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A DNA replicon system for rapid high-level production of virus-like particles in plants

Zhong Huang[1], **Qiang Chen**[1,2], **Brooke Hjelm**[1], **Charles Arntzen**[1], and **Hugh Mason**[1]

[1]The Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ 85287

[2]Department of Applied Biological Sciences, Arizona State University, Mesa, AZ 85212

Abstract

Recombinant virus-like particles (VLPs) represent a safe and effective vaccine strategy. We previously described a stable transgenic plant system for inexpensive production and oral delivery of VLP vaccines. However, the relatively low level antigen accumulation and long time frame to produce transgenic plants are the two major roadblocks in the practical development of plant-based VLP production. In this paper, we describe the optimization of geminivirus-derived DNA replicon vectors for rapid, high-yield plant-based production of VLPs. Co-delivery of bean yellow dwarf virus (BeYDV)-derived vector and Rep/RepA-supplying vector by agroinfiltration of *Nicotiana benthamiana* leaves resulted in efficient replicon amplification and robust protein production within five days. Co-expression of the P19 protein of tomato bush stunt virus, a gene silencing inhibitor, further enhanced VLP accumulation by stabilizing the mRNA. With this system, hepatitis B core antigen (HBc) and Norwalk virus capsid protein (NVCP) were produced at 0.80 and 0.34 mg/g leaf fresh weight, respectively. Sedimentation analysis and electron microscopy of transiently expressed antigens verified the efficient assembly of VLPs. Furthermore, a single replicon vector containing a built-in Rep/RepA cassette without p19 drove protein expression at similar levels as the three-component system. These results demonstrate the advantages of fast and high-level production of VLP-based vaccines using the BeYDV-derived DNA replicon system for transient expression in plants.

Keywords

Replicon; Viral vector; Virus-like particle; Transient expression; Vaccine

INTRODUCTION

A number of viral structural proteins, when over-expressed in recombinant expression systems, can self-assemble into organized macromolecular particulate structures that morphologically mimic authentic viruses and are therefore termed virus-like particles (VLPs). Recombinant VLPs represent a safe and effective vaccine strategy, because they lack viral nucleic acid and are thus absolutely noninfectious, they can be produced at large scales using recombinant expression systems that do not rely on viral replication, and most importantly, they are very potent immunogens. It has been well documented that VLPs of different origin are capable of eliciting potent humoral and cellular responses (Boisgerault et al., 2002; Garcea and Gissmann, 2004; Warfield et al., 2005). The strong immunogenicity of VLPs results from their efficient capture by and interaction with professional antigen presenting cells (APC) (Beyer et al., 2001; Boisgerault et al., 2002; Gamvrellis et al., 2004;

Correspondence to: Hugh Mason.

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Warfield et al., 2003), high-density repetitive display of B-cell epitopes on their surfaces, inclusion of potent helper T-cell epitopes, and direct activation of B cells (Milich and McLachlan, 1986).

Recombinant VLP for a variety of viruses has been produced in different prokaryotic and eukaryotic heterologous expression systems, including plants. The production of recombinant VLP vaccines in plants has many potential advantages over traditional microbial or animal systems, including (1) potential risks of contamination with animal or human pathogens (such as viruses and prions) and toxins are minimal (Daniell et al., 2001; Koprowski and Yusibov, 2001; Thanavala et al., 2006); (2) plant systems are more cost-effective than industrial facilities using fermentation or bioreactor systems (Koprowski and Yusibov, 2001); (3) vaccine production can be easily scaled up and the technology is already available for harvesting and processing plants and plant products on a large scale (Streatfield et al., 2001; Streatfield et al., 2003); (4) the purification step can be eliminated if the plant tissue containing vaccine antigen is used for oral immunization (Mason et al., 2002; Tacket et al., 1998).

The traditional approach to the plant production of VLPs has used stable transgenic plants. VLP-forming antigens of different origins expressed in transgenic plants have been shown to assemble into VLPs and their immunogenicity have been demonstrated in experimental animals when delivered by injection of purified forms or by oral consumption of unprocessed plant tissues (reviewed in (Santi et al., 2006; Thanavala et al., 2006). Phase I clinical trials using transgenic plant-derived hepatitis B surface antigen (HBsAg) and Norwalk virus capsid protein (NVCP) VLPs showed safety and oral immunogenicity in humans (Tacket et al., 2000; Thanavala et al., 2005). However, long generation time and modest level antigen accumulation (<1% total soluble protein or <0.1mg/g fresh weight) are two main factors limiting the practical application of transgenic plants for commercial production of VLPs.

As an alternative approach, plant virus-based transient expression has the potential of achieving high level antigen accumulation in a short period of time (≤ two weeks) [reviewed in (Canizares et al., 2005; Gleba et al., 2007; Lico et al., 2008; Yusibov et al., 2006)]. However, the difficulty in genetic manipulation of large full-length or near-full-length viral genomes and inconvenient infection procedures, which some times involve *in vitro* transcription of DNA to infectious RNA and the co-delivery of multiple DNA/RNA segments, represent major challenges in commercial application of this technology. For example, even the new generation tobacco mosaic virus (TMV)-based “deconstructed” vector system, requires simultaneous co-introduction of three vector modules into same cells for *in planta* assembly of the RNA replicon (Marillonnet et al., 2004). Thus, further development of simple, easily manipulated viral vectors is warranted.

Bean yellow dwarf virus (BeYDV), a Mastrevirus of the *Geminiviridae* family, has a single-stranded circular DNA genome that can replicate to very high copy number by a rolling circle mechanism. This genome is comprised of a long intergenic region (LIR), a short intergenic region (SIR) and four open reading frames (ORFs): V1, V2, C1 and C2 (Liu et al., 1997). These ORFs encode four proteins: the movement protein, the coat protein, and replication associated proteins Rep and RepA (Liu et al., 1997). For viral replication, both LIR and SIR are necessary *cis*-acting elements, and the Rep is the only viral protein required but can be supplied in trans (Mor et al., 2003). BeYDV-based vectors replicated efficiently and boosted protein expression in bombarded plant cell cultures (Mor et al., 2003) and in transgenic plants (Zhang and Mason, 2005). In this paper, we demonstrate further improvement of the BeYDV-derived DNA replicon vector system to allow rapid high-yield protein production in plants via transient expression. Through a series of optimization

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procedures, we ultimately developed a single-vector system capable of producing VLPs at 0.8 mg/g leaf fresh weight within four days after vector delivery. The rapidity, simplicity, and high-yield potential of this vector system thus greatly enhance the commercial feasibility of VLP vaccine production in plants.

MATERIALS AND METHODS

Vector construction

A plasmid pBY036 containing the native BeYDV C1/C2 genes between the 35S promoter/TEV 5' UTR and the VSP terminator was described previously (Mor et al., 2003). The C1/C2 expression cassette was released from pBY036 by XhoI/SacI digestion and inserted into pPS1(Huang and Mason, 2004) from the same site, making binary vector pREP110 for expression of both Rep and RepA (Fig. 1). The binary vector pREP111 was similarly generated using the intron-deleted Rep gene from pBY037 (Mor et al., 2003) for expression of Rep only. The P19 gene from tomato bushy stunt virus was amplified from pTBSV (H. Scholthof, Texas A&M University) using primers P19-Bam (5'-TCAAGGATCCATGGAACGAGCTATACA) and P19-Sac (5'-AGAGGAGCTCTTACTCGCCTTCTTTTTC), digested with NcoI and SacI and inserted in pPS1, yielding binary vector pPSP19.

Replicon vectors were constructed based on backbone vector pBY023 (Mor et al., 2003). The NVCP replicon in pBYsNV410 (Zhang and Mason, 2005) was released by digestion with AscI and FseI and ligated with pBY023 at the same sites to make pBYNVCP. The GFP gene was amplified from pICH7410 (Marillonnet et al., 2004)(Icon Genetics) using primers P-GFP/NcoI.F (5-GTCACCATGGTGAGCAAGGGCGAG) and P-GFP/SacI.R (5'-ATTAGAGCTCTTACTTGTACAGCTCGTC), digested with NcoI and SacI, and inserted into pIBT210 (Haq et al., 1995) to make pGFPi210. The XhoI-SacI fragment containing TEV 5'UTR-GFP was ligated into the same sites of pBYsNV110 (Zhang and Mason, 2005) to make pBYGFP. The hepatitis B core antigen gene (HBc) was obtained from pICH-HBc (Huang et al., 2006) by digestion with NcoI-SacI, ligated into pIBT210, and then subcloned via XhoI-SacI into pBYGFP to make pBYHBc. Rep gene-containing replicons were made by insertion of the 727 bp BamHI fragment of BeYDV C1/C2 gene from pBY002 (Mor et al., 2003) into pBYGFP and pBYHBc to make pBYGFP.R and pBYHBc.R, respectively.

Agroinfiltration procedure

Binary vectors were separately introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. The resulting strains were verified by restriction digest of plasmids, grown overnight at 30° and used to infiltrate leaves of 6 to 8 week-old greenhouse-grown *Nicotiana benthamiana* plants. Briefly, the bacteria were pelleted by centrifugation for 5 min at 5,000×g and then resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH5.5 and 10 mM $MgSO_4$) to $OD_{600} = 0.2$. The resulting bacterial suspensions were injected by using a syringe without needle, either alone or as a mixture of several strains, into fully expanded leaves through a small puncture (Huang and Mason, 2004).

Plant DNA extraction and Southern blotting

Total DNA from plant leaves was extracted as described (Zhang and Mason, 2005). DNA (~3 μg) was either undigested or digested with XhoI, resolved in 0.8% agarose gel, stained with ethidium bromide, transferred to a nylon membrane (Zeta-probe, Bio-Rad, Hercules, CA). Membranes were then hybridized with a digoxygenin (DIG)-labeled HBc-specific probe, which was synthesized by PCR with primers anchored to 5' (-TAGCCATGGACATTGACCCTT-) and 3' (-TTAACATTGAGATTCCCT-) ends of the

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HBc gene, respectively, according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN).

Plant RNA extraction and Northern blotting

Total RNA isolation and RNA blot analysis were performed as described (Zhang and Mason, 2005). Briefly, RNA was extracted from plant leaves with Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, resolved in 0.8% agarose gel, and transferred to a nylon membrane (Zeta-probe, Bio-Rad, Hercules, CA). The transcripts of HBc on the membrane were detected using DIG-labeled HBc-specific probes prepared as for DNA blots.

Protein analysis

For HBc analysis, total soluble protein from *N. benthamiana* leaf was extracted and measured as previously described (Huang et al., 2005). Total HBc was quantified by a sandwich ELISA as previously described (Huang et al., 2006) with slight modification. Briefly, *E. coli*-derived recombinant HBc (ViroGen, Watertown, MA) was used as the reference standard; a rabbit polyclonal anti-HBc (Cat# D44410130, BiosPacific, Emeryville, CA) and mouse polyclonal anti-HBc (generated by i.p. injection of mice with *E. coli*-derived recombinant HBc) diluted 1:5000 in 1× PBS buffer were used as capture and detection antibodies, respectively. Dot blotting, Western blotting and sucrose gradient sedimentation were performed as previously described (Huang et al., 2006).

For NVCP analysis, total leaf protein was extracted with ice-cold acid extraction buffer (25mM sodium phosphate buffer, pH 5.75, 100mM NaCl, 50mM sodium ascorbate and 10 μg/ml leupeptin) using a FastPrep machine (Bio101), and cleared supernatants were assayed for NVCP by ELISA as described (Mason et al., 1996). Briefly, insect cell-derived recombinant NVCP (Jiang et al., 1992) (provided by Dr. Mary Estes, Baylor College of Medicine) was used as the reference standard; a rabbit polyclonal anti-NVCP and a guinea pig polyclonal anti-NVCP (provided by Dr. Mary Estes) were used as capture and detection antibodies, respectively. Western blotting was performed as previously described (Mason et al., 1996) except probing with a rabbit anti-rNV serum. Sucrose gradient sedimentation of NVCP extracts followed a modified protocol (Mason et al., 1996). Briefly, plant extracts or insect cell-derived NVCP standard were layered onto linear 5ml 10−60% sucrose gradients dissolved in modified phosphate buffer (25mM sodium phosphate, pH5.75, 100mM NaCl). After centrifugation in a Beckman SW55Ti rotor at 45,000rpm for 3 h at 4°C, 15 fractions (0.333 ml each) were taken and assayed for NVCP content by ELISA.

Electron microscopy

HBc was partially purified from infiltrated leaves as previously described (Huang et al., 2006). Partially purified HBc was subjected to negative staining with 0.5% aqueous uranyl acetate, and transmission electron microscopy was performed with a Philips CM-12S microscope.

Visualization of GFP

Leaves expressing GFP were viewed under UV illumination generated by a B-100AP lamp (UVP, Upland, CA).

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RESULTS

Agrobacterium-mediated co-delivery of a replicon vector and a Rep/RepA-supplying vector into plant leaf leads to efficient replicon formation and high-level protein production

It was previously shown that formation of BeYDV-based replicons in bombarded tobacco NT-1 cells resulted in increased transient expression of target proteins (Mor et al., 2003). To develop a scalable high-yield transient expression system, we first tested if *Agrobacterium*-mediated delivery of replicon vectors into plant leaf can lead to replicon formation and increased target protein expression. *N. benthamiana* leaves were infiltrated with an Agrobacterium culture containing a replicon vector pBYGFP (Fig. 1) encoding the green fluorescent protein, or co-infiltrated with a mixture of two Agrobacterium cultures containing pBYGFP and one of the Rep-supplying vectors (pREP110 encoding both Rep and RepA or pREP111 encoding Rep only, Fig. 1). At 5 days post infiltration (dpi), very faint green fluorescence was observed from leaf areas infiltrated with pBYGFP alone or co-infiltrated with pBYGFP and pREP111 (abbreviated as BYGFP/REP111), whereas no green signal was found from the control infiltrated with the infiltration buffer only (Fig. 2A). In contrast, co-infiltration with BYGFP/REP110 resulted in intensive green fluorescence within the entire infiltrated area (Fig. 2A).

Similarly, *N. benthamiana* leaves were infiltrated with another replicon vector pBYHBc (Fig. 1) encoding the hepatitis B core antigen (HBc), a VLP-forming protein, alone or in combination with pREP110 or pREP111. HBc expression was monitored over a 7 day period by dot blotting (data not shown) and polyclonal sandwich ELISA (Fig. 3A). The BYHBc/REP110 combination produced the highest expression, averaging ~0.18 mg HBc per gram leaf mass at 2 and 4 dpi (measured by ELISA with an *E.coli*-derived recombinant HBc standard). No significant difference was found between the BYHBc and the BYHBc/REP111 treatments, in agreement with the results from the GFP study.

To determine whether this increase is associated with the replicon formation, DNA extracted from infiltrated leaves was resolved in 0.8% agarose gel. A high molecular weight band representing plant chromosome DNA was observed in all samples, serving as an internal loading control. Two additional bands of ~3.0kb and ~2.0Kb were present in the BYHBc/REP110 lane, but not in the BYHBc or BYHBc/REP111 lanes (Fig. 3B). Southern blotting with HBc-specific probe confirmed that the ~3.0kb and ~2.0Kb bands were indeed the HBc replicons (Fig. 3B), probably representing the open and covalently-closed circular forms, respectively. Digestion of plant DNA resulted in only one major band of ~3.0kb (Fig. 3C), as expected for the linearized replicon. Taken together, the above results demonstrate a tight correlation between replicon amplification and HBc accumulation for the BYHBc/REP110 treatment, strongly suggesting the higher protein yield is due to high copy number of replicons.

Post-transcriptional gene silencing (PTGS) suppressor P19 enhanced accumulation of target-specific mRNA and protein

PTGS suppressor P19 from tomato bushy stunt virus enhanced recombinant protein accumulation in *N. benthamiana* leaf by suppressing gene silencing (Voinnet et al., 2003). To test if expression of P19 in our replicon system can further elevate protein accumulation, we co-infiltrated a P19 vector (Fig. 1) with combinations of replicon and Rep-supplying vectors and found that BYGFP/REP110/P19 yield the most intensive GFP fluorescence (Fig. 2B). Similarly, the BYHBc/REP110/P19 combination yielded the highest HBc expression, averaging 0.8 mg/g at 4dpi, ~4-fold more than without p19 (Fig. 3A). Co-delivery of P19 only marginally increased the replicon copy number as demonstrated by Southern blotting (Fig. 3B and 3C). In contrast, Northern blot showed that accumulation of HBc-specific

mRNA increased by 2- to 4-fold when P19 was co-expressed (Fig. 3D, BYHBc/REP110 vs. BYHBc/REP110/P19). These results indicate that P19 indeed can increase target mRNA and protein accumulation, most likely by suppressing post-transcriptional silencing of the transgene.

Transiently expressed HBc assembled into VLPs

Plant extracts from BYHBc/REP110/P19 infiltrated leaves were subjected to Western blot analysis. As expected, plant HBc (p-HBc) was identified as a ~21kDa band co-migrating with the *E. coli*-derived HBc (e-HBc) standard under denaturing and reducing conditions (Fig. 4A). Sucrose gradient sedimentation of BYHBc/REP110/P19 leaf extracts, in comparison with *e*-HBc (which is known to assemble into VLPs), showed that both p-HBc and e-HBc were strongly detected in particulate fractions (Fig. 4B and 4C). Examination of the p-HBc peak sucrose gradient fractions by electron microscopy conclusively revealed the presence of typical HBc VLPs with a diameter of ~30 nm (Fig. 4D and 4E).

Transient expression and VLP formation of Norwalk Virus capsid protein (NVCP)

To confirm the effectiveness of this system in producing VLP vaccines, we also tested the replicon system for the transient expression of NVCP, another VLP forming antigen. Fig. 5A shows a time course study of NVCP expression with different vector combinations. The BYsNV/REP110/P19 was the best combination, yielding NVCP at levels ~0.34 mg/g FW (measured by ELISA with insect cell-derived NVCP (Jiang et al., 1992) as the standard). These results are consistent with the findings for GFP and HBc.

The integrity of NVCP was verified by Western blotting. As shown in Fig. 5B, plant-derived NVCP showed a ~58KDa band as did the insect cell-derived NVCP standard (Jiang et al., 1992).

BYNVCP leaf extracts were subjected to sucrose gradient sedimentation to determine the VLP assembly. Purified insect cell-derived NVCP was present primarily in fractions 10−12 as VLPs (Fig. 5C); whereas the plant-derived NVCP was detected in these three VLP fractions and also in fractions 1−3 representing non-VLP forms.

Single-vector replicon system

We investigated the potential to simplify the 3-vector replicon system without compromising yields. Replicon vectors containing a native C1/C2 coding region under the control of the viral LIR promoter were constructed for the expression of GFP (pBYGFP.R) and HBc (pBYHBc.R), respectively (Fig. 1). UV illumination of infiltrated leaves at 5dpi revealed that the BYGFP.R or the BYGFP.R/P19 samples exhibited fluorescence with intensity similar to that of BYGFP/REP110/P19 but higher than that of BYGFP/REP110 (Fig. 2C). In addition, BYHBc.R and BYHBc.R/P19 samples expressed HBc at levels comparable with those of BYHBc/REP110/P19 at 4 or 7 dpi (Fig. 6). No BYHBc/REP110/P19 sample was collected at 7 dpi due to severe necrosis, which occurred repeatably with this treatment. These results collectively demonstrate that the simpler single-vector replicon system is as efficient as the three-component system in terms of the final yield of protein of interest.

DISCUSSION

The BeYDV-derived DNA replicon vector system

In this study, we exploited the replication mechanism of BeYDV, a geminivirus, to develop a rapid single-vector replicon-based, high-yield transient expression system. Geminiviruses possess single-stranded circular DNA genomes that replicate to very high-copy number in

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the nuclei of infected cells, making them very attractive in gene amplification strategies to increase the copy number and enhance the expression levels of foreign genes that are linked to the viral replicon (Palmer and Rybicki, 1998). DNA replicons provide a potential advantage over RNA replicons (TMV, PVX) in that DNA replication in the nucleus using host-supplied replication factors (Palmer and Rybicki, 1998) is likely to occur with higher fidelity than RNA replication in the cytosol. Moreover, there is no obvious size limit for geminiviral replicons, while RNA replicons with large inserts often suffer instability.

Previously, Mor et al. showed that co-delivery of BeYDV-derived replicon vector and a Rep/RepA-supplying vector into tobacco NT-1 cells by bombardment resulted in replicon formation and elevated expression of target proteins (Mor et al., 2003). However, this bombardment process is not practically scalable. Therefore, in this study we first showed that agroinfiltration, a readily scalable procedure, can be used to introduce a replicon and a Rep/RepA-supplying vector into the same leaf cells to achieve efficient replicon formation and amplification. Indeed, the replicon DNA is so abundant that its presence can be visualized by EtdBr staining (Fig. 3B and 3C). The data also clearly showed that the high-copy numbers of replicons correlated with greatly increased accumulation of recombinant protein HBc and mRNA (Fig. 3). Although we did not accurately quantify replicon or mRNA abundance in this study, it seems clear that the DNA replicon was amplified to a greater extent than the mRNA (Fig. 3C, 3D), which is consistent with our findings in transgenic plants using inducible expression of Rep/RepA (Zhang and Mason, 2005). This may reflect a saturation of the transcription factors when the transcription template (replicon) is extremely abundant.

Next we showed that transient expression using the BeYDV replicon system can be further increased by co-expression of the PTGS suppressor P19, which has been previously shown to enhance target RNA accumulation and protein expression directed by conventional plant binary vectors (Voinnet et al., 2003) or potato virus X (PVX) amplicon (Mallory et al., 2002). Our results showed that P19 substantially improved HBc mRNA accumulation 2- to 4-fold (estimated from Fig. 3D) and enhanced HBc protein yield >4-fold at 4 dpi (Fig. 3A).

Moreover, this three-component BeYDV replicon system can be reduced to a single vector without sacrificing the yield of target protein. We incorporated a native Rep/RepA expression cassette driven by the viral LIR promoter into the replicon, such that the relatively weak promoter activity of LIR for the complimentary sense C1/C2 gene is boosted by replicon amplification. Single vector Rep-containing replicons like pBYGFP.R (Fig. 1) produced replicon amplification (Southern blot, data not shown). GFP and HBc expression directed by the Rep-containing single replicon vectors (pBYGFP.R and pBYHBc.R) produced levels similar to those obtained by infiltration with three vectors (Figs. 2C and 6), indicating that the simpler single-vector replicon is as efficient as the three-component system in driving the expression of protein of interest.

While the expression levels of target proteins by our system are very high, further optimization might be possible. For example, we found that necrosis triggered by pBYHBc.R occurred later (starting at 8 dpi) than that by the pBYHBc/REP110/P19 combination (starting at 5 dpi), perhaps because the weak LIR promoter in pBYHBc.R directs relatively lower Rep/RepA expression. However, necrotic phenotypes were not observed in either the pBYGFP.R or the pBYGFP/REP110/P19 infiltrated leaves. These results suggest the necrosis is associated with the recombinant protein HBc and not the Rep/RepA. However, the degree of necrosis might be increased by higher Rep/RepA expression. Therefore, an optimal Rep/RepA level should be defined in order to maximize the expression potential of this replicon system for any particular target protein.

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Rapid, high-yield production of VLPs using the replicon system

In order to show the usefulness of our replicon system in rapidly producing vaccines at high levels, we tested two antigens: HBc and NVCP. Recombinant HBc and NVCP have been previously expressed in transgenic plants, however, at relatively low levels (Mason et al., 1996; Tsuda et al., 1998). For example, transgenic plants expressed up to 24 μg HBc per gram of leaf fresh weight (Tsuda et al., 1998). In contrast, much higher HBc expression can be achieved using plant viral vectors within a short period of time (one to two weeks). For example, about fifty to several hundred μg HBc per g of leaf fresh weight were obtained with a potato virus X (PVX) vector (Mechtcheriakova et al., 2005). Recently, Sainsbury and Lomonossoff reported HBc expression at ~1 mg/g FW using a non-replicating vector that contains a mutated 5' UTR derived from cowpea mosaic virus RNA-2, co-delivered with a p19 expression vector (Sainsbury and Lomonossoff, 2008). Our replicon system produced HBc at very similar levels (~0.8 mg/g FW) without p19 (Fig. 6). Thus, since P19 expression can be omitted, the BeYDV replicon is potentially more convenient and simple to use.

In addition to the results reported here, we have used this system to express several other vaccine antigens, including Narita104 norovirus capsid protein and human papillomavirus L1 capsid protein (unpublished data). In general, the speed and levels of antigen accumulation are comparable to those produced by the state-of-art magnICON transient expression system (Huang et al., 2006; Santi et al., 2008). More significantly, we showed that a Rep-expressing single replicon vector produced HBc at the same levels as the three-vector combination (Fig. 6). The ability of a simplified single vector to drive the high-level expression of protein of interest provides a key advantage over other plant expression systems in its commercial production feasibility.

VLPs can be used as stand-alone vaccines or platforms for epitope presentation (Garcea and Gissmann, 2004). Previously, we have extensively studied the expression of several VLP-forming antigens in transgenic plants and in the magnICON transient expression system (reviewed in (Santi et al., 2006)) and demonstrated the latter system efficiently produced immunogenic VLPs (Huang et al., 2008; Huang et al., 2006; Santi et al., 2008). In this study, we examined if the replicon system produced HBc and NVCP were also capable of VLP formation. For HBc, affirmative results were obtained by sucrose gradient analysis, ELISA, dot blot analysis, and electron microscopy (Fig. 4). Using gradient separation of proteins in NVCP crude extract, two peaks were detected on sucrose gradients which we assign as partially assembled subunits (Fractions 1−3, Fig. 5C) and assembled VLPs (Fractions10−12, Fig. 5C). The latter peak co-migrated with VLPs produced in insect cells (Fig. 5C). This "two-peaks" pattern is similar to that observed for the magnICON system produced NVCP, which was very immunogenic in mice (Santi et al., 2008). Thus, we conclude that VLP-forming antigens produced by this replicon system can effectively assemble into high-order oligomeric VLPs.

In conclusion, this paper describes a rapid and robust geminivirus-based expression system that produces high-level VLP within 4−5 days after vector delivery. Moreover, multiple DNA replicons can be linked in tandem in a single vector for co-expression of two or more recombinant proteins (data to be published elsewhere). The simplicity and high-yield potential of the single-vector replicon system greatly enhance the feasibility of realistic commercial application of this system for vaccine and other recombinant protein production.

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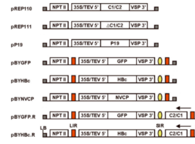

Figure 1.

Diagrams of the T-DNA region of the vectors used in this study. 35S/TEV 5’, CaMV 35S promoter with tobacco etch virus 5’ UTR; VSP 3’, soybean vspB gene 3’ element; NPT II, expression cassette encoding nptII gene for kanamycin resistance; LIR, long intergenic region of BeYDV genome; SIR, short intergenic region of BeYDV genome; C1/C2, BeYDV ORFs C1 and C2, encoding Rep and RepA; ΔC1/C2, intron-deleted C1 and C2 encoding Rep only; LB and RB, the left and right borders of the T-DNA region.

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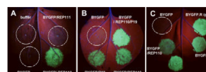

Figure 2.
Visualization of GFP expression. *N. benthamiana* leaves were infiltrated with a single *Agrobacterium* strain or mixtures of strains harboring expression vectors as indicated. Infiltrated leaves were examined at 5 days post infiltration (dpi) with UV lamp as described in Materials and Methods.

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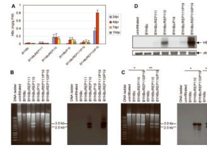

Figure 3.

Expression of HBc in *N. benthamiana* leaves with replicon vectors. **A:** Time course of HBc expression with different vector combinations. Infiltrated leaf extracts were analyzed by ELISA with *E.coli*-derived recombinant HBc as standard. Data are means ± S.D. from three independently infiltrated samples. **B**: Southern blot of undigested plant DNA from leaves infiltrated with vector combinations. The left panel is an EtdBr-stained agarose gel, and the right panel is a blot detected with an HBc specific probe. **C:** Southern blot of plant DNA undigested (indicated by **) or digested with XhoI (indicated by *). The left panel is an EtdBr-stained agarose gel, and the right panel is a blot detected with an HBc specific probe. **D**: Northern blot for HBc transcripts (upper panel), the low panel shows methylene blue-stained plant 25S rRNA as internal loading control.

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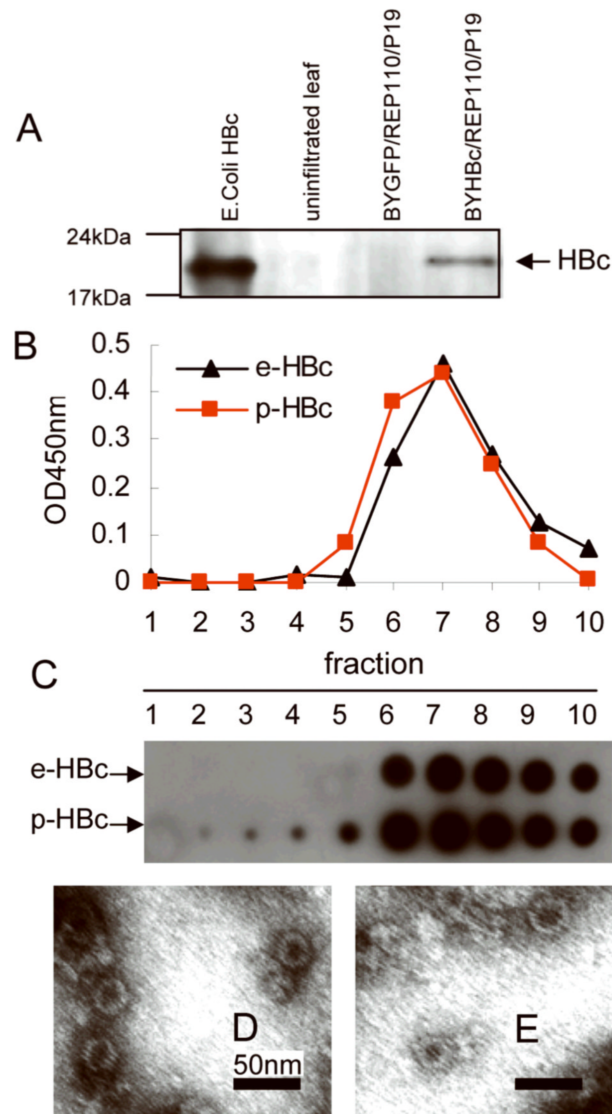

Figure 4.

Characterization of plant-expressed HBc. **A**: Western blot of HBc from *E. coli* derived reference standard (5 μg) or crude extracts of *N. benthamiana* leaves (representing 1 mg of leaf tissue) infiltrated with the indicated constructs, probed with a mouse monoclonal anti-HBc that recognizes amino acid residues 130−140 of HBc. **B**: Sucrose gradient sedimentation of leaf extracts; p-HBc, BYHBc/REP110/P19 infiltrated leaves; e-HBc, *E.coli*-derived HBc. Sedimentation is left to right; fractions were assayed by HBc ELISA. **C**: Dot blot of sucrose gradient fractions probed with rabbit polyclonal anti-HBc. **D** and **E:** Electron microscopy of partially purified HBc negatively stained with 0.5% uranyl acetate, two representative fields shown. Bar = 50 nm.

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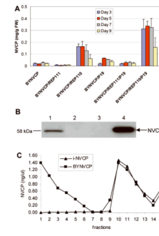

Figure 5.

Transient expression of NVCP using replicon vectors. **A**: Time course of NVCP expression with different vector combinations. Infected leaf extracts were analyzed by ELISA with insect cell-produced NVCP as the standard. Data are means ± S.D. from four independently infiltrated samples. **B**: Western blot for NVCP expression. Lane 1, insect-derived NVCP standard (100 ng); lanes 2, 3, and 4 are 5 μg TSP each from uninfiltrated, BYGFP-infiltrated and BYNVCP-infiltrated leaf extracts, respectively. **C**: Sucrose gradient profile of insect-derived (i-NVCP) and plant-expressed NVCP (BYNVCP). Sedimentation is left to right; fractions were analyzed by polyclonal ELISA.

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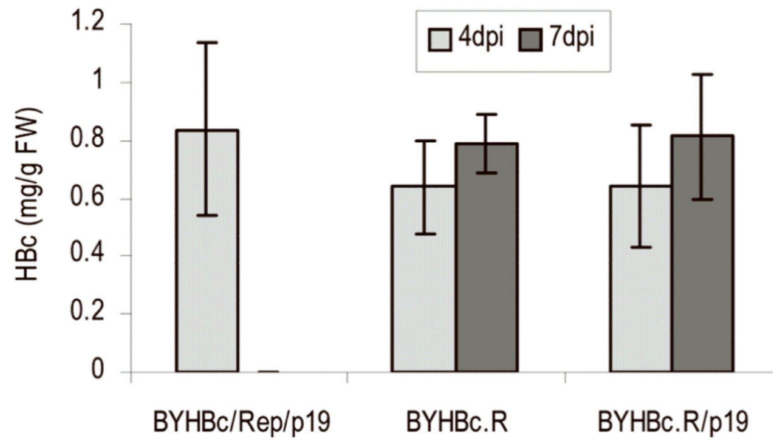

Figure 6.

Expression of HBc using a single replicon vector. Extracts from *N. benthamiana* leaves infiltrated with BYHBc/REP110/P19, BYHBc.R, or BYHBc.R/P19 were analyzed for HBc expression by polyclonal ELISA. Data are means ± S.D. from four independently infiltrated samples.