinase from Tobacco Etch Virus
PTGS	post transcriptional gene silencing
PVX	potato virus X
SiRNA	small interfering RNA

Introduction

Plant biotechnology, including the production of pharmaceutical proteins in plants, typically relies on one of two different systems for delivery and expression of heterologous genes: stable genetic transformation and transient expression using plant viral vectors (Marillonnet et al., 2005). Since the first expression in transgenic plants of pharmaceutically relevant proteins, a human growth hormone (Barta et al., 1986), and an antibody (Hiatt et al., 1989), many different pharmaceutical proteins have been expressed successfully in plants (Canizares et al., 2005; Grevich and Daniell, 2005; Ma et al., 2003).

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Remarkable progress has been made in recent years toward understanding and improving viral vectors used to express recombinant proteins in plants (Canizares et al., 2005; Marillonnet et al., 2005; Pogue et al., 2002). Viral-based transient gene expression of recombinant proteins has several advantages over stable transformation, most notably, the ability to produce proteins without time-consuming transformation and regeneration of plants. In some cases, useful amounts of recombinant proteins can be recovered from treated tissue in as little as 1–2 weeks (Canizares et al., 2005; Gleba et al., 2005; Pogue et al., 2002). Several proteins destined to be used as vaccines have been successfully expressed using viral vectors such as those based on tobacco mosaic virus (TMV) and potato virus X (PVX) (Canizares et al., 2005). However, productivity in viral vector expression systems can be greatly reduced by post transcriptional gene silencing (PTGS) (Angell and Baulcombe, 1997), by what is thought to be a plant defensive response against virus infection (Lindbo et al., 2001; Marathe et al., 2000a). Specific RNAs are targeted for degradation (Lindbo et al., 2001; Marathe et al., 2000a), greatly reducing the amount of translatable message present. This limitation was effectively addressed by the amplicon-plus system (Mallory et al., 2002). In that system, after crossing stably-transformed tobacco plants (line 'TEV-B') expressing the mutated P1/HC-Pro suppressor of PTGS with a stably-transformed line carrying an amplicon encoding a replicating PVX virus vector exhibited very high levels of replication. A β-glucuronidase (GUS) protein encoded by the amplicon accumulated to very high levels in the progeny plants.

In the current study, the amplicon-plus system has been modified in several important ways that permit expression and accumulation of significant amounts of a labile recombinant protein in as little as 1–2 weeks. First, a replicating amplicon vector carrying our gene of interest, *vcp* (a synthetic version of the COPV L1 gene; see below), was introduced into tobacco plants (transgenic line TEV-B, see below) by infiltrating leaves with a suspension of *Agrobacterium* carrying the amplicon, instead of delivering the amplicon by stable transformation. Further, initial experiments indicated that use of the amplicon-plus system alone did not result in accumulation of appreciable amounts of the very labile L1 protein despite the presence of large amounts of viral RNA. Therefore, the system was modified to target L1 to various cellular compartments by creating fusions between the protein of interest and different targeting peptides. With these modifications, L1 protein accumulated in the plants most successfully when the protein was targeted to the chloroplasts. We have termed this system Amplicon-plus Targeting Technology (APTT).

Existing agroinfiltration systems were inefficient and unlikely to be economically viable (Pogue et al., 2002). In order to address this shortcoming, and to improve the utility and economic value of the APTT system for large-scale production applications, a high-throughput "wound and agrospray" method was developed for *Agrobacterium* inoculation of tobacco plants, and an "agrospray" only method was developed to facilitate large-scale inoculation of the alternative host *Nicotiana benthamiana.*

L1, the primary coat protein from canine oral papillomavirus (COPV) was chosen as the model protein with which to develop and test APTT for several reasons. First, effective vaccines against papillomaviruses can be made using the L1 coat protein of the target virus, and COPV has been shown to be an excellent model for this type of vaccine (Suzich et al., 1995). Importantly, recombinant L1 is broadly susceptible to factors, such as enzymatic proteolysis, that result in its degradation, thus reducing recovery of the recombinant protein from various sources (Biemelt et al., 2003; Warzecha et al., 2003). In our hands, it is significantly more labile than commonly-used reporter proteins, such as β-glucuronidase (GUS) or various green fluorescent proteins (Weissinger et al., unpublished), and is far more difficult to isolate from stably transformed plants than bovine lysozyme (Wilcox et al., 1997). Because the native coding sequence includes a nuclear localization signal, the L1 protein is targeted to the nuclei of oral mucosal cells in dogs infected with the virus. However, it is unclear that this nuclear localization sequence is operative in plant cells (data not shown). L1 therefore serves as a useful model with which to test the efficacy of targeting the protein to various sub-cellular locations as a means of increasing yield of recombinant product from plants.

Materials and methods

Expression vectors

The L1 gene from COPV (Suzich et al., 1995), which encodes the primary capsid protein L1, was modified to use plant-preferred codons, and to eliminate potential cryptic splicing sites and polyadenylation signal-like internal sequences (Perlak et al., 1991) by Aptagen, Inc, Herndon, VA. The custom-synthesized version of the COPV L1 gene was designated "*vcp*" (GenBank accession no. DQ508357; Weissinger et al., in preparation).

The PVX amplicon vector pGR106 (Lu et al., 2003) was kindly provided by Dr. D. Baulcombe. Four constructs based on pGR106 were made to express the L1 protein in cytosol, and to target the protein to the chloroplast, the endoplasmic reticulum (ER), or the apoplast (Fig. 1). The *vcp* gene was inserted at the blunted *Not*I site in pGR106 to make ''pKA19'' (Fig. 1). For chloroplast targeting, the chloroplast transit peptide (CTP) coding sequence of ribulose-1,5-bisphosphate carboxylase oxidase (rubisco) small subunit from tobacco (GenBank accession No. X02353) was obtained by PCR amplification of the sequence from tobacco DNA. The sequence was then fused with the 5' end of the *vcp* coding sequence. PCR based precise in-frame fusion of the CTP and the *vcp* gene was carried out using the three primer approach (Yon and Fried, 1989). The resulting fusion gene (CTP::*vcp*, 1.7 kb) was inserted into vector pGR106 at the blunted *Not*I site and the resulting construct was designated ''pKA20'' (Fig. 1). In a similar approach, the TEV 5' untranslated leader sequence (for translation enhancement) and the ER-targeting signal peptide (SP) from the tobacco PR1a gene (Xu et al., 2002) was fused in-frame with the 5' end of the *vcp* coding sequence. The sequence encoding the ER retrieval signal KDEL was included as a part of the reverse PCR primer so that it was fused in frame with the 3' end of the *vcp* gene. The resulting fusion sequence (TEV leader::SP::*vcp*::KDEL) was inserted at the blunted *Not*I site in vector pGR106, resulting in the construct designated ''pKA21'' (Fig. 1). A similar construct was made without the KDEL to target the protein to the apoplast. The fusion construct (TEV leader::SP::*vcp*), cloned in pGR106 as described above, was designated ''pKA22'' (Fig. 1). These constructs were used to transform *Agrobacterium tumefaciens* strain GV3101 containing the helper plasmid pJIC SA_Rep (≤http://www.jic.ac.uk/sainsbury-lab/dcb/Services/vigsprotocol.htm≥) using the freeze-thaw method (An et al., 1988).

Agroinfiltration of plants

Experiments were carried out using *N. tabacum* cv. Xanthi, *N. benthamiana*, and homozygous transgenic TEV-B plants (made in the tobacco cv. Xanthi, courtesy of Dr. V. Vance) containing a mutated P1/HC-Pro gene from TEV that suppresses post-transcriptional gene silencing (Mallory et al., 2002). Preparation of *Agrobacterium* cultures and infiltration of tobacco plants were carried out as described (English et al., 1997) with the following modifications. The recombinant bacteria were grown overnight in 50 ml of LB medium containing 100 μM acetosyringone and 10 μM MES (pH 5.6), and subsequently were pelleted by centrifugation at 4000 *g* for 5 min. The pellets were resuspended in the infection medium [Murashige and Skoog salts with vitamins, 2% sucrose, 500 μM MES (pH 5.6), 10 μM $MgSO_4$, and 100 μM acetosyringone] to OD_{600} = 0.4–1.0 and subsequently held at 28°C for 2–3 h. Infiltration of individual leaves was carried out on 4–5 weeks old recipient plants using a 2 ml syringe by pressing the tip of the syringe (without a needle) against the abaxial surface of the leaf. Infiltrated plants were maintained at 21–22°C with a photoperiod of 16 h light and 8 h dark.

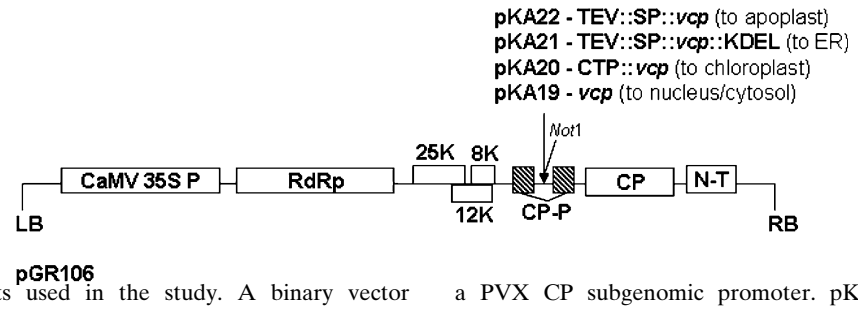

Fig. 1 Gene constructs used in the study. A binary vector containing potato virus X (PVX) amplicon, pGR106 Lu et al. (2003), was the basis for all constructs. In pGR106, ''CaMV 35S P'' is the CaMV 35S promoter; ''RdRp'' is the sequence encoding RNA-dependent RNA polymerase from PVX; ''25 K'', ''12 K'', and ''8 K'' are, collectively, the ''triple gene block'' encoding three specific movement proteins of PVX; ''CP-P'' designates a duplicated coat protein subgenomic promoter from PVX; ''CP'' designates a sequence encoding the PVX coat protein; and, ''N–T'' designates the *nos* terminator. ''LB'' and ''RB'' designate the left and right border sequences of the T-DNA of *A. tumefaciens*. The codon optimized *vcp* cDNA (Weissinger et al., in preparation) and its derivatives were inserted at the *Not*I site of a polylinker within pGR106, driven by a PVX CP subgenomic promoter. pKA19 contains the *vcp* coding sequence. pKA20 carries a CTP coding sequence fused with 5' terminus of *vcp*, thereby encoding a fusion protein to target L1 to the chloroplasts. pKA21 incorporates sequences encoding an untranslated TEV leader sequence (labeled ''TEV''), a signal peptide (SP) for endoplasmic reticulum (ER) targeting, *vcp* and the ER retrieval signal, KDEL. The translation product of this construct is a fusion protein in which the SP is fused to the amino terminus of L1, and the KDEL sequence is fused with its carboxyl terminus. Expression of this construct is expected to result in retention of the entire L1 fusion protein within the ER. pKA22 is identical to pKA21 except that it does not include the KDEL coding sequence, resulting in expulsion of the protein into the apoplast

Inoculation of plants by agrospray

Agrobacterium harboring pKA20 was prepared as described, but the infection medium was supplemented with Tween 20 [0.01% (v/v)]. The suspension of *Agrobacterium* was sprayed onto plants using an airbrush, Model 200 NH, connected to a compressor that produced 20–50 PSI (Badger Air-Brush Co, Illinois, USA). Before agrospray, plants were wounded by slightly scoring the adaxial surfaces of upper leaves with the edge of a plastic pot label.

RNA analysis

Total RNA was extracted using Trizol Reagent (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA (5–10 μg/lane) was separated by 1.2% formaldehyde-agarose gels, and blotted onto Hybond-N nylon membrane (Amersham Biosciences, Piscataway, NJ, USA) by the manufacturer's protocols. Blots were hybridized with a probe consisting of the full-length *vcp* coding sequence labeled with ^{32}P-[dCTP] using the Prime-It II® random priming kit (Stratagene, Cedar Creek, TX, USA), and washed according to the manufacturer's instruction.

Isolation and detection of siRNAs

Short interfering RNAs (siRNA) were detected as described by Dalmay et al. (2000) with modifications as described below. For Northern-blot analysis, 10 μg of low molecular weight RNA was mixed with loading dye containing formamide and bromophenol blue (final concentration 0.025%), and subjected to electrophoresis in a 15% polyacrylamide/7 M urea Ready Gel (Bio-Rad, Hercules, CA, USA) in 0.5 × TBE buffer at 180 V. The RNA was electroblotted onto a Hybond XL nylon membrane at 100 V for 1 h in 0.5 × TBE. Single-stranded ^{32}P-UTP labeled RNA probes were made from the *vcp* gene cloned in a pBSSKII vector using Promega (Madison, WI, USA) Riboprobe system according to the manufacturer's instructions. Antisense *vcp* RNA was transcribed in vitro using T3 polymerase. The probes were hydrolyzed and hybridization was carried out as described (Dalmay et al., 2000).

Immuno-blot analysis

Total soluble protein was extracted, using a buffer described by Weissinger et al. (in preparation) modified from that of Biemelt et al. (2003). Total soluble protein was quantified using the Protein Assay Dye Reagent Concentrate® (Bio-Rad). Samples from infiltrated and systemically infected leaves were collected separately for analyses involving comparison of L1 accumulation in these tissues from a single plant. In other experiments, in which the goal was measurement of the recombinant protein within the whole plant, quantitative L1 assays were carried out on extracts prepared from an aggregate of all leaves from a single whole plant. Crude protein extracts (11–25 μg per lane) were separated on 10% Tris–HCl gels and transferred to 0.45 μM nitrocellulose membranes. Bound L1 protein was detected by incubating the blots with a primary antibody "B1", which is a monoclonal antibody raised against an epitope present on the linear L1 protein (A. B. Jenson, personal communication), followed by incubation with a secondary antibody and chemiluminescence reagents from the WesternBreeze™ kit (Invitrogen), according to the manufacturer's protocol.

Chloroplast isolation and detection of L1 protein

Chloroplasts were isolated from infiltrated leaves essentially as described (Mills and Joy, 1980) with the following major modification. TEV-B and Xanthi tobacco plants were infiltrated with *Agrobacterium* containing the pKA20 construct. Twelve days after infiltration, the infiltrated and control plants were maintained in the dark for 48–60 h to reduce chloroplast starch levels. Approximately 4 gm of leaf tissue comprising a bulk sample of infiltrated and systemically infected leaves was harvested, washed with ice-cold sterile water, and sliced into small pieces (2×2 cm^2). The sliced leaf pieces were placed in 50 ml ice-cold chloroplast isolation buffer (CIB) [330 mM sorbitol, 50 mM Tris–HCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.1% BSA, and 25 mM β-mercaptoethanol, pH 8.0] and were homogenized using a small Warring commercial Blender (Fisher, Pittsburgh, PA, USA) with two bursts of 3 s each, at the low speed setting. The homogenate was filtered through three layers of cheesecloth and three layers of Miracloth (Calbiochem, La Jolla, CA, USA). The filtrate was aliquoted into 50 ml Falcon centrifuge tubes and centrifuged at 1500 *g* for 7 min at 4°C. The supernatant was discarded and the pellets were re-suspended in 1.5 ml CIB per tube. The suspension was carefully layered on 40–80% discontinuous Percoll (MP Biomedicals LLC, Aurora, OH, USA) gradients (5 and 4 ml, respectively) and centrifuged at 2000 *g* for 7 min at 4°C. Intact chloroplasts were collected at the interface of 40 and 80% Percoll layers and suspended with 1 ml CIB without BSA and β-mercaptoethanol, and centrifuged at 2500 *g* for 6 min at 4°C. The

supernatant was discarded and three 2.5 mm glass beads (Biospec, Bartlesville, OK, USA) were added to the pellet in 1.5 ml Eppendorf tube, which was then flash frozen in liquid nitrogen. The pellet was thawed on ice and homogenized in a Silamat S5 amalgamator (Ivoclar Vivadent, Amherst, NY, USA). Finally, the pellet containing the chloroplast fraction was resuspended in a modified protein extraction buffer (Biemelt et al., 2003). Eleven micrograms of total soluble protein was separated on a 10% Tris–HCL gel, transferred to nitrocellulose, and immuno-blot analysis was carried out as described above.

Results

Agroinfiltration of TEV-B plants to express COPV L1 protein

Agrobacterium infiltration was used to infect TEV-B plants with various PVX amplicon constructs carrying the *vcp* gene (Fig. 1). TEV-B, a tobacco line derived from *N. tabacum* cv. 'Xanthi' that has been stably transformed with a mutated form of the P1/HC-Pro gene from tobacco etch virus, was chosen for agroinfiltration experiments because PTGS is effectively eliminated, without the deleterious phenotypic changes associated with use of the un-mutated form of P1/HC-Pro (Mallory et al., 2002). Both targeted and non-targeted constructs were tested in an attempt to maximize L1 accumulation. Targeted amplicon gene fusion constructs (Fig. 1) included targeting sequences that directed the L1 protein to the chloroplast, ER, or apoplast. Non-transgenic tobacco (control) plants were also infiltrated with *Agrobacterium* strains containing these constructs.

All infiltrated TEV-B plants showed symptoms similar to those of virus infection on both infiltrated leaves and other leaves of the plants, indicating ''systemic infection'' (see Fig. S1). Symptoms were observed on both infiltrated and systemically infected leaves 7–12 days post infiltration (d.p.i.) (Fig. S1b). Symptoms similar to those of systemic infection were only observed at the site of infiltration on the leaves of non-transgenic control plants of tobacco cv. 'Xanthi' (Fig. S1d). RNA (Northern blot) analysis revealed the presence of large amounts of viral RNAs with homology to *vcp* in the infiltrated leaves of both TEV-B and Xanthi plants when they were infiltrated with each of the constructs (Fig. 2A). Similar levels of RNA were detected in systemically infected leaves of the TEV-B plants but not in control Xanthi plants at 12 d.p.i. (Fig. 2B). By 18 d.p.i. RNA levels had dropped significantly, with the highest levels observed in plants

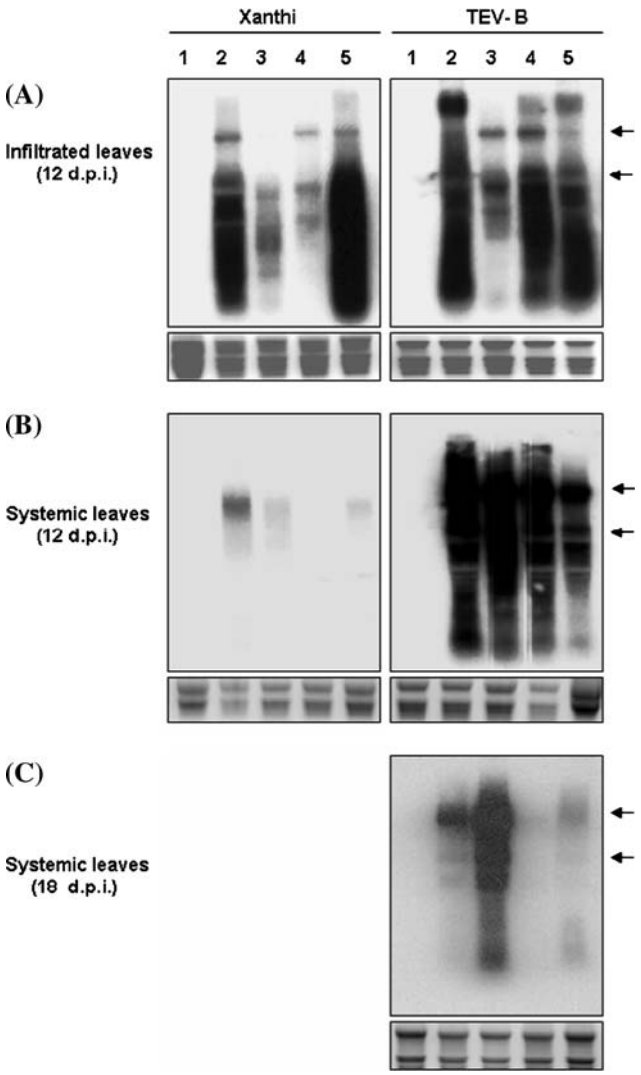

Fig. 2 Northern-blot analysis of RNA from agroinfiltrated plants. All blots probed with full-length *vcp*. Lanes 1, 2, 3, 4, and 5 correspond to RNA isolated from tissues either infiltrated or systemically infected with pG106, pKA19, pKA20, pKA21, and pKA22, respectively (Fig. 1). (**A**) Accumulation of viral RNA in infiltrated leaves of non-transgenic Xanthi and TEV-B transgenic plants 12 d.p.i. . (**B**) Accumulation of viral RNAs in systemically infected leaves 12 d.p.i. (Xanthi and TEV-B). (**C**) Accumulation of viral RNAs in systemically infected leaves 18 d.p.i. (TEV-B only). Arrows indicate the positions of genomic and sub-genomic *vcp*-containing viral RNAs. The lower panel in part figures a, b and c show the ribosomal RNA stained with ethidium bromide as a loading control

infected with pKA20, which incorporated a coding sequence expressing a fusion of L1 with a chloroplast targeting protein (Fig. 2C).

Immuno-blot analysis revealed significant accumulation of chloroplast-targeted L1 protein in both infiltrated leaves and systemically infected leaves from TEV-B plants (Fig. 3A), but L1 accumulated only in the infiltrated leaves of the control Xanthi plants

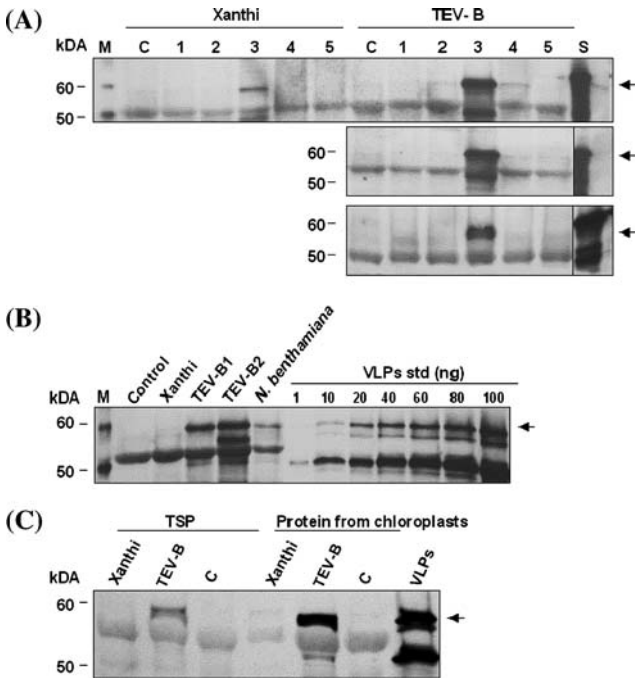

Fig. 3 Immuno-blot analysis of L1 protein expression in tobacco plants. Lane M, molecular weight standard; Lane C, TEV-B un-inoculated (negative) control plants; lanes 1–5, extracts of leaves from plants infiltrated with constructs pGR106, pKA19, pKA20, pKA21, and pKA22, respectively. Lane S, antibody binding control consisting of crude protein extract from *E. coli* expressing L1. The arrow indicates the positions of the intact L1 protein. L1 was detected using ''B1'' a monoclonal antibody raised against an epitope in the linear form of L1. The bands observed at approximately 50 kDa, below the authentic L1 protein band indicated by arrows in all immuno-blots, are the large subunit of ribulose bisphosphate carboxylase/oxidase (RuBisCO). (**A**) Detection of L1 in protein from infiltrated (upper panel) and systemically infected leaves (two lower panels) isolated from plants 12 and 18 d.p.i., respectively. Twenty-five micrograms total soluble protein per lane was used. (**B**) Immuno-blot used in quantification of L1 protein isolated from plants infiltrated with pKA20, which targets the L1 to the chloroplasts. TEV-B1, an aggregate of all leaves from a single whole plant. TEV-B2, an aggregate of all leaves from four different plants. Twenty-five micrograms total soluble protein per lane was used. VLPs, virus like particles consisting of COPV L1 subunits in a higher-order structure, were produced in baculovirus infected insect cells for use as a quantitative standard. (VLPs were the kind gift of Dr. A. B. Jenson.) (**C**) Enrichment of L1 protein in isolated chloroplasts from tobacco plants infiltrated with pKA20. Eleven micrograms of total soluble protein (TSP) from plants or chloroplast-enriched fractions from Xanthi and TEV-B plants were used. C; control, uninfiltrated TEV-B plants

(Fig. 3A see upper panel). Only very small quantities of L1 protein accumulated in the infiltrated leaves of TEV-B or Xanthi plants infiltrated with *Agrobacterium* carrying constructs other than pKA20 (Fig. 3A).

The level of L1 protein in un-inoculated control Xanthi plants, and in Xanthi, TEV-B and *N. benthamiana* plants infiltrated with pKA20 was measured by image analysis of immuno-blots of extracts from whole plants. The level of L1 protein in TEV-B plants was estimated to be about 3 ng/μg total soluble protein or 0.3% TSP (Fig. 3B) while L1 was barely detectable in the non-transgenic Xanthi plants. In *N. benthamiana*, the level of protein was estimated to be 0.04 ng/μg or 0.04% TSP (Fig. 3B). It is interesting to observe that in experiments in which leaf samples from four different infiltrated plants were pooled, two bands binding B1 antibody were observed (see TEV-B2, Fig. 3B). Although we are uncertain about the origin of the second band, both its size and antibody binding are consistent with the hypothesis that L1 is being degraded, and that this is a degradation product. It is worthy of note that this product is observed only when L1 is present in relatively high concentrations. Our results are consistent with the hypothesis that the L1 protein was targeted to the chloroplasts when pKA20 was used to infiltrate TEV-B plants. Results of an immuno-blot comparing L1 levels in total leaf protein with that in protein isolated from leaf extracts in which the chloroplast content had been enriched were consistent with this hypothesis, because L1 appeared to accumulate preferentially in the chloroplasts. The chloroplast-targeted L1 protein and an authentic L1 standard exhibited essentially identical electrophoretic mobility, suggesting, further, that the transit peptide was correctly cleaved after the fusion protein entered the chloroplasts (Fig. 3C).

The lack of L1 protein accumulation in Xanthi plants was thought to be due to PTGS of the viral amplicon RNA in these plants. RNA-blot analysis of low molecular weight RNA showed that PTGS-associated siRNAs corresponding to the PVX vector did accumulate in the infiltrated leaves of Xanthi plants, but not in TEV-B plants (Fig. 4).

The time course of systemic infection was investigated in the TEV-B plants following *Agrobacterium* infiltration of the two bottom leaves of the plants. Northern-blot analysis detected the presence of viral RNA in the infiltrated leaves 3 d.p.i.. There was no substantial difference in the amount of viral RNA accumulated over the period between 4 d.p.i. and 12 d.p.i. in these leaves (Fig. S2a). In systemically infected leaves, the viral RNA was first detected 6 d.p.i., and the greatest accumulation was observed by 12 d.p.i. (Fig. S2b).

Expression of COPV L1 protein with PVX amplicon in Nicotiana benthamiana

PVX amplicon RNA replication and movement, and L1 expression were evaluated in model plant

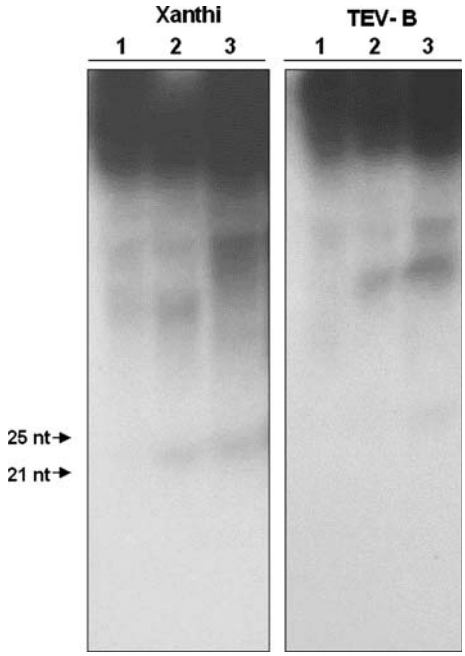

Fig. 4 siRNA analysis in Xanthi and TEV-B plants infiltrated with different constructs. Lane 1, pGR106 (as a control); Lane 2, pKA19; Lane 3, pKA20. Ten micrograms low molecular weight RNA per lane. siRNAs were detected using in vitro-transcribed ^{32}P-UTP labeled antisense *vcp* RNA as probes. Arrows indicate positions of molecular weight standards

N. benthamiana to compare function of the APTT system in this species with that in tobacco. Four-week-old, un-transformed *N. benthamiana* plants were infiltrated with *Agrobacterium* carrying each of the various constructs shown in Fig. 1. In contrast to *N. tabacum* cv. Xanthi plants infiltrated with these vectors, symptoms of systemic infection were observed in all infiltrated *N. benthamiana* plants by 7 d.p.i.. Viral RNAs that hybridized with a *vcp* probe were detected in both infiltrated and systemically infected leaves. However, significant degradation of the viral RNA was observed in Northern blots of extracts from systemically infected leaves, while most of the viral RNA appeared to be intact in the infiltrated leaves (Fig. S3a).

Interestingly, even though the viral RNA appeared to be degraded in the systemically infected leaves, L1 protein accumulated in both infiltrated and systemically infected leaves from plants infiltrated with the chloroplast-targeting construct, pKA20 (Fig. S3b). Infiltration with other constructs (Fig. 1) produced no detectable L1 protein. This result is similar to the L1 protein expression data obtained from TEV-B plants infiltrated with this construct, although less L1 protein accumulated in *N. benthamiana* than in TEV-B plants (Fig. S3b online, also see the quantitation data in Fig. 3B). Northern-blot analysis revealed that systemic movement occurred in *N. benthamiana* (see Fig. S3a), despite the presence of siRNAs, which accumulated at high levels in plants infected with each construct (Fig. S3c).

High throughput inoculation of tobacco and N. benthamiana by ''agrospray''

To apply APTT to a large-scale commercial production, a simple and efficient method was developed with which it is possible to inoculate a large number of tobacco plants simultaneously. Leaves of the recipient plants were wounded by scratching them lightly with the edge of a plastic pot label, and were then sprayed with an *Agrobacterium* solution using an airbrush (wound and agrospray). Alternatively, leaves were sprayed without preliminary wounding (agrospray only). Without the use of any mechanized apparatus, these procedures allowed inoculation of plants at a rate of approximately 100 plants/min/person, which is much faster than could be achieved by infiltrating individual plants using a syringe. Symptoms of systemic infection were observed at 12 d.p.i. in TEV-B plants infected with pKA20 using this method (agrospray), but not in Xanthi control plants (data not shown). Northern-blot analysis revealed that TEV-B plants infected using agrospray accumulated viral RNAs with homology to *vcp* at levels comparable to that observed in TEV-B plants infiltrated using a syringe (Fig. 5A), but infection only occurred when the plants were wounded prior to infiltration (data not shown). Imuno-blot analysis showed that recombinant COPV L1 protein accumulated in these plants at levels similar to those observed in plants infiltrated individually with the same *Agrobacterium* strain using the syringe method (Fig. 5B).

Similar experiments were carried out with and without mechanical wounding on *N. benthamiana* plants using *Agrobacterium* harboring the pKA20 vector. Plants exhibited symptoms of systemic infection at 7 d.p.i. (Fig. S4). No difference was observed in systemic symptoms regardless of whether or not plants were wounded prior to application of the agrospray (Fig. S4b, c) suggesting that simply applying an *Agrobacterium* suspension to leaves as a spray is sufficient to cause infection of *N. benthamiana*, with subsequent replication and systemic movement of the virus. Northern blot (Fig. 6A) and immuno-blot analyses (Fig. 6B) confirmed that comparable amounts of viral RNAs and L1 protein accumulated in both wounded and unwounded *N. benthamiana* plants.

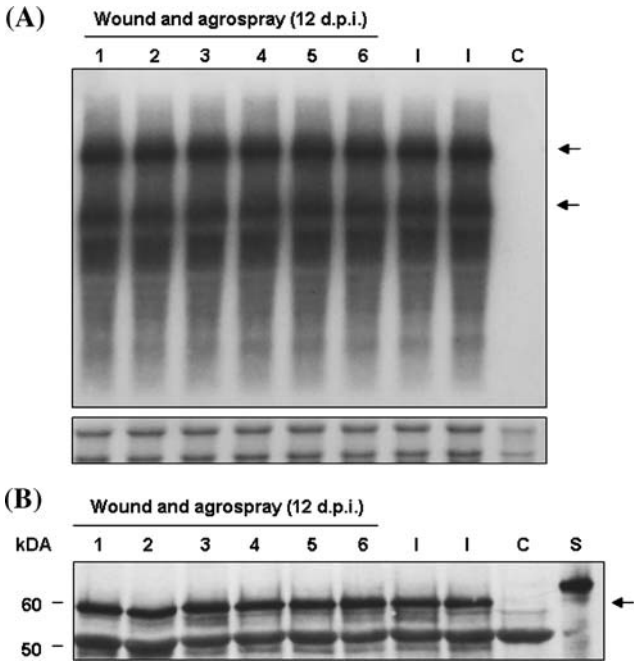

Fig. 5 Accumulation of *vcp* viral RNA and L1 protein from TEV-B plants treated by the ''wound and agrospray'' protocol. *Agrobacterium* carrying pKA20 was sprayed onto 4-week-old, slightly wounded, TEV-B plants. (**A**) Northern analysis. Five microgrmas RNA per lane. Lanes 1–6, samples from six individual ''agrosprayed'' TEV-B plants. For comparison, lanes ''I'' contain extract from agroinfiltrated TEV-B plants. Lane C, non-infected TEV-B plant negative control. RNA blots were probed with ^{32}P-dCTP labeled full-length *vcp*. The lower panel shows the ribosomal RNA bands stained with ethidium bromide as a loading control. (**B**) Immuno-blot analysis of L1 accumulation the same plants analyzed in Fig. 5a. Twenty-five micrograms total soluble protein was loaded in each lane. Antibody binding control (lane S), crude protein extract from *E. coli* expressing L1. The bands observed at approximately 50 kDa, below the authentic L1 protein band indicated by arrows in all immuno-blots, are the large subunit of ribulose bisphosphate carboxylase/oxidase (RuBisCO)

Discussion

We report here on the development of a novel technology for production of high-value recombinant proteins in plants, which we refer to as APTT. This new technology is comprised of several components that work together to overcome limitations of previous plant based transient protein production systems. These include: the use of a suppressor of PTGS to enhance systemic infection of tobacco, resulting in rapid production of expressing tissue from which protein can be recovered; inclusion of various targeting strategies to permit accumulation of the recombinant protein in specific sub-cellular locations, an important approach for the protection of highly-labile proteins; development of a simple and efficient ''wound and agrospray'' protocol that permits rapid inoculation of tobacco with amplicon constructs; and an ''agrospray only'' method that is effective for high-throughput inoculation of *N. benthamiana*, a possible alternative host for use with APTT.

APTT has two marked advantages over the original amplicon-plus system (Mallory et al., 2002), from which it was developed. First, in APTT the self-replicating amplicon is introduced into recipient plants by infiltration of one or a few leaves on a recipient plant with an *Agrobacterium* suspension harboring T-DNA encoding the amplicon. Transient expression of this sequence produces self-replicating RNA that can then move systemically to the remainder of the plant. This differs from the original system (Mallory et al., 2002) in which T-DNA encoding the amplicon was stably transformed into the recipient plants. Because stable transformation is avoided, APTT allows production of plants expressing the protein in very little time. Very importantly, infiltration of only a small number of leaves is sufficient to achieve systemic infection with the amplicon, causing expression of the recombinant protein in essentially all tissues of the plant. This makes it possible to generate a large, reliable supply of uniformly expressing biological material from which the protein of interest can be extracted, a critical requirement for scale-up.

We have further improved the utility of this system by the development of the ''agrospray'' protocol for inoculation of large numbers of plants. In this procedure, tobacco plants are wounded very slightly, and then sprayed with a suspension of *Agrobactrium* carrying the amplicon of interest. Such a system could readily be mechanized to inoculate very large numbers of plants quickly, to produce a high throughput production system for commercial purposes.

PTGS can severely limit accumulation of recombinant proteins in plants. However, APTT includes the use of a constitutively expressed P1/HC-Pro gene, known to eliminate the accumulation of the viral siRNAs that direct PTGS/VIGS in both tobacco and *N. benthamiana* (Mallory et al., 2001; Marathe et al., 2000b; Roth et al., 2004). Data reported here indicate that the mutated P1/HC-Pro element present in the TEV-B tobacco line effectively reduces PTGS in this system. In tobacco cv. Xanthi, which lacks this element, systemic infection was essentially eliminated, an observation consistent with PTGS. However, systemic infection occurred routinely following infiltration of one or a few leaves of the TEV-B plants.

Further, in the experiments reported above, expression of the recombinant protein occurred quickly following infiltration. A time course experiment revealed

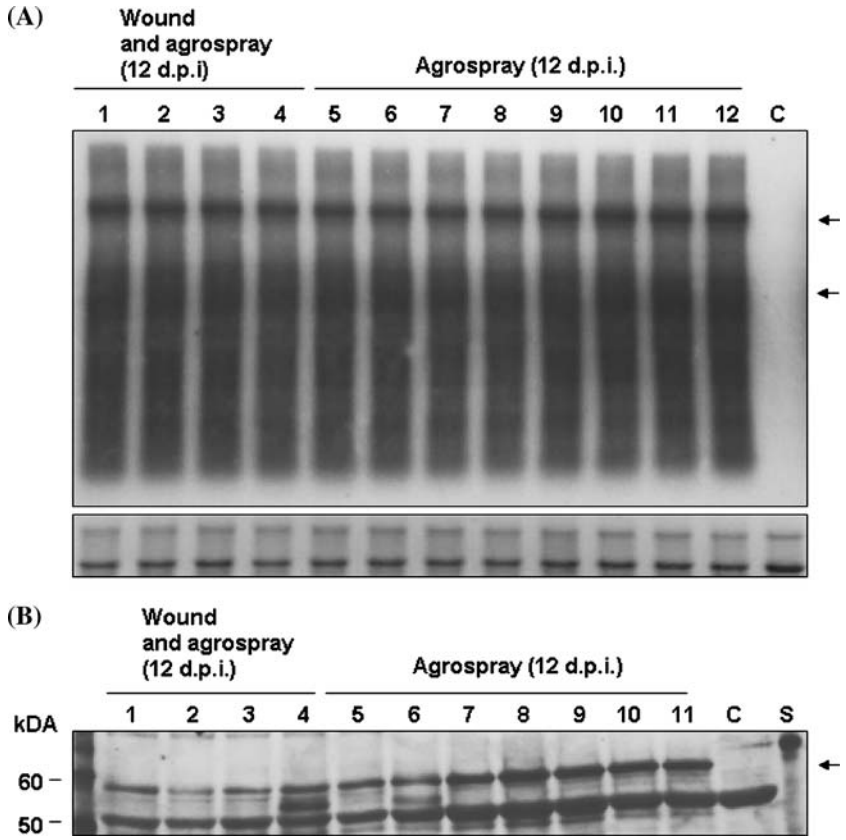

Fig. 6 Accumulation of *vcp* viral RNA and L1 protein from *N. benthamiana* plants treated by the agrospray protocol. (**A**) Northern analysis. Lanes 1–4, individual plants wounded and agrosprayed. Lanes 5–12, individual plants agrosprayed only without wounding. The lower panel shows the ribosomal RNA bands stained with ethidium bromide as a loading control. (**B**) Immuno-blot analysis of L1 accumulation in the same plants analyzed in Fig. 6a. Twenty-five micrograms total soluble protein was loaded in each lane. Antibody binding control (lane S), crude protein extract from *E. coli* expressing L1. The bands observed at approximately 50 kDa, below the authentic L1 protein band indicated by arrows in all immuno-blots, are the large subunit of ribulose bisphosphate carboxylase/oxidase (RuBisCO)

peak accumulation of viral RNA and L1 protein in TEV-B plants by 12 days after infiltration, so protein harvest could begin less than 2 weeks after plants are treated. Further, RNA replication and protein expression persisted to at least 18 days post inoculation, implying that protein recovery could be maximized by allowing additional biomass to accumulate as the plant continues to grow after peak expression levels have been achieved.

Our results indicate that high levels of recombinant protein expression can be achieved quickly in large masses of treated tobacco plants. Although there is a large literature attesting to the utility of tobacco in the production of various recombinant proteins, these data suggest that use of APTT makes tobacco an especially appropriate choice for commercial production of labile, high-value proteins. This is especially true when one also considers that tobacco can quickly produce significant amounts of biomass in a greenhouse (Weissinger et al., unpublished).

A second advantage of APTT is that the amplicons can be engineered to incorporate sequences that target the recombinant protein to specific sub-cellular compartments. This results in a marked increase in recombinant protein yield, presumably by stabilizing the protein and/or protecting it from cellular proteases. Concentration of the protein in specific organelles might also be used to facilitate purification of the recombinant protein.

Poor protein folding, and resulting instability has been shown to reduce accumulation of heterologous proteins in plants (Ma et al., 2003). This problem has been effectively addressed by targeting the proteins to specific organelles (Twyman et al., 2003). Our experiments revealed that L1 protein, chosen for these experiments because it is especially labile, accumulated to quite different levels when targeted to different subcellular locations, and achieving a maximum when targeted to chloroplasts. A possible explanation for this phenomenon is that microenvironments vary between

the organelles and cytosol, and among organelles, especially with regard to the protein hydrolyzing machinery, and this could result in differences in protein degradation. However, these experiments do not rule out alternative explanations. For example, a chaperone present in the chloroplast could assist in the correct folding of the L1 protein, increasing its stability. Further, the chloroplast targeting sequence may act as a translational enhancer, and/or it could be protecting nascent proteins from degradation in the cytosol (Jang et al., 1999). A recent report indicated that accumulation of a human growth hormone protein increased when it was targeted to apoplast, rather than to the chloroplast (Gils et al., 2005). Interestingly, a high level of viral RNAs was present at 18 d.p.i. in plants infiltrated with the pKA20 construct, but only a low level of RNA was present in plants infected with the other constructs. This suggests that the chloroplast targeting peptide sequence may in some way stabilize or affect the replication of the PVX amplicon (Fig. 2C).

The level of accumulation of a heterologous protein in plant cells could be affected by both the nature of the specific protein, and by factors inherent to specific locations in which the protein might be sequestered. It seems likely, therefore, that the sub-cellular target that is ideal for each protein, or class of proteins, must be determined empirically, and the appropriate APTT constructs developed to optimize accumulation.

Although the focus of this study was the development of an expression system for use with tobacco expressing the P1/HC-Pro suppressor of PTGS, other host systems might also be employed. *N. benthamiana*, which has often been used as a host for plant virus studies because of their susceptibility to infection by RNA viruses, is an interesting candidate. Many recombinant proteins have been expressed in this species in recent years (Canizares et al., 2005). Importantly, in our experiments systemic infection of *N. benthamiana* with a replicating amplicon could be achieved without the inclusion of an exogenous suppressor of PTGS. Although siRNA was observed, clearly indicating that PTGS was occurring in these plants, this did not prevent replication and systemic movement. Although PTGS appears to have degraded most of the viral RNAs in systemically infected leaves (see Fig. S3), L1 protein accumulation was observed in systemically infected tissues. This contrasts sharply with observations made in normal Xanthi tobacco plants, where PTGS prevented systemic infection and prevented accumulation of L1 in any but directly infiltrated leaves. Although recombinant protein expression was observed in *N. benthamiana* without the aid of P1/HC-Pro, systemic infection and overall expression of a recombinant protein in this host could likely be improved by the inclusion of P1/HC-Pro or other suppressor of PTGS (Voinnet et al., 2003).

Another factor that makes *N. benthamiana* an especially promising alternative host for the APTT system is its readiness with which it is infected by *Agrobacterium*. In this study, we demonstrated that infection could be achieved by spraying the plants with the bacterial suspension. Unlike tobacco, *N. benthamiana* became infected without preliminary wounding. This fact could further streamline the process of generating large, highly-expressing plant populations for commercial protein production.

This study clearly showed that heterologous protein expression could be limited by PTGS and rapid protein degradation. APTT offers a means for overcoming both of these obstacles in tobacco, by including both the P1/HC-Pro suppressor of PTGS and targeting sequences that permit accumulation of protein in the chloroplast where it is apparently protected from factors that reduce its stability in plant cells.

APTT is superior to previous plant-based transient protein expression systems that also use agroinfiltration to deliver expression constructs into recipient plants. For example, Voinnet et al. (2003) described a system in which *Agrobacterium* binary vectors carrying the p19 PTGS suppressor and a gene of interest are infiltrated into *N. benthamiana*. Icon Genetics has also recently developed a ''magnifection'' system (Marillonnet et al., 2005) in which entire plants are infiltrated with *Agrobacterium* carrying a TMV vector with a gene of interest. Unlike APTT, neither of these systems uses a replicating vector, which can spread throughout the plant from a small inoculation point. Further, neither of these other systems has the capacity to inoculate the large numbers of plants using the highly efficient ''wound and agrosparay'' or ''agrospray'' method that characterizes the APTT system. APTT therefore requires far less labor than either of these other methods to produce an equivalent amount of biomass expressing a recombinant protein. APTT is also better suited to scale-up needed to meet the demands of commercial production.

APTT effectively addresses three of the most important criteria required of an effective plant-based transient protein production system. First, suppression of PTGS by HC-Pro prevents accumulation of siRNAs, thus permitting accumulation of large amounts of viral RNA. It also permits systemic movement in tobacco of the self-replicating amplicons carrying the gene of interest. Second, through targeting of the protein to specific sub-cellular locations, recombinant protein, including those known to be highly unstable, can accumulate to high levels, greatly increasing overall

efficiency of the system. Third, by delivering a self-replicating amplicon into the plants *via* the ''wound and agrospray'' or ''agrospray'' procedures, it permits production of large amounts of biomass expressing the recombinant protein at minimal cost in a short period of time. This system is ideal not only for routine production of pharmaceutical proteins, but it is also well suited to production of proteins such as vaccines that may be needed quickly and in large amounts in response to health emergencies of natural origin, or those which might result from attack with biological weapons.

Acknowledgements We thank David Baulcombe for supplying us with the PVX amplicon vector pGR106, and *A. tumefaciens* strain GV3101 containing the helper plasmid pJIC SA_Rep, Vicki Vance for supplying us with seeds of the transgenic TEV-B line, and Nancy Magnuson for providing us with the vector pZW containing the TEV and SP sequences. We also thank A. Bennett Jenson and Shin-je Ghim for providing the B1 monoclonal antibody used in this study, and for helpful discussions regarding COPV L1. We thank Rebecca Boston and Raymond Long for useful discussions, and Jussi Joensuu and Mirkka Kotiaho for information about the chloroplast extraction buffer used in this study. We especially thank Lesley Corbin for her work with the immuno-blot analysis. We also thank Yan Liu and Tan Tuong for growing plants and collecting leaf samples for some experiments. This work was supported by a grant to A.K.W. from the United States Department of Agriculture (USDA-CSREES, Contract No. 2001-38712-10592).

References

An G, Ebert P, Mitra A, Ha S (1988) Binary vector. In: Gelvin SB, Schilproot RA (eds), Plant molecular biology manual. Kluwar Academic Publishers, Dordrecht, pp A3 1–19

Angell SM, Baulcombe DC (1997) Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. EMBO J 16:3675–3684

Barta A, Sommergruber K, Thompson D, Hartmuth K, Matzke MA, Matzke AJM, (1986) The expression of a nopaline synthase—human growth-hormone chimeric gene in transformed tobacco and sunflower callus-tissue. Plant Mol Biol 6:347–357

Biemelt S, Sonnewald U, Gaimbacher P, Willmitzer L, Muller M (2003) Production of human papillomavirus type 16 virus-like particles in transgenic plants. J Virol 77:9211–9220

Canizares MC, Nicholson L, Lomonossoff GP (2005) Use of viral vectors for vaccine production in plants. Immunol Cell Biol 83:263–270

Dalmay T, Hamilton A, Mueller E, Baulcombe DC (2000) Potato virus X amplicons in arabidopsis mediate genetic and epigenetic gene silencing. Plant Cell 12:369–379

English JJ, Davenport GF, Elmayan T, Vaucheret H, Baulcombe DC (1997). Requirement of sense transcription for homology-dependent virus resistance and trans-inactivation. Plant J 12:597–603

Gils M, Kandzia R, Marillonnet S, Klimyuk V, Gleba Y (2005) High-yield production of authentic human growth hormone using a plant virus-based expression system. Plant Biotechnol J 3:613–620

Gleba Y, Klimyuk V, Marillonnet S (2005) Magnifection—a new platform for expressing recombinant vaccines in plants. Vaccine 23:2042–2048

Grevich JJ, Daniell H (2005) Chloroplast genetic engineering: recent advances and future perspectives. Crit Rev Plant Sci 24:83–107

Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. Nature 342:76–78

Jang IC, Nahm BH, Kim JK (1999) Subcellular targeting of green fluorescent protein to plastids in transgenic rice plants provides a high-level expression system. Mol Breeding 5:453–461

Lindbo JA, Fitzmaurice WP, della-Cioppa G (2001) Virus-mediated reprogramming of gene expression in plants. Curr. Opin. Plant Biol. 4:181–185

Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, Wu AJ, Rathjen JP, Bendahmane A, Day L, Baulcombe DC (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. EMBO J 22:5690–5699

Ma JKC, Drake PMW, Christou P (2003) The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 4:794–805

Mallory AC, Ely L, Smith TH, Marathe R, Anandalakshmi R, Fagard M, Vaucheret H, Pruss G, Bowman L, Vance VB (2001) HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. Plant Cell 13:571–583

Mallory AC, Parks G, Endres MW, Baulcombe D, Bowman LH, Pruss GJ, Vance VB (2002) The amplicon-plus system for high-level expression of transgenes in plants. Nat Biotechnol 20:622–625

Marathe R, Anandalakshmi R, Smith TH, Pruss GJ, Vance VB (2000a) RNA viruses as inducers, suppressors and targets of post-transcriptional gene silencing. Plant Mol Biol 43:295–306

Marathe R, Smith TH, Anandalakshmi R, Bowman LH, Fagard M, Mourrain P, Vaucheret H, Vance VB (2000b) Plant viral suppressors of post-transcriptional silencing do not suppress transcriptional silencing. Plant J 22:51–59

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 23:718–723

Mills WR, Joy KW (1980) Rapid method for isolation of purified, physiologically active chloroplasts, used to study the intracellular-distribution of amino-acids in Pea leaves. Planta 148:75–83

Perlak FJ, Fuchs RL, Dean DA, Mcpherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA 88:3324–3328

Pogue GP, Lindbo JA, Garger SJ, Fitzmaurice WP (2002) Making an ally from an enemy: Plant virology and the new agriculture. Annu Rev Phytopathol 40:45–74

Roth BM, Pruss GJ, Vance VB (2004) Plant viral suppressors of RNA silencing. Virus Res 102:97–108

Suzich JA, Ghim SJ, Palmerhill FJ, White WI, Tamura JK, Bell JA, Newsome JA, Jenson AB, Schlegel R (1995) Systemic immunization with Papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci USA 92:11553–11557

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J 33:949–956

Warzecha H, Mason HS, Lane C, Tryggvesson A, Rybicki E, Williamson AL, Clements JD, Rose RC (2003) Oral immunogenicity of human papillomavirus-like particles expressed in potato. J Virol 77:8702–8711

Wilcox CP, Weissinger AK, Long RC, Fitzmaurice LC, Mirkov TE, Swaisgood HE (1997) Production and purification of an active bovine lysozyme in tobacco (*Nicotiana tabacum*): Utilization of value-added crop plants traditionally grown under intensive agriculture. J Agr Food Chem 45:2793–2797

Xu H, Montoya FU, Wang ZP, Lee JM, Reeves R, Linthicum DS, Magnuson NS (2002) Combined use of regulatory elements within the cDNA to increase the production of a soluble mouse single-chain antibody, scFv, from tobacco cell suspension cultures. Protein Expres Purif 24:384–394

Yon J, Fried M (1989) Precise gene fusion by PCR. Nucleic Acids Res 17:4895–4895

Plant Mol Biol (2007) 64:619
DOI 10.1007/s11103-007-9168-5

ERRATUM

Amplicon-plus Targeting Technology (APTT) for rapid production of a highly unstable vaccine protein in tobacco plants

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Published online: 24 April 2007

Erratum to: Plant Mol Biol (2007) 63: 393–404
DOI 10.1007/s11103-006-9096-9

Due to an unfortunate misunderstanding, the co-corresponding author and the electronic supplementary materials were not properly identified in the original publication. The correct representation of the authors and their affiliations is listed below.

The online version of the original article can be found at http://dx.doi.org/10.1007/s11103-006-9096-9

Electronic supplementary material The online version of this erratum (doi:10.1007/s11103-007-9168-5) contains the original supplementary material, which is available to authorized users.

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