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# Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce

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## Summary

Pharmaceutical protein production in plants has been greatly promoted by the development of viral-based vectors and transient expression systems. Tobacco and related *Nicotiana* species are currently the most common host plants for generation of plant-made pharmaceutical proteins (PMPs). Downstream processing of target PMPs from these plants, however, is hindered by potential technical and regulatory difficulties due to the presence of high levels of phenolics and toxic alkaloids. Here, we explored the use of lettuce, which grows quickly yet produces low levels of secondary metabolites, and viral vector-based transient expression systems to develop a robust PMP production platform. Our results showed that a geminiviral replicon system based on the bean yellow dwarf virus permits high-level expression in lettuce of virus-like particles (VLP) derived from the Norwalk virus capsid protein and therapeutic monoclonal antibodies (mAbs) against Ebola and West Nile viruses. These vaccine and therapeutic candidates can be readily purified from lettuce leaves with scalable processing methods while fully retaining functional activity. Furthermore, this study also demonstrated the feasibility of using commercially produced lettuce for high-level PMP production. This allows our production system to have access to unlimited quantities of inexpensive plant material for large-scale production. These results establish a new production platform for biological pharmaceutical agents that is effective, safe, low-cost, and amenable to large-scale manufacturing.

### Keywords

Geminiviral replicon system; lettuce; plant vaccine; plant antibody; West Nile virus; Ebola virus

## Introduction

Plants have been used as bioreactors to express proteins of pharmaceutical importance such as vaccines and mAbs (De Muynck et al., 2010; Paul and Ma, 2010; Rybicki, 2010). Historically, plants have been proposed as vehicles for protein production of biosimilars because of their capacity to generate large volumes of proteins at low cost, ability to make appropriate post-translational modification of recombinant proteins, and low contamination risks by animal or human pathogens (Chen, 2011a; Faye and Gomord, 2010). More recently, plants also have been recognized as a promising system to make safer and more effective

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pharmaceuticals (biobetters) due to the development of transgenic plant lines that offer a portfolio of specific and unique properties in protein post-translational modification (Castilho et al., 2010; Chen, 2011b; Faye and Gomord, 2010; Gomord et al., 2010).

Pharmaceutical proteins can be produced either in transgenic plants or by expressing a target transgene transiently in plant materials (Chen, 2008). The former strategy requires an extensive time requirement to generate transgenic plants and many of these, unfortunately have had low protein yields (Davies, 2010). In contrast, transient expression can produce the target proteins rapidly within 1 to 2 weeks (Komarova et al., 2010). While transient expression with traditional non-viral based vectors driven by the 35S cauliflower mosaic virus promoter produces relatively low protein yields, plant viral vectors promote high-levels accumulation of foreign protein due to their efficient replication, which results in high copy numbers of transgene and their mRNA transcripts (Canizares et al., 2005; Lico et al., 2008). One of the more robust transient expression vectors is the “deconstructed” viral vector system. The MagnICON system is based on replication-competent tobacco mosaic virus (TMV) and potato virus X (PVX) genomes under the control of plant promoters. Once delivered to plant cells, the TMV or PVX genome is transcribed and spliced to generate a functionally infective replicon (Giritch et al., 2006). Another example is the geminivirus-based expression system: a DNA replicon system derived from the bean yellow dwarf virus (BeYDV) that allows rapid high-yield production of proteins in tobacco plants (Chen et al., 2011). In these systems, *Agrobacterium tumefaciens* is used to deliver the “deconstructed” viral vectors to plant cells to eliminate the need for systemic viral spread within the plant and the *in vitro* process of generating RNA-based vectors. This approach also prevents transgene loss during systemic spread, and allows the technology to be applied to a diversity of plant species beyond the natural host(s) of the virus. The deletion of viral coat protein genes in these systems also facilitates the high protein yield of a viral system without the concern of generating infectious virions. Thus, the deconstructed viral vector system provides the flexibility of nuclear gene expression with the speed and yield of viral vectors.

The most common host plants for transient expression of proteins are tobacco and related *Nicotiana benthamiana* owing to their high biomass yield and the availability of expression vectors for these species (Chen, 2011a). However, most tobacco and other *Nicotiana* plant leaves contain high levels of phenolics and toxic alkaloids, which foul purification resins and are difficult to remove from the protein target (Platis and Labrou, 2008; Roque et al., 2004). Similar to tobacco, lettuce (*Lactuca sativa*) is a robust-growing plant that is cultivated readily and produces large quantities of biomass rapidly. Unlike tobacco, it produces negligible quantities of phenolics and alkaloids, and thus, would simplify the protein purification process and reduce the overall cost of goods. Furthermore, the availability of large-scale lettuce growing and processing technology and infrastructure in the agricultural and food industry provides the advantage of rapid adaptation for pharmaceutical protein production.

Lettuce has been explored for protein production with transgenic lines (Kapusta, 1999; Rosales-Mendoza et al., 2010; Webster et al., 2006) or transient expression with conventional non-viral vectors (Negrouk et al., 2005), both of which produced relatively low levels of transgenic proteins. However, the effectiveness of deconstructed viral vectors such as geminiviral or MagnICON systems in facilitating the expression and production of pharmaceutical proteins in lettuce has not been examined. Here, we investigated the feasibility of using deconstructed viral vectors and commercially-produced lettuce to produce two groups of biological candidates of pharmaceutical importance: a virus-like particle (VLP) vaccine and two therapeutic monoclonal antibodies (mAbs). Our results showed that the geminiviral replicon system permits high-level expression of VLP derived from the Norwalk virus capsid protein (NVCP) and therapeutic humanized mAbs against

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Ebola (EBV) (6D8) or West Nile (WNV) (hE16) viruses in lettuce. These vaccine and therapeutic mAb candidates can be readily purified in high-yield from lettuce leaves with scalable processing methods while retaining functional activities. In addition, this study also demonstrated the feasibility of using commercially produced lettuce for high-level PMP protein production. This allows our production system to have access to potentially unlimited quantities of inexpensive plant material for large-scale production. The robustness and scalability of the protein expression system and product purification scheme, coupled with the virtually unlimited nature of plant material generation, provide a platform that is effective, safe, low-cost and amenable to large-scale manufacturing.

## Results

### Expression of green fluorescent protein (GFP) with transient expression vectors in lettuce

It was previously reported that transient expression of proteins with non-viral vectors resulted in relatively low level of product accumulation in lettuce (Kapusta, 1999; Rosales-Mendoza et al., 2010; Webster et al., 2006). To identify an optimal vector for protein expression, we compared two deconstructed viral vectors, the TMV or PVX-based MagnICON vector and BeYDV-based geminiviral vector, in promoting expression of a reporter protein, GFP. Grocery store purchased lettuce (red leaf) was infiltrated with an *Agrobacterium* culture containing a GFP-expressing geminiviral replicon vector (pBYGFP) alone, or co-infiltrated with cultures containing pBYGFP and a replication associated protein (Rep)-supplying vector (pREP110) that is required for pBYGFP replication (Fig. 1) (Huang et al., 2009). Another group of lettuce was co-infiltrated with three *Agrobacteria* cultures containing pBYGFP, pREP110, and the pP19 vector that encodes for a suppressor of post-transcriptional gene silencing (Fig. 1). We also infiltrated lettuce with three cultures containing the three vector modules of the MagnICON system (Giritch et al., 2006). As expected, no green fluorescence was observed from negative control lettuce leaves that were infiltrated with infiltration buffer (Fig. 2a). Dim fluorescence was detected from leaves infiltrated with pBYGFP alone due to the absence of the required Rep protein for replicon replication (Fig. 2b). In contrast, co-infiltration with pBYGFP/pREP110 resulted in intense green fluorescence over the entire lettuce leaf area (Fig. 2c) similar to the positive control, *N. benthamiana* (Fig. 2d). Inclusion of pP19, the suppressor for gene silencing in the co-infiltration, however, did not enhance the intensity of green fluorescence (data not shown). In comparison, we did not observe green fluorescence from leaves co-infiltrated with MagnICON vectors (Fig. 2e). The relative GFP intensity between leaves infiltrated with different expression vectors remained unchanged between days 4 and 6 post infiltration (dpi) and a similar trend was also observed with laboratory-grown lettuce (data not shown).

### High-level transient expression of Norwalk virus capsid protein

To examine the effectiveness of geminiviral vector in producing pharmaceutically relevant proteins in lettuce, we investigated the transient expression of NVCP, which self-assembles into an empty VLP and is a vaccine candidate (Jiang et al., 1992). Western blot analysis of pBYNVCP/pREP110 infiltrated lettuce leaves confirmed that NVCP was produced in lettuce leaves with the expected molecular weight of 58 kDa (Fig. 3a) as previously characterized when produced in insect cells (Santi et al., 2008). Using an ELISA, kinetic analysis revealed that NVCP accumulation could be detected 3 dpi and reached highest levels at 4 dpi, with an average expression level of ~0.2 mg/g leaf fresh weight (LFW) with or without the presence of P19 (Fig. 3b). This level is comparable to that of NVCP expression in *N. benthamiana* (Huang et al., 2009) and is the highest expression level of any non-chloroplast-derived vaccine component ever reported in lettuce plants. TMV-based MagnICON vectors were also tested for transient expression, but NVCP was not detected by

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Western blot or ELISA (data not shown), consistent with our findings for expression of GFP.

## Purification and Characterization of NVCP

Downstream processing is an important component of pharmaceutical protein production. The ease of purifying target proteins from lettuce therefore, is essential for an expression system to be considered a viable production platform candidate. NVCP was extracted from lettuce leaves and purified by low pH precipitation and anion exchange chromatography. The low pH treatment was designed to precipitate the most abundant plant protein, the photosynthetic enzyme RuBisCo, and other host proteins. This two-step purification process effectively removed most of the host proteins and enriched NVCP to greater than 95% purity (Fig 4a). To examine if lettuce-derived NVCP self-assembled into VLPs as previously observed from insect cells (Jiang et al., 1992), leaf extracts were analyzed by sucrose gradient centrifugation. The majority of lettuce-derived NVCP was detected in particulate fractions, and sedimented at the same density as did NVCP VLP derived from insect cells (Fig. 4b). Electron microscopy of purified lettuce-derived NVCP confirmed assembly into VLPs with a diameter of ~ 30nm, typical of NVCP VLPs (Fig. 4c).

## High-yield transient expression of therapeutic mAbs against EBV and WNV

To demonstrate further the utility of our geminiviral-lettuce expression system as a platform for production of pharmaceutical proteins, we assessed production of mAbs, another important group of pharmaceutically relevant molecules. The light chain (LC) and heavy chain (HC) coding sequences of 6D8, a protective mAb against EBV glycoprotein GP1 (Huang et al., 2010) and hE16, a therapeutic mAb against WNV envelope (E) protein (Lai et al., 2010; Oliphant et al., 2005) were cloned into geminiviral dual replicon vectors pBY-HL(6D8).R and pBY-HL(hE16).R (Fig. 1). For both mAbs, the “KDEL” tetrapeptide ER-retention signal was added to the C-terminus of the HC. In addition to the two replicons for the LC and HC, these single vectors also contain the expression cassette for Rep (C1/C2) (Huang et al., 2010). *Agrobacterium* strains containing these multi-replicon single vectors were infiltrated into commercially-produced or laboratory-grown lettuce leaves. Western blot analysis revealed that the LC and HC of 6D8 and hE16 were expressed in lettuce leaves with the expected molecular weights of 25 kDa and 50 kDa, respectively (Fig. 5a and b). The full tetrameric (2HC + 2 LC) assembly of these mAbs was confirmed by Western blotting under non-reducing conditions (Fig. 5c). The Western blots also established that proteolytic degradation of the LC or HC did not occur, since only the expected bands were observed. Both mAbs were produced rapidly in lettuce and reached the highest level of accumulation on 4 dpi, with an accumulation of ~0.23 to 0.27 mg/g LFW (Fig. 5d). This expression level is comparable to the highest expression level of mAbs reported by geminiviral vectors in tobacco (Huang et al., 2010). To investigate the variability of mAb yield between different batches of commercially produced lettuce material, we compared 6D8 mAb production in three groups of lettuce purchased at monthly intervals as well as three groups of laboratory-grown lettuce generated in parallel. While commercially produced lettuce had greater variability in 6D8 mAb expression than the laboratory-grown counterpart (Fig. 5d), the mean accumulation was similar between the lettuces produced from different sources. Consistent with the results for GFP and NVCP VLPs, co-infiltration with pP19 did not enhance mAb accumulation, and we could not detect mAb expression in lettuce when TMV and PVX-based MagnICON expression vectors were used (data not shown). Overall, these results demonstrate that a geminiviral-lettuce system can produce rapidly fully-assembled mAbs at high levels.

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### Purification of lettuce-derived mAbs

To assess whether the geminiviral-lettuce system has advantages both in upstream protein expression and downstream processing, we extracted and purified the two mAbs from infiltrated lettuce leaves. We previously reported that purification of mAbs from *N. benthamiana* requires an ammonium sulfate precipitation step prior to affinity chromatography (Lai et al., 2010; Phoolcharoen et al., 2011). This step is necessary to eliminate phenolics and alkaloids from tobacco extract to prevent subsequent fouling of the protein A or G affinity chromatography resins. Since lettuce leaves produce negligible amounts of phenolics and alkaloids, we evaluated whether we could bypass the precipitation step and load the clarified lettuce extract directly onto the protein A affinity column. SDS-PAGE and Coomassie blue staining analysis showed that 6D8 and hE16 mAbs produced in lettuce can be purified to > 95% purity with intact HC and LC by a single affinity chromatography step (Fig. 6). A similar analysis under non-reducing conditions confirmed that both mAbs remained fully assembled after purification (data not shown). Moreover, direct loading of lettuce extract onto Protein A resin did not foul the resin over 20 purification cycles, which contrasts with that observed with tobacco leave extracts (Chen, 2008). When 6D8 and hE16 were further polished with a DEAE-ion exchange chromatography, the level of residual DNA, endotoxin, and Protein A in the final purified mAb preparations was below the United States Food and Drug Administration specifications for injectable mAb pharmaceuticals (Table 1); this supports the production system's potential for mAb production under current Good Manufacture Practice (cGMP) regulations.

### Geminiviral – lettuce system-derived mAbs are functional

We performed additional functional characterization to determine if mAbs produced by the geminiviral-lettuce system retained biological and pharmaceutical activity. We tested the antigen-binding capacity of lettuce-derived 6D8 by incubating various concentrations of this mAb with irradiated EBV immobilized on an ELISA plate. As shown, the binding to inactivated EBV increased with the concentration of 6D8 in the reaction, whereas, the negative control IgG (a generic human IgG) showed no specific binding (Fig. 7a). This result indicates that the specific avidity for Ebola virus GP1 protein is retained by the lettuce-derived 6D8. For lettuce-derived hE16, we examined its binding activity both by ELISA against recombinant E protein and using a flow cytometry assay in which domain III of the E protein is displayed on the surface of yeast (Oliphant et al., 2005). ELISA analysis indicated that lettuce-derived hE16 bound to WNV E protein in a similar manner as the mammalian cell-derived hE16 (data not shown). Flow cytometric analysis showed that the percentage of yeast cells expressing domain III and the mean fluorescence intensity of binding by lettuce-derived hE16 were indistinguishable compared to the positive control hE16 that was derived in mammalian cells (Fig. 7b). The results from these assays confirm that lettuce-produced hE16 retains its antigen binding activity and specificity compared to its mammalian cell-derived counterpart. As further corroboration, we evaluated the neutralizing activity of hE16 produced in lettuce using a focus reduction neutralization assay that measures antibody inhibition of WNV infection (Fuchs et al., 2011). hE16 derived in lettuce neutralized WNV infection equivalently compared to mammalian cell-derived hE16 (Fig. 7c, EC50 of 6.9 and 6.6 ng/ml, respectively, $P > 0.8$). Thus, hE16 produced by the geminiviral-lettuce system retained its potent neutralizing activity against infectious WNV.

## Discussion

Tobacco and related *Nicotiana* species are the most common host plants for production of PMPs. Downstream processing of target PMPs from these plants, however, is limited by potential technical and regulatory difficulties due to the presence of high levels of phenolics and toxic alkaloids. While low-alkaloid *Nicotiana* varieties naturally exist and more have

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been created through breeding, these varieties are not always optimal hosts for PMP production due to the lack of correlation between varieties that accumulate high levels of target protein and those with low alkaloid content (Conley et al., 2010). In this study, we explored the use of lettuce, a plant that grows rapidly yet produces low levels of toxic secondary metabolites, for viral vector-based transient expression system as a robust PMP production platform. Our results show that the BeYDV-based geminiviral replicon system can efficiently promote high-level expression of NVCP VLP vaccine and anti-EBV or WNV mAb therapeutic candidates in lettuce. Using the geminiviral-lettuce system, the VLP and the two therapeutic mAbs accumulated to levels that were comparable to that observed in tobacco (Huang et al., 2010; Lai et al., 2010), but higher than previously reported in lettuce using non-viral vectors (Kapusta, 1999; Rosales-Mendoza et al., 2010; Webster et al., 2006). For example, hE16 and 6D8 mAbs accumulated to 0.27 mg/g fresh leaf weight 4 dpi (Fig. 5), up to 13.5-fold higher than that produced by non-viral expression vectors in lettuce (Negrouk et al., 2005). To our knowledge, this is the highest level of mAb accumulation ever reported in lettuce. Recently, two novel transient expression vector systems that are based on a plastocyanin promoter or the 5′ and 3′-untranslated region of Cowpea mosaic virus RNA-2 have been developed. These vector systems do not require viral replication yet allow the high level expression of mAbs from a single plasmid in *N. Benthamiana* (Sainsbury et al., 2009; Vézina et al., 2009). As such, it will be interesting to explore the utility of these vectors in lettuce as well.

In addition to targeting transgenes to the nuclear genome, certain pharmaceutical proteins can be expressed via a chloroplastic genome (Daniell, 2006). As a result, higher level of target protein accumulation can be achieved by expression in transplastomic lettuce (Davoodi-Semiromi et al., 2010). Nonetheless, because chloroplasts lack posttranslational modification machinery, transplastomic plant lines cannot efficiently produce complex hetero-oligomeric proteins such as mAbs or simple proteins that require posttranslational modifications (e.g., N-linked glycosylation) for pharmaceutical function (Chen, 2008). In contrast, the geminiviral-lettuce system relies on transient expression of nuclear-targeted transgenes, and can efficiently express and assemble complex proteins including mAbs.

P19, a suppressor of gene silencing from tomato bushy stunt virus (TBSV), has been reported to enhance recombinant protein expression by suppressing post-transcriptional gene silencing (PTGS) (Voinnet et al., 2003). In prior research with *N. benthamiana*, we demonstrated that co-infiltration of pP19 with BeYDV replicons elevated target mRNA and protein accumulation (Huang et al., 2009; Huang et al., 2010). However, inclusion of the pP19 construct in the co-infiltration of replicon vector did not enhance the accumulation of the NVCP VLP in lettuce (see Fig 3b) or the two target mAbs in lettuce (Q. Chen, unpublished results). It seems unlikely that this is caused by a poor interaction between P19 and the heterologous RNA-silencing machinery in lettuce. P19 prevents gene silencing by sequestering the small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Lakatos et al., 2004), and thus should not be plant species-specific. Indeed, P19 has been shown to inhibit RNA interference in a broad range of organisms including human cells (Dunoyer et al., 2004). Moreover, lettuce is susceptible to TBSV infection, suggesting P19's effectiveness as a suppressor of gene silencing in this plant (Obermeier et al., 2001). It remains possible that under the conditions tested in lettuce, PTGS occurred at a low level or not at all. This hypothesis is corroborated by the results of a previous study in which P19 and beta-glucuronidase constructs were co-introduced into lettuce leaves by non-replicon expression vectors (Simmons and VanderGheynst, 2007).

In addition to the geminiviral replicon system, we examined the ability of TMV and PVX-based MagnICON deconstructed viral vectors to promote transgene expression in lettuce. Notably, we did not observe accumulation of GFP in lettuce leaves infiltrated with TMV or

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PVX-based vectors or expression of NVCP or mAbs. Similar to other positive-stranded RNA viruses, TMV and PVX RNA viral genomes are templates for both translation and replication, which leads to complex interactions at multiple levels between plant host factors and viral RNA replication and translation (Ahlquist et al., 2005). Such host-virus interaction is one of the critical factors in determining the host range of the infecting virus. Several such plant host factors have been identified for TMV and PVX (Park et al., 2009). For example, a 56-Kda tomato protein was found to be related to the RNA-binding subunit of yeast translational initiation factor, eIF3 and important for the translation of RNA-dependent RNA polymerase (RdRp) of TMV (Buck, 1999). Therefore, for MagnICON vectors to work in a plant species, host factors from this particular plant species must recognize and interact with TMV and/or PVX elements/factors to assist the translation of RdRp and the replication of the deconstructed viral genome. In contrast, geminiviruses have a small, single-stranded DNA genome that replicates using the well-conserved cellular apparatus and a virus-encoded protein Rep (Chen et al., 2011). For some geminivirus species, they can even replicate in non-host plant cells, but their host-ranges are limited instead by their inability to move from cell to cell for systemic infection (Teng et al., 2010). BeYDV has a broad host range in dicotyledonous plants and remains competent for replication as long as Rep and its cis-binding element (LIR) are present (Liu et al., 1999). Thus, the BeYDV-based geminiviral vectors may be functional in a wide range of plant species. We speculate that our finding reflects the host specificity of the viral elements/factors of TMV and PVX; host factors in lettuce may be sufficiently different from those in tobacco and other *Solanaceae* plants such that they cannot interact with the viral elements/factors and assist the translation of RdRp and/or for the replication of deconstructed TMV or PVX genome. In contrast, LIR and the gene coding for Rep are readily engineered as parts of the BeYDV replicon vectors, thereby, allowing its replication in lettuce and potentially a broader range of other host plant species.

Downstream processing can account for up to 80% of the total pharmaceutical protein production cost (Chen, 2011a). The development of effective, scalable, economical and cGMP compliant purification methods for recovering target proteins from lettuce is crucial. We developed robust and effective downstream processing protocols to purify our target vaccine and mAbs from lettuce. Our procedures can efficiently isolate the NVCP vaccine candidate and the two therapeutic mAbs to high (>95%) purity, in a scalable and cGMP compatible format. Direct loading of clarified lettuce extracts onto Protein A affinity columns did not adversely affect the integrity of the resin over 20 purification cycles. Thus, in contrast to tobacco, the precipitation step for reducing phenolics and alkaloids from the feed-stream becomes unnecessary and can be eliminated. This simplifies purification processing, prolongs resin life, and reduces the cost of downstream processing, and the overall cost of goods. The ease of purifying PMPs from lettuce and its cost-saving benefit support the feasibility of the geminiviral-lettuce expression system as a viable large-scale PMP production platform.

The geminiviral-lettuce derived NVCP and mAbs had the expected structural and functional properties. Similar to NVCP produced in tobacco, our preliminary results suggest that it accumulates in the cytosol of lettuce leaf cells (Q. Chen, unpublished results), although its precise subcellular location requires further confirmation. Nevertheless, NVCP derived from lettuce assembled into 30 nm VLPs in a manner indistinguishable from NVCP VLP derived from insect cells. Moreover, lettuce-derived mAbs readily assembled into tetramers without degradation, establishing that the geminiviral-lettuce system can assemble intact mAbs efficiently. In general, the minor difference in protein N-glycosylation between plant and mammalian cells could be an issue for plant-derived mAbs, since the possibility of inducing plant-glycan specific antibodies could reduce stability and therapeutic efficacy by accelerating clearance from plasma, or cause potential adverse effects through immune

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complex formation. However, since the HCs of our mAbs were tagged with the ER-retention signal KDEL at their C-termini, we speculate that they would be retained in ER and have a high-mannose form of glycans at their Fc region. Our N-glycan analyses confirmed that both mAbs produced in lettuce have the expected high-mannose glycoform (Q. Chen, unpublished results). Since high-mannose is common between plant and mammalian cells, the concern for the immunogenicity of lettuce-derived mAbs in humans is diminished. Previously, we demonstrated that a tobacco-derived hE16 mAb with the same high-mannose N-glycosylation pattern had no *in vivo* stability issue and showed potent therapeutic activity against lethal WNV challenge in a mouse model (Lai et al., 2010). The structural equivalency of lettuce and tobacco-derived mAbs suggests their similarity in stability and functional activities. Indeed, these lettuce-derived mAbs showed specific binding to their respective antigens, and for hE16 retained equivalent functional activity. To the best of our knowledge, this is the first demonstration of functional activity for a lettuce-produced mAb. Our success in producing fully-assembled VLP and mAbs with the geminiviral-lettuce system strongly suggest its utility in producing a broad range of pharmaceutical proteins.

This study serves as a proof-of-principle for using commercially produced lettuce in high-level PMP production. While a greater variation in expression levels of mAbs was observed between different batches of lettuce possibly due to differences in growing or post-harvest storage conditions, surprisingly, the overall accumulation levels of mAbs was similar between grocery stored-purchased lettuce and the laboratory-grown lettuce. Lettuce is a fast-growing plant that is already cultivated year-round in commercial greenhouses for mass production. In addition, processing technologies for commercial-scale harvesting, washing, and processing of lettuce exist. Therefore, biomass production could be subcontracted to existing commercial growers to provide lettuce with consistent growth and post-harvest storage conditions, which likely would reduce variation in target protein accumulation. The existence of large-scale processing technology and commercial infrastructure in the agricultural industry will facilitate adaptation for PMP production. Our success in using commercially produced lettuce and geminiviral expression vectors for high-level PMP production allows access to potentially unlimited quantities of inexpensive plant material for large-scale production.

Since lettuce is a food crop, there may be environmental and regulatory concerns for its use in PMP production. While this is an issue for production systems using transgenic lettuce cultivated in open fields, our geminiviral transient expression system does not require transgenic lettuce, but rather, uses non-transgenic lettuce that can be purchased from commercial sources. In addition, operations for agroinfiltration and downstream processing can be performed in a contained cGMP controlled environment as required for all pharmaceutical production systems. As a result, there would be no exposure of transgenic lettuce or vectors to the environment, essentially eliminating the risk of contaminating food supply. This safety feature will minimize biosafety and regulatory concerns and costs associated with transgenic crops and genetically modified plants.

In summary, the use of geminiviral replicon expression system and commercially produced lettuce as plant production host provides a new platform for pharmaceutical protein production that is robust, low-cost, safe, and scalable to commercial manufacturing.

## Experimental Procedures

### Construction of expression vectors

The construction of geminiviral vectors pREP110, pBYGFP, pBYNVCP, pBY-HL(6D8).R and non-replicon vector pP19 has been described previously (Huang et al., 2009; Huang et al., 2010). The dual-replicon vector pBY-HL(hE16).R was constructed with a similar

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cloning strategy as for pBY-HL(6D8).R. Briefly, the coding sequences of hE16 HC or LC (Lai et al., 2010) were used to replace that of 6D8 HC or LC as a XbaI-KpnI fragment in vector pBY-H2KDEL210 (Huang et al., 2010) to create pBYhE16HC-KDEL or pBYhE16LC. The AscI and KpnI fragment of pBYhE16LC was used to replace that of 6D8-K3 in vector pBYK3R (Huang et al., 2010) to create pBYhE16LC.R. The replicon in pBYhE16HC-KDEL was then inserted into the vector pBYhE16LC.R to create the dual-replicon vector pBY-HL(hE16).R as described previously (Huang et al., 2010). MagnICON vectors for GFP, NVCP and hE16 expression were described previously (Lai et al., 2010; Santi et al., 2008).

## Lettuce agroinfiltration

Plant expression vectors were transformed into *Agrobacterium tumefaciens* GV3101 individually by electroporation as described (Huang et al., 2010). Wild-type lettuce (*L. sativa, red leaf*) plants were either grown with 16/8 hr light/dark cycle at 25°C for 6 weeks in a growth chamber or obtained commercially from a local grocery store. The store-purchased lettuce heads were rinsed with distilled water, put on wet paper towels, covered with saran wrap, and conditioned for two days in the growth chamber described above. Lettuce heads were vacuum infiltrated with GV3101 strains containing the targeted expression vectors as described previously (Negrouk et al., 2005). Agroinfiltrated lettuces were incubated in growth chamber for 3, 4, 5, and 6 days after infiltration before harvested for protein extraction.

## Protein extraction

Lettuce leaves were harvested 3, 4, 5 and 6 days dpi for time-course experiments of evaluating temporal expression pattern of the target proteins. For other experiments, leaves were harvested 4 dpi. Lettuce leaves were ground in extraction buffer (phosphate-buffered saline (PBS), 1 mM EDTA, 10 μg/ml leupeptin, and 0.3 mg/ml PMSF) with a Fastprep (Bio101) machine to extract the total leave soluble protein. The crude leaf extract was processed by centrifugation at 14,000 × *g* for 10 minutes at 4°C to yield “lettuce extract”. To obtain the “clarified lettuce extract”, the “lettuce extract” was further clarified by filtration through a 0.2 micron filter.

## Protein analysis

SDS-PAGE, Western blot, and ELISA analysis for NVCP, 6D8 mAb and hE16 mAb, sucrose gradient centrifugation and electron microscopy for NVCP VLP, antigen binding assays for 6D8 and hE16 mAbs, and GFP visualization were all performed according to published protocols (Huang et al., 2009; Huang et al., 2010; Lai et al., 2010). Irradiated, inactivated Ebola virus for the antigen binding assay of 6D8 mAb was prepared as described in (Huang et al., 2010) and generously provided by Dr. William Pratt of USAMRIID.

## WNV neutralization

The neutralizing activity of hE16 against WNV was assessed using a focus reduction neutralization assay as previously described (Fuchs et al., 2011). WNV ($2.4 \times 10^2$ PFU in DMEM with 5% FBS) was pre-incubated with serial dilutions purified hE16 mAbs at 37°C for 30 minutes. Virus-mAb mixtures were added in duplicate to individual wells of tissue culture 96-well microtiter plates containing Vero cells at ~90% confluency. Virus was incubated with Vero cells for 1 hour at 37°C, after which wells were overlaid with 1% carboxymethylcellulose (Sigma Chemical) in MEM with 4% FBS. After culture at 37°C for 24 hours, overlay media was removed and wells were washed with PBS. Cells were fixed with 1% paraformaldehyde in PBS (10 minutes at room temperature) and permeabilized with 0.1% saponin and 0.1% BSA in PBS (saponin buffer; 5 minutes at room temperature).

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Cells were stained with a mouse E16 mAb (Oliphant et al., 2005) (50 μl at 200 ng/ml in saponin buffer) for 2 hours at room temperature. Following several washes, wells were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma; 250 ng/ml in saponin buffer) for 1 hour at room temperature. Wells were washed and foci were visualized with TrueBlue Substrate (KPL) after 5 to 10 minutes of incubation at room temperature. Wells were rinsed with water and dried prior to analysis with a Biospot counter (Cellular Technology) using Immunocapture software. Neutralization curves (percent reduction of spot numbers in samples pre-incubated with mAb compared to wells with virus pre-incubated with medium alone) were graphed using Prism software, and EC50 values calculated.

### Protein Purification

Lettuce leaves infiltrated with 6D8 or hE16 MAb construct were harvested on 4 dpi and homogenized with a blender in extraction buffer (PBS, 1 mM EDTA, 10 mg/ml sodium ascorbate, 10 μg/ml leupeptin, 0.3 mg/ml PMSF). Leaf extract was clarified by filtering through Miracloth, followed by centrifugation at 17,700 × *g* for 30 min at 4°C and filtration through a 0.2-micron filter. Clarified leaf extracts were applied directly to a MAbSelect Protein A column (GE Healthcare, Piscataway, NJ). The column was eluted with 50 mM sodium citrate, pH 2.5 after washing with PBS. Tris-base (1M) was added immediately to the eluate to attain a final pH of 7.0. MAbs were further purified by DEAE anion exchange chromatography with DEAE Sepharose FF 26/20 resin (GE Healthcare) as described previously (Lai et al., 2010). The purity of 6D8 and hE16 mAbs, and levels of residual DNA, Protein A and endotoxin in the final purified samples were determined using a densitometer and commercial PicoGreen dsDNA quantitation (Invitrogen), protein A ELISA (Cygnus Technologies), and QCL-1000 Chromogenic LAL Endpoint assay kits (Lonza), respectively as described preciously (Huang et al., 2010; Lai et al., 2010).

NVCP VLP was purified from lettuce leaves with a low pH (pH 5.2) precipitation step followed by a DEAE ion exchange chromatography as described for NVCP purification from *N. benthamiana* leaves (Chen, 2011a).

## Acknowledgments

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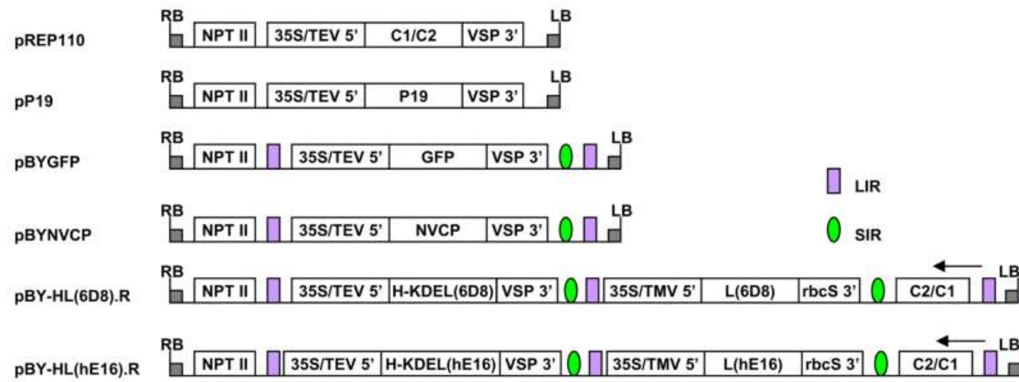


**Figure 1.**

The T-DNA region of the vectors used in this study. 35S/TEV5′: CaMV 35S promoter with tobacco etch virus 5′UTR; VSP3′: soybean vspB gene 3′ element, NPTII: expression cassette encoding nptII gene for kanamycin resistance, C2/C1: BeYDV ORFs C1 and C2 which encode for replication initiation protein (Rep) and RepA, P19: a gene silencing suppressor gene from tomato bushy stunt virus, LIR (purple box): long intergenic region of BeYDV genome, SIR (green oval): short intergenic region of BeYDV genome, rbcS3′: pea rbcS gene 3′ element, LB and RB: the left and right borders of the T-DNA region, L(6D8) and L(hE16): LC of mAb 6D8 and mAb hE16, H-KDEL(6D8) and H-KDEL(hE16): HC of mAb 6D8 and mAb hE16 with the ER-retention signal “KDEL” fused to its C-terminus.

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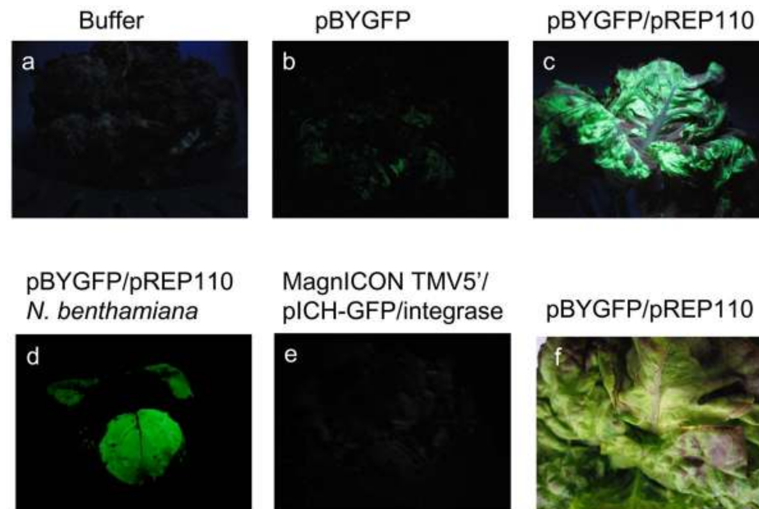


**Figure 2.**

Visualization of GFP expression in lettuce. Commercially produced lettuce heads were infiltrated with a single Agrobacterium culture, or co-infiltrated with two or three cultures containing the indicated expression vector(s). Leaves were examined and photographed 4 days post infiltration under UV (a–e) or regular light (f). Lettuce infiltrated with the infiltration buffer (a) was used as a negative control. *N. benthamiana* was used as a positive control (d). MagnICON vectors were described in (Santi et al., 2008). One representative of at least three independent experiments is shown.

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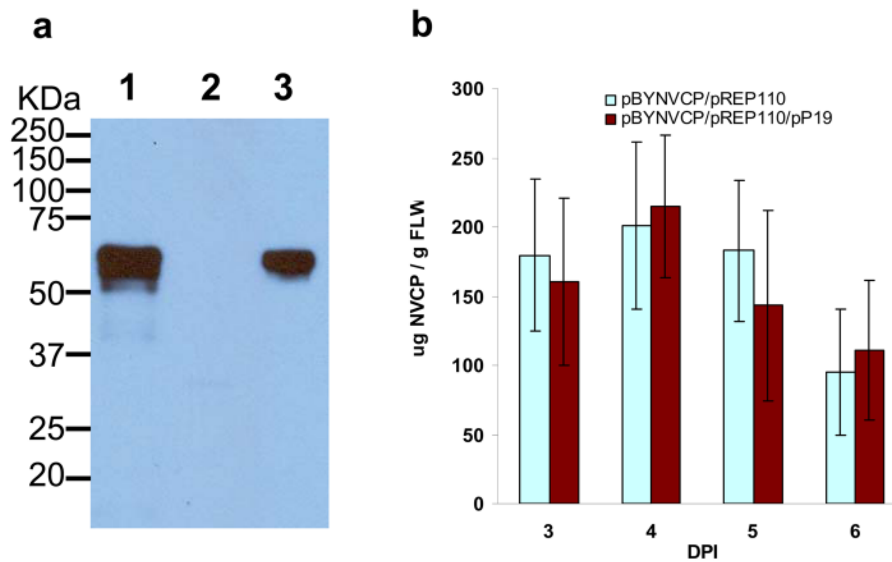


**Figure 3.**

Expression of NVCP in lettuce leaves with geminiviral vectors. (a) Western blot analysis of NVCP. Leaf protein extracts were separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes. The membranes were probed with a rabbit polyclonal antibody against NVCP. Lane 1: insect cell-derived NVCP standard; lane 2: protein extract from uninfiltrated lettuce leaves (negative control); lane 3: extract from pBYNVCP/pREP110 infiltrated lettuce leaves. (b) Time course of NVCP expression. Total proteins from lettuce leaves infiltrated with pBYNVCP/pREP110 or pBYNVCP/pREP110 + pP19 were extracted on days 3–6 post infiltration and analyzed by ELISA with insect cell-produced NVCP as the standard, a rabbit polyclonal anti-NVCP as the capture antibody, and a guinea pig polyclonal anti-NVCP as the detection antibody as described previously (Huang et al., 2009). Mean ± SD of samples from three independent infiltrations are presented.

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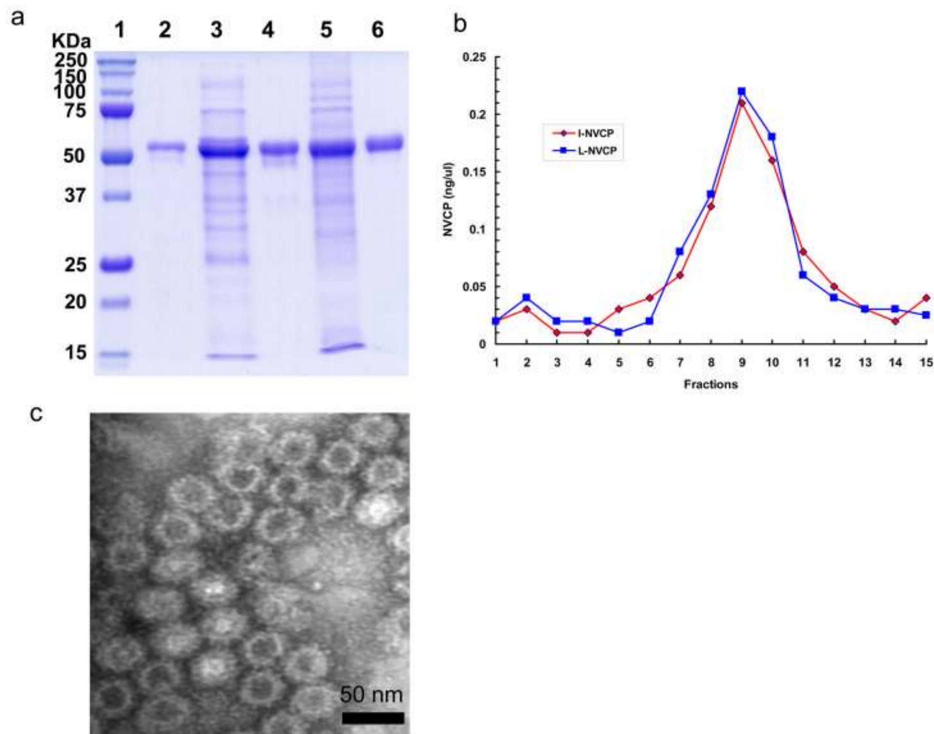


**Figure 4.**

Purification and characterization of NVCP transiently expressed in lettuce leaves. (a) Purification of lettuce-derived NVCP. Leaf protein samples were purified and analyzed on a 4–20% gradient SDS-PAGE gel under reducing condition and visualized with Coomassie stain. Lane 1: Molecular weight marker; lane 2: insect cell-derived NVCP reference standard; lanes 3 and 4: crude protein extract and purified NVCP from *N. benthamiana* leaves as a comparison; lane 5: crude extract from pBYNVCP/pREP110 infiltrated lettuce leaves; lane 6: purified NVCP from lettuce leaves. (b) Sucrose gradient sedimentation profile of NVCP. Purified NVCP from lettuce leaves (L-NVCP) and insect cell-derived NVCP reference standard (I-NVCP) were analyzed on linear 10 to 60% sucrose gradients. After sedimentation, 15 fractions were taken from top to bottom (from left to right in the figure) and assayed for NVCP by ELISA. (c) Electron microscopy of lettuce-derived NVCP negatively stained with 0.5% uranyl acetate. Bar = 50 nm. One representative experiment of three is shown.

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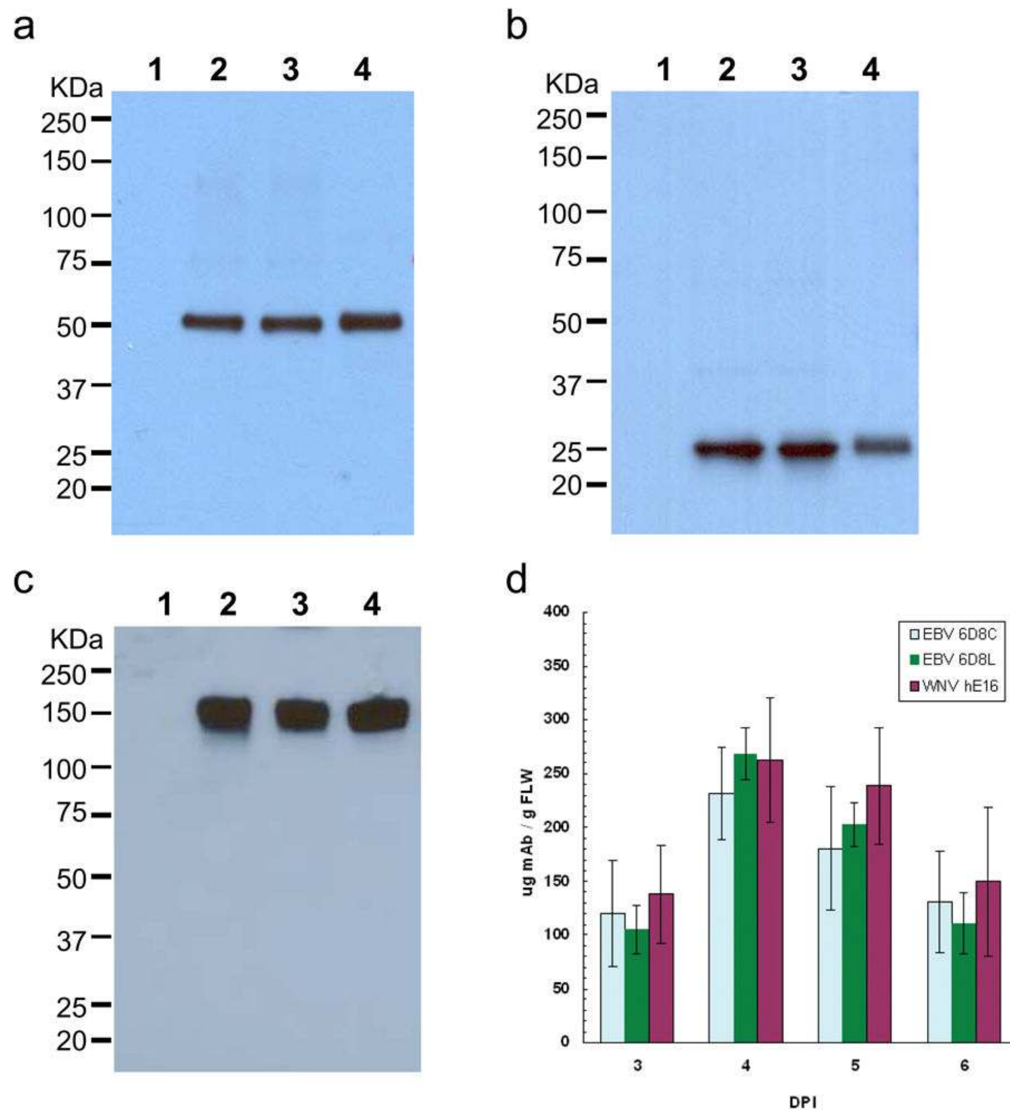


**Figure 5.**

Expression of MAbs against EBV and WNV in lettuce with geminiviral vectors. Commercially produced (a–d) or laboratory-grown (d) lettuce were infiltrated with dual replicon geminiviral vector pBY-HL(6D8).R or pBY-HL(hE16).R and harvested at 4 dpi (a–c) or 3–6 dpi (d). (a–c): Western blot analysis. Total protein extracts of lettuce leaf were separated on 4–20% SDS-PAGE gradient gels under reducing (a and b) or non-reducing (c) condition and transferred to PVDF membranes. The membranes were incubated with a goat anti-human-gamma chain antibody to detect HC (a) or a goat anti-human-kappa chain antibody to detect LC (b and c). Lane 1: extract from uninfiltrated lettuce leaves; lanes 2 and 3: protein samples from lettuce infiltrated with pBY-HL(6D8).R or pBY-HL(hE16).R construct; lane 4: human IgG reference standard. (d) ELISA analysis of 6D8 or hE16 mAb expression. Goat anti-human gamma and kappa chain antibodies were used as capture and detection reagents, respectively to confirm the assembled forms of 6D8 or hE16 mAb. Mean ± SD of three batches of lettuce in three independent infiltration experiments are presented. EBV 6D8C: samples from commercially produced lettuce infiltrated with pBY-HL(6D8).R; EBV 6D8L: proteins from laboratory-grown lettuce infiltrated with pBY-HL(6D8).R; WNV

hE16: protein extracts from commercially produced lettuce infiltrated with pBY-HL(hE16).R.

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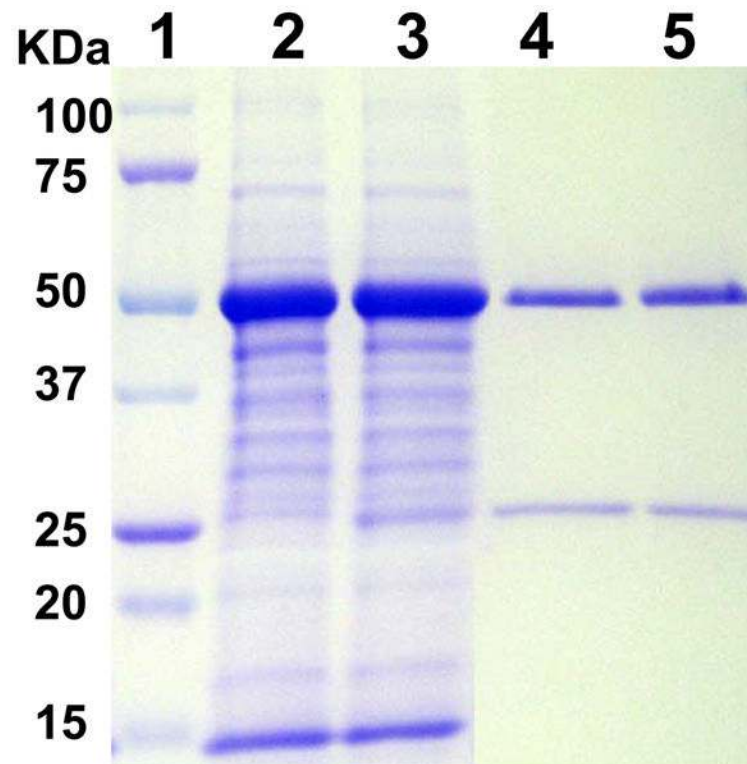


**Figure 6.**

Purification of geminiviral-lettuce-produced mAbs. Protein extracts from lettuce leaves infiltrated with pBY-HL(6D8).R or pBY-HL(hE16).R were purified and analyzed on a 4–20% SDS-PAGE gel under reducing condition. Lane 1: Molecular weight marker; lane 2: total leaf proteins from uninfiltrated lettuce leaves; lane 3: total leaf protein from lettuce leaves infiltrated with pBY-HL(6D8).R; lane 4: purified 6D8 mAb eluted from protein A column; lane 5: hE16 mAb purified from pBY-HL(hE16).R infiltrated lettuce leaves by protein A chromatography. One representative of several independent experiments is shown.

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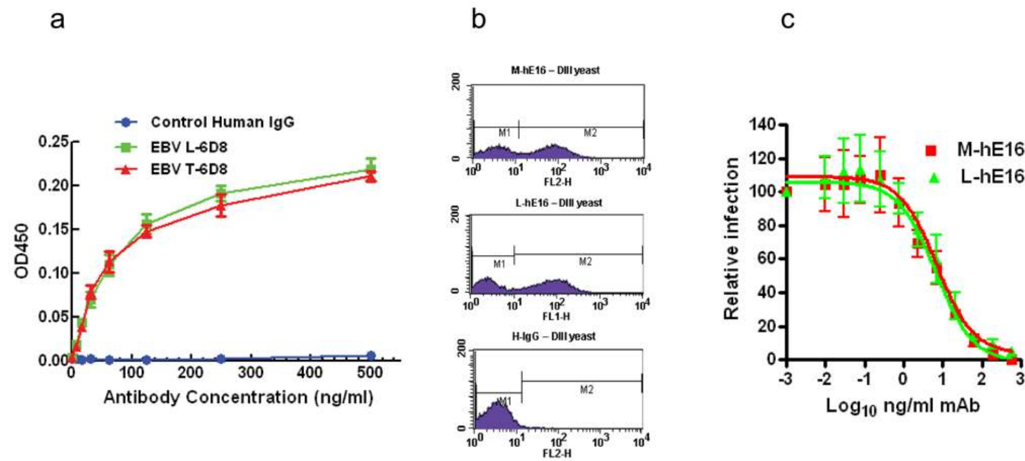


**Figure 7.**

Functional characterization of geminiviral-lettuce-produced mAbs. (a) Specific binding of 6D8 mAb to EBV. Irradiated, inactivated EBV was immobilized on an ELISA plate and incubated with increasing dilutions of lettuce-derived 6D8 mAb (EBV L-6D8), tobacco-derived 6D8 (EBV T-6D8, positive control), or a negative control generic human IgG. A horseradish peroxidase-conjugated anti-human IgG was used to detect MAbs bound to EBV virus. Mean ± SD of $OD_{450}$ from three independent experiments are presented. (b) Binding of lettuce-derived hE16 to domain III of WNV E displayed on the cell surface of yeast. Lettuce-produced hE16 mAb (L-hE16), mammalian cell-derived hE16 (M-hE16, positive control), or a generic human IgG (h-IgG, negative control) was used to stain yeast cells displaying domain III of WNV E protein, which were then processed by flow cytometry. Representative data from three independent experiments are shown. (c). Neutralization of WNV by lettuce-produced hE16 mAb. WNV (strain New York 1999) was incubated with serial dilutions of hE16 derived from lettuce (L-hE16) or mammalian cells (M-hE16) (positive control) and used to infect Vero cells. Cells were then fixed, permeabilized, analyzed by focus reduction assay and quantitated by Biospot analysis. Mean ± SEM is shown from one of two independent experiments.

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## Table 1

Analysis of purified 6D8 and hE16 mAbs produced in lettuce

| MAb | Purity | Endotoxin (EU/ml) | Residual Protein A (ng/ml) | Residual DNA (ng/ml) |
|---|---|---|---|---|
| 6D8 | > 95% | 3.40±1.83 | 13.54±4.67 | < 1 |
| hE16 | > 95% | 2.86±2.74 | 8.65±3.81 | < 1 |

Data (mean ± SD) from three independent purification batches for each mAb are presented.