Vaccines Against West Nile Virus

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Abstract Despite the availability of two veterinary vaccines against West Nile virus (WNV), there remains a desperate need for a more efficient, safer, cheaper WNV vaccine that can be delivered conveniently to animals. The global threat of WNV epidemics with increasingly severe neuroinvasive infections makes this need even more urgent. To date, vaccine candidates based on inactivated, live-attenuated, or chimeric virus, and viral DNA and protein subunits have been developed. However, commercialization of a WNV veterinary vaccine may largely depend on the economics of vaccine production, as only novel low-cost production platforms would produce vaccines that outcompete the cost of clinical treatment for animals. In this chapter, we review the progress of using plants to develop effective WNV vaccines and address the economic challenges of WNV vaccine production. The status of current WNV vaccine development is summarized. The advantages of plant-based platforms for WNV vaccine production in cost, speed and scalability are briefly discussed. The progress in developing WNV subunit vaccines in plants is reviewed within the context of their expression, characterization, downstream processing, and potency in animal models. The development of WNV vaccines based on virus-like particles is also highlighted. We are confident that plants are one of the platforms that offer potent, safe and affordable veterinary vaccines against WNV.

Keywords West nile virus (WNV) · Flavivirus · Zika virus (ZIKV) Plant-made vaccine · Virus-like particle (VLP) · Plant-made biologics (PMB) Plant-made pharmaceutical (PMP) · Downstream processing · Oral vaccine

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1 Introduction

West Nile virus (WNV) is a single-stranded RNA virus that belong to the genus *Flavivirus* of the family *Flaviviridae*. This genus includes many arthropod-borne pathogens such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), Dengue virus (DENV1-4) tick-borne encephalitis virus (TBEV), and Zika virus (ZIKV). In nature, WNV is mostly maintained in sylvatic cycles between birds and ornithophilic arthropods, i.e. mosquitos and occasionally ticks. Although not all bird species develop illness from WNV infection, high mortality rate has been observed in some species (Komar et al. 2003). WNV can easily spread to humans and domestic mammals, especially horses. Epidemics of WNV fever were first reported in the 1950s in Israel (Zeller and Schuffenecker 2004). Since then, WNV outbreaks both in humans and horses have occurred in Africa, Europe and eventually North America in 1999 (Zeller and Schuffenecker 2004). WNV has become an important health concern for both humans and horses, including pet horses, because of the increased frequency of outbreaks and increased neurological disease cases (Castillo-Olivares and Wood 2004).

Due to its global health impact, WNV infection has been under intensified surveillance in humans, horses, birds and mosquitos since the 1990s. This has led to the identification of many WNV strains. WNV strains can be classified in up to seven different genetic lineages based on phylogenetic analysis (Rizzoli et al. 2015). Among all the known WNV strains, Lineage 1 is the most popularly spread lineage which was responsible for the outbreak in New York in 1999 (Rizzoli et al. 2015). Lineage 1 can be further divided into three sublineages: 1a, 1b and 1c. WNV-1a has been found in Africa, Europe, North America and Asia. WNV-1b contains Kunjin virus strains identified in Australia and WNV-1c was only found in India (Rizzoli et al. 2015). Lineage 2 is considered the oldest WNV strain and the second most widely spread (Rizzoli et al. 2015). Lineage 3, also known as Rabensburg virus, was isolated from mosquitos found in Czech Republic in 1997 (Rizzoli et al. 2015). Lineage 4 includes three sublineages with 4a first isolated from ticks then in mosquitos and frogs in Russia while 4b and 4c were detected in mosquitos from Spain and Australia, respectively. Kunjin virus KUN MP502-66 isolated from Malaysia, Koutango virus and a putative new lineage of WNV isolated from Senegal in Africa were classified as Lineage 5, 6 and 7 (Rizzoli et al. 2015). Most circulating WNV strains belong to either lineage 1 or 2 and are associated with outbreaks among humans and horses, while other lineages have been identified, so far, mainly in mosquitos and birds (Rizzoli et al. 2015).

In humans, most of the WNV infections are asymptomatic, but 15–20% of the cases reported mild symptoms including fever, nausea, headache and vomiting and, in less than 1% cases, it leads to neuroinvasive diseases, even death (Zeller and Schuffenecker 2004). In horses, various clinical signs have been reported for WNV encephalomyelitis that includes fever, ataxia, partial paralysis, recumbency, and behavioral changes. Compared to humans, the incidence of WNV infection in horses is much higher and the disease is more severe with almost one third of

infections resulting in fatality and 40% of the surviving horses have neurological sequelae (Aharonson-Raz et al. 2014). Unlike in humans, the severity of neurological diseases in horses is not associated with aging (Castillo-Olivares and Wood 2004).

WNV has an 11 kb single-stranded RNA genome that encodes three structural proteins: Capsid protein (C), Pre-membrane/membrane protein (prM/M) and envelope protein (E), as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The functions of the non-structural proteins are not yet fully determined, but they are known to be involved in WNV RNA replication, translation, and the assembly of viral replication complexes in infected cells (Brinton 2013). In addition to its role for viral RNA replication and packaging, the WNV Capsid protein was found to interact with multiple host cell proteins (Yang et al. 2002; van Marle et al. 2007; Medigeshi et al. 2009; Brinton 2013) that determines virulence and plays an important role in pathogenesis. The prM and E protein are the two main immunogens and they form heterodimers in immature viral particles (Xu et al. 2011) while the prM acts as chaperone for the maturation of E protein. Cleavage of the pr peptide from PrM by the cellular protease furin and the resulting conformational change of E protein yields mature virions. Co-expression of the prM and E proteins in vitro has been shown to generate noninfectious virus like particles (VLPs) that have been tested as WNV vaccine candidates (Ohtaki et al. 2010). The E protein is the major surface component of the mature WNV viral particle and it is responsible for receptor binding and viral entry into host cells through membrane fusion. Similar to DENV and TBEV, the WNV E protein consists of three distinct domains DI, DII and DIII (Kanai et al. 2006). The DI contains a β barrel core structure and connects the DII and DIII. DII is built mostly of β strands and contains the well-conserved fusion loop among flaviviruses. DIII has an Ig-like structure and contains the putative receptor binding sites and epitopes for WNV-specific neutralizing antibodies (Kanai et al. 2006). There is no definitive conclusion on which WNV E domain(s) displays the most diversity across WNV strains, while it is clear that DIII is the most diverse domain among the E protein of different flaviviruses.

2 Current Development of WNV Vaccines

Currently, there is no effective therapeutic for WNV related diseases; all treatments for both humans and horses are supportive, which include providing fluid, nutritional care and reducing inflammation in neurological diseases (Castillo-Olivares and Wood 2004). Several vaccine candidates are under clinical investigations, but no WNV vaccine has been yet approved for human use (Table 1). For veterinary applications, vaccines currently available are poorly immunogenic, unsafe, or not economical to produce (Table 1). Therefore, development of effective, safe, and cost-effective vaccines is urgently needed for preventing WNV infection and controlling its outbreaks in human or horse populations.

Table 1 Current WNV veterinary vaccines and vaccines under clinical trials

Vaccine name and sponsor	Vaccine type	Antigen	Development stage	References
West Nile innovator (Pfizer)	Inactivated whole virus vaccine	Formalin inactivated WNV-NY99	Licensed for veterinary use	Ng et al. (2003)
Recombitek (Merial)	Chimeric/ recombinant vaccine	Canarypox expressing PrM/E	Licensed for veterinary use	Minke et al. (2004)
Prevenile (Intervet)	Chimeric/ recombinant vaccine	YF17D backbone expressing PrM/E	Licensed for veterinary use in 2005 then recalled in 2010	Arroyo et al. (2004)
West Nile innovator DNA (Pfizer)	DNA vaccine	DNA plasmid encodes PrM/E	Licensed for veterinary use in 2005, discontinued	Martin et al. (2007)
ChimeriVax-WN02	Chimeric/ recombinant vaccine	YF17D backbone expressing PrM/E	Phase II trial	Monath et al. (2006), Dayan et al. (2013)
rWN/DEN4Δ30	Chimeric/ recombinant vaccine	DV4 expressing PrM/E	Phase I trial	Durbin et al. (2013)
VRC-WNVDNA017-00-VP	DNA vaccine	DNA plasmid encodes PrM/E	Phase I trial	Martin et al. (2007), Ledgerwood et al. (2011)
WN-80E (Hawaii Biotech)	Recombinant subunit vaccine	Truncated WNV E protein	Phase I trial	Coller et al. (2012)

2.1 Inactivated Whole WNV Vaccines

Using an inactivated whole virus as a vaccine is well established and has been continuously used for a variety of viral diseases. In fact, the first WNV vaccine was based on a formalin-inactivated WNV-NY99 that was developed by Fort Dodge Animal Health (commercialized by Pfizer) shortly after the 1999 outbreak in New York (Ng et al. 2003). This inactivated whole virus vaccine induces protective immunity against WNV infection in horses and other animal models (Ng et al. 2003). However, the requirement of handling highly pathogenic WNV strains in a Biosafety Level 3 (BSL-3) environment in large-scale during the manufacturing process is costly and labor intensive. Moreover, the risk of incomplete inactivation of the live virus presents another public safety concern. Recently, a new WNV vaccine candidate has been developed by inactivation of a naturally attenuated

WNV Kunjin strain using hydrogen peroxide (Amanna et al. 2012). Compared to formalin, inactivation with hydrogen peroxide minimizes the damage to WNV antigenic structures. As a result, this vaccine candidate may have improved immunogenicity over the first WNV vaccine, as well as a better safety profile due to the attenuated nature of the Kunjun strain. Animal studies have shown that two doses of this WNV vaccine candidate can protect mice and non-human primates from WNV infection by inducing broadly neutralizing WNV specific antibody responses (Pinto et al. 2013; Poore et al. 2017).

2.2 Chimeric/Recombinant Vaccines

Chimeric/recombinant vaccines are another important approach for WNV vaccine development. These vaccines use a non-WNV virus as the backbone and replace the PrM/E coding sequences of the carrier virus with the corresponding WNV genes to create chimeric viruses. Two such vaccines have been licensed for veterinary use using the canarypox and YFV 17D as the backbone, respectively (Arroyo et al. 2004; Minke et al. 2004). Further mutations in the E protein were introduced to reduce the potential side effect of neurovirulence in immunized subjects (Arroyo et al. 2004; Monath et al. 2006). The YFV-based vaccine (ChimeriVax-WN02) has been evaluated in numerous pre-clinical studies and three clinical trials (Dayan et al. 2013). The potential safety risk is a major disadvantage of these chimeric vaccines. Since they are attenuated live viruses, there is always a concern for their potential reversion to virulent strains. In addition, new viruses could be generated as the result of recombination between the chimeric virus and another flavivirus, raising further safety concerns. A similar strategy was used to develop another live and attenuated chimeric/recombinant vaccine for WNV using DENV4 backbone with 30 neucleotides deleted in the 3′ non-coding region (rWN/DEN4Δ30) (Durbin et al. 2013). This vaccine candidate is well tolerated and immunogenic. However, it can replicate in a mosquito vector that carries wild-type WNV and DENV, which raises safety concerns for its further development.

2.3 DNA Based WNV Vaccines

DNA based vaccine approaches can provide efficient and cost-effective vaccine development using modern genetic technology. The first WNV DNA vaccine was developed in 2001 with one single plasmid encoding the WNV prM/E region (Davis et al. 2001). This DNA vaccine was shown to protect against WNV by inducing neutralizing antibodies in both mice and horses. It was licensed by USDA for preventing WNV infection in horses in 2005 (Martin et al. 2007), but later

discontinued by Pfizer (Brandler and Tangy 2013). Subsequently, a similar DNA plasmid vaccine and its updated version with a modified promoter were assessed in phase I clinical trials (Martin et al. 2007; Ledgerwood et al. 2011). These studies showed that these DNA-based vaccines were safe and somewhat immunogenic in both young (18–50) and elder (50–65) human groups. However, this vaccine candidate required three 4-mg doses via intramuscular injection to achieve detectable immunogenicity, rendering it a suboptimal vaccine candidate due to its poor immunogenicity. Another DNA vaccine for WNV used a plasmid vector to direct in vivo transcription of the full length Kunjin viral RNA in mice (Hall et al. 2003). Attenuated Kunjin virus was detected in the sera of immunized mice after 3–4 days. The vaccinated mice showed full protection against a lethal challenge of virulent WNV strain NY99.

For most DNA vaccines, the biggest challenge is their low immunogenicity. Over the years several approaches have been applied to improve the stimulation of immune responses. One approach was to co-express the capsid protein from a separate promoter along with a capsid-deleted DNA vaccine to allow the formation of secreted single-round infectious particles (SRIPs) (Chang et al. 2008), thereby mimicking the live virus infection to induce protective immune response but without the usage of infectious virus. Other approaches include co-immunization of an E protein DIII-based DNA vaccine with an optimized IL-15 plasmid to stimulate antibody secreting B cells, which enhanced the overall immune response by four to five folds (Ramanathan et al. 2009). While promising, these strategies use more than one plasmid in their formulation, complicating the manufacturing process and increasing the production cost. More recently, nanoparticles were used as carriers for DNA vaccine delivery (De Filette et al. 2014); however, it failed to induce the desired humoral immune response when applied alone. Furthermore, efforts have been made to develop infectious yet safe WNV DNA vaccines by designing chimeric WNV W1806 DNA with mutations in the E protein to further attenuate its virulence (Yamshchikov et al. 2017).

2.4 Recombinant Subunit Protein Vaccines

Recombinant subunit WNV proteins have been researched as vaccine candidates against WNV infection since 2001. The WNV E protein was expressed in *E. coli* and purified and used to immunize mice, which protected mice against a lethal WNV infection (Wang et al. 2001). However, Flavivirus E or its subdomains produced in *E. coli* are often recovered in insoluble inclusion bodies, requiring a cumbersome and unscalable refolding process to obtain native antigenic structures (Yang et al. 2017a). Later, a truncated form of the E protein was expressed in Drosophila S2 cells to produce an antigen that resembled its native conformation (Ledizet et al. 2005). Vaccination of mice with this truncated E protein, using

aluminum hydroxide as an adjuvant, protected animals from a WNV lethal challenge. Similarly, another truncated WNV E protein without the C-terminal membrane anchor (WN-80E) was also expressed in Drosophila S2 cells. When co-delivered with adjuvants, this subunit vaccine candidate elicited both humoral and cellular immune responses in mice and a nonhuman primate model (Lieberman et al. 2007, 2009). WN-80E was eventually tested in a Phase I clinical trial for safety and immunogenicity. Human subjects were vaccinated with three injections of 5, 15 or 50 μg of WN-80E with adjuvant or 50 μg of WN-80E without adjuvant (Coller et al. 2012). The vaccine was well tolerated and all subjects developed neutralizing antibodies after the third injection with the PRNT50 (plaque reduction neutralization test) ranging from 1:10 to 1:100. These results indicate that insect cells provide an improved production platform for producing soluble antigens. Like all cell culture-based systems, however, it has limitations in high production cost and scalability (Chen 2011a, b).

VLPs are another group of vaccine candidates in the recombinant subunit protein vaccine category. VLPs containing the WNV prM and E protein were first produced in the baculovirus expression system and elicited neutralizing antibody responses in immunized BALB/c mice (Qiao et al. 2004). Subsequent studies in a Chinese hamster ovary (CHO) cell expression system with serum free culture media showed that two types of VLPs differing in size and maturation stage were produced. The larger VLP was shown to contain the mature M protein and have better protective efficacy in immunized mice (Ohtaki et al. 2010). In another study, WNV prM-E VLPs were expressed with a herpes simplex virus 1 recombinant vector. Furthermore, they were efficiently released from host cells and induced a protective immune response against WNV upon vaccination in mice (Taylor et al. 2016). While efficacious, these mammalian cell-produced vaccine candidates are costly. Further development and production of these candidates in a more cost-effective system will facilitate the realization of their full potential in preventing WNV global epidemics (Chen 2011a, b).

Since DIII of the E protein has been shown to contain the receptor-binding motif and epitopes for WNV-specific neutralizing antibodies, VLPs with DIII displayed on the surface were also explored (Spohn et al. 2010; Chua et al. 2013; Taylor et al. 2016). A conjugated vaccine with recombinant DIII chemically cross-linked to bacteriophage AP205-derived VLPs induced higher titers of DIII-specific neutralizing antibodies in mice compared to those induced by recombinant DIII protein alone (Spohn et al. 2010). Similarly, DIII-carrying mosaic AP205 VLPs were also developed by genetic fusion of the DIII sequence to the C-terminal of AP205 coat protein under codon suppression condition, which potently induced the production of WNV-neutralizing antibodies (Cielens et al. 2014). Another variation of DIII-displaying VLPs were designed by fusing DIII with the gamma chain of IgE receptor. When this fusion construct was expressed in insect cells, VLPs were actively secreted by host cells into the media and DIII protein was found on the surface of these extracellular VLPs (Chua et al. 2013). However, these DIII displayed VLPs only generated modest neutralizing immune responses in mice.

3 Plants as an Optimal Platform to Produce WNV Veterinary Vaccines

As WNV has become a serious health concern for humans and animals, with effective therapeutics still not available, there is an urgent need to develop vaccines not only for humans but also for other susceptible animals. WNV has been found in about 300 bird species and many non-avian vertebrates including horses, who are also susceptible to and commonly infected by WNV (Iyer and Kousoulas 2013). Low cost and effective vaccines that can be delivered via flexible routes would protect animals against WNV infection and significantly reduce its transmission. While two WNV vaccines are currently commercially available for use in horses, these vaccines require at least two injections and an annual boost to ensure protection. Adverse effects have been widely-reported for these vaccines in horses, indicating the current veterinary vaccines are far from ideal for WNV prevention.

Besides the difficulty in balancing the immunogenicity and safety of the vaccines, development of new WNV candidate vaccines is often haunted by the high cost of large-scale recombinant protein expression, purification and storage. However, plants represent an alternative system that may address these limitations. Tobacco and sunflower plants have been used to express recombinant proteins for more than 30 years (Barta et al. 1986). Plant based production systems have drawn more attention as Ebola virus-infected patients showed dramatic improvement after receiving ZMapp, a plant-made antibody cocktail against EBOV (Lyon et al. 2014). Plant-based expression systems may yield large amounts of recombinant proteins in a relatively short production period without the risk of contamination by animal pathogens, compared to using mammalian cell culture (Chen 2011a, b). Unlike bacteria or other prokaryotic systems, plants share similar endomembrane and secretory pathways with mammalian cells, thereby allowing proper assembly of recombinant proteins and the necessary post translational modifications. Moreover, plant-made recombinant proteins are more cost effective than mammalian cell expressed recombinant proteins (Tuse et al. 2014; Chen and Davis 2016) and can be easily scaled for manufacturing (Chen 2018; Chen and Lai 2015; Chen et al. 2016). For orally delivered drugs, the cost of production can be further reduced when edible plants are used for production as no intensive purification steps are needed (Chen and Davis 2016). Therefore, plant based expression systems may serve as a useful alternative for developing low cost WNV recombinant veterinary vaccines (Chen et al. 2018).

3.1 Plant Expression System

Traditionally, foreign genes were incorporated into the plant nuclear genome to generate transgenic plants for recombinant protein production (Chen 2008, 2011a, b). This typically includes using *Agrobacterium tumefaciens* to deliver the gene of interest randomly into the plant genome and selection of positive clones. While

establishing a permanent genetic source of an antigen is attractive, this procedure is often time consuming and can be complicated by unexpected gene silencing (Takeyama et al. 2015). More recently, transplastomic plants have been developed for recombinant protein production. This approach uses particle bombardment to deliver the gene of interest to the chloroplast of the plants. While it can produce high protein yield and prevent transgene outcrossing through pollen, this has been limited to the production of subunit vaccines containing one polypeptide and those that do not need post-translational modification (Takeyama et al. 2015).

Recent advances in plant viral vectors made it possible for transient vaccine expression in plants with high protein yield in a short time period. Many plant viruses have been used for this purpose, including tobacco mosaic virus (TMV), Potato virus X (PVX), Cucumber mosaic virus (CMV), Cowpea mosaic virus (CPMV), geminivirus and Cauliflower mosaic virus (CaMV) (Lico et al. 2008; Hefferon 2014). Historically, full virus vectors carrying the gene of interest were used; these vectors retain infectivity and produce viral particles from host cells that may spread to other plants. The second generation of plant viral vectors use a "deconstructed virus" strategy in which minimal sections of the viral genome important for replication and non-viral sections were integrated together to form a complete replicon (Peyret and Lomonossoff 2015). One such popularly used viral vector system is the magnICON system, which contains a 5′ module that has the TMV-based elements necessary for replication, a 3′ module that has the gene of interest, and a recombinase module containing a streptomyces phage 31 integrase (Peyret and Lomonossoff 2015). The magnICON system also relies on the co-infiltration of the three Agrobacterium strains with each containing one of the three modules. Once delivered to plant cells, the integrase fuses the 5′ and 3′ modules into a complete replicon and targets protein production (Peyret and Lomonossoff 2015).

Another example of the destructed virus vectors is the geminivirus-based expression systems. Unlike the single stranded RNA viruses, geminivirus-based vectors have a circular DNA genome that is replicated through a rolling-circle mechanism using double stranded DNA as intermediates (Chen et al. 2011). The geminivirus genome contains the long and short intergenic regions (LIR and SIR). The DNA can be circularized by joining two LIRS by Rep/RepA proteins which is particularly useful for handling large gene sequence or expressing multiple genes at the same time. Using a bean yellow dwarf virus (BeDV) based replicon system, DIII of WNV E protein was fused to Hepatitis B core antigen (HBcAg) gene and successfully produced in *Nicotiana benthamiana* plants (Chen et al. 2011).

The pEAQ vectors are another series of small binary vectors that use the CPMV hyper-translational expression system to facilitate recombinant protein expression in the plants (Peyret and Lomonossoff 2013). The vector backbone has the RNA-2 which encodes the viral coat protein and movement protein deleted from the original CPMV genome and it was replaced with foreign gene of interest. P19, a suppressor of gene silencing from Tomato bushy stunt virus was inserted into the pEAQ vector to enhance the recombinant protein expression. After the genes of interest are inserted into the pEAQ vectors, the plasmid DNA is transformed into *A.*

tumefaciens and then inoculated into *N. benthamiana* through infiltration. The pEAQ vectors have been successfully used for producing VLPs as candidate vaccines.

3.2 Plant-Produced WNV Subunit Vaccines

Since the first report of using a plant system to express the DIII of DENV E protein (Saejung et al. 2007), plant expression systems have been used to successfully express several flavivirus subunit vaccine candidates against DENV (Saejung et al. 2007; Martinez et al. 2010; Coconi-Linares et al. 2013; Kim et al. 2016), JEV (Appaiahgari et al. 2009; Wang et al. 2009) and WNV (He et al. 2012; He et al. 2014). Our laboratory has been interested in developing plant made flavivirus proteins as vaccine candidates and diagnostic reagents. The WNV E protein DIII sequence was cloned into a pICH11599 vector of the MagnICON system and then transformed into *A. tumefaciens* prior to the agroinfiltration of *N. benthamiana* using the syringe method (Leuzinger et al. 2013). The leaves were harvested 3–8 days following infiltration (dpi). Similar to the *E. coli*-expressed WNV DIII protein (Chu et al. 2005), a 13.5 kD DIII protein band from samples of plant extracts infiltrated with the DIII construct was detected by western blot analysis using an anti-WNV DIII polyclonal antibody (He et al. 2012, 2014). The expression of the plant-derived WNV DIII protein was quantified by ELISA using two WNV DIII specific antibodies. The expression level reaches its peak four days after infiltration to an average about 0.1 mg/g of leaf fresh weight (LFW), which was the highest expression level of a flavivirus antigen ever reported. Subsequent studies demonstrated that the WNV DIII expression level is subcellular compartment dependent (He et al. 2014). Using three different 5′ modules of the MagnICON system to specifically direct the DIII into ER, chloroplast or the cytosol, Western blot detected the 13.5 kD DIII protein band only from leaves infiltrated with ER targeted DIII construct, but not from leaves infiltrated with chloroplast or cytosol targeted DIII constructs (Fig. 1a). ELISA then showed that the ER targeted DIII reached an average level of 73 μg/g LFW, while the maximum level of DIII targeted to chloroplast or cytosol is only about 1.16 μg/g LFW (Fig. 1b). However, the level of the ER targeted WNV DIII is lower than the levels of other viral antigens or antibodies we produced in *N. benthamiana* using MagnICON-based plant expression vectors ranging from 100 to 700 μg/g LFW (Santi et al. 2008; Lai et al. 2010; Phoolcharoen et al. 2011; Dent et al. 2016). This could be the result of leaf necrosis observed after 4 dpi which significantly shortened the accumulation time for DIII expression. Nevertheless, the plant-produced WNV DIII was rapidly expressed as a soluble protein in the ER and can be directly extracted and purified to more than 95% purity using a simple procedure based on metal chelation chromatography (He et al. 2014). Recombinant WNV E protein DIII has been expressed in *E. coli* (Chu et al. 2005) and in insect cells (Alonso-Padilla et al. 2011). In *E. coli*, DIII is expressed as an insoluble protein in the inclusion bodies

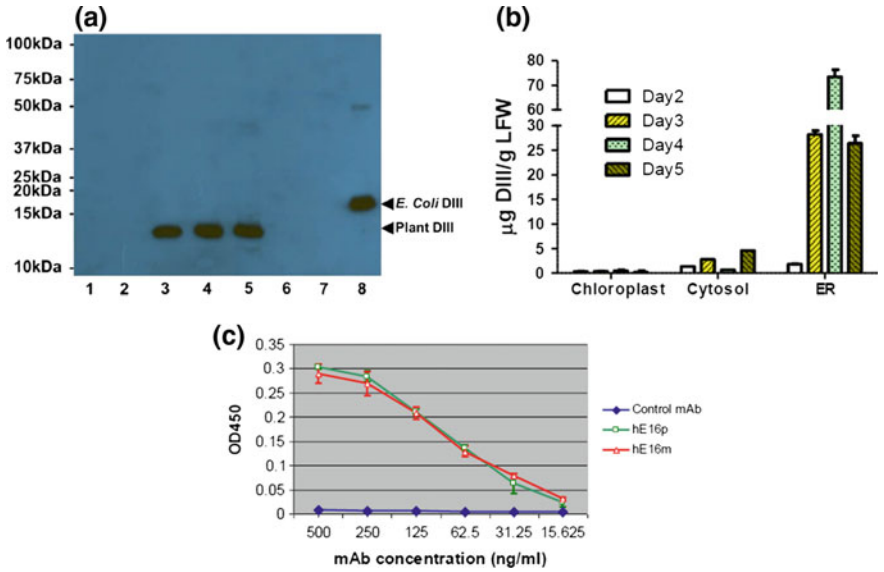

Fig. 1 Expression and characterization of plant-produced WNV E DIII. **a** WNV E DIII was extracted from *N. benthamiana* leaves and separated on 15% SDS-PAGE gels and transferred onto PVDF membranes. A WNV E DIII-specific antibody was used to detect DIII. Lane 1, Protein sample extracted from un-infiltrated leaves as a negative control; Lanes 2–5, Sample collected 2, 3, 4, and 5 days post Agro-infiltration (DPI) from leaves infiltrated with ER-targeted DIII construct; Lane 6: Sample collected 5 DPI from leaves infiltrated with chloroplast-targeted DIII construct; Lane 7: Sample collected 5 DPI from cytosol-targeted DIII leaves; Lane 8: *E. coli*-produced DIII as a positive control. **b** Total protein from plant leaves infiltrated with chloroplast, cytosol or ER-targeted DIII construct was extracted 2–5 DPI and analyzed by an ELISA with an anti-WNV E DIII antibody. Mean ± SD of samples from several independent experiments are presented. **c** Serial dilutions of hE16 purified from mammalian or plant cells were incubated in sample wells coated with plant-produced WNV E DIII and detected with an HRP-conjugated anti-human gamma antibody. A commercial generic human IgG was used as a negative control. Mean ± SD of samples from three independent experiments is presented

and it requires a time-consuming solubilization and refolding process to produce the recombinant DIII in its native conformation. The insect expression system needs several weeks to generate recombinant baculovirus of high titer prior to infection of insect cells for protein production. Our plant-produced DIII is folded properly as it can be recognized by WNV DIII specific antibodies (Oliphant et al. 2005; He et al. 2014). Moreover, the production and purification procedure for plant-produced DIII is fast, cost-effective and highly scalable.

ELISA was used to measure the binding affinity of plant-produced DIII to the mammalian monoclonal neutralizing antibody E16 (hE16m) against WNV E protein (Nybakken et al. 2005; Oliphant et al. 2005) and the plant-made E16 (hE16p) which has been shown to protect mice from lethal WNV infection (Lai et al. 2010). The results indicate plant-produced-DIII resembles the native DIII conformation displayed on WNV viral particles (Fig. 1c). BALB/c mice were treated with four

doses of plant-produced DIII subcutaneously over 8 weeks to evaluate the immunogenicity of plant-produced DIII protein (He et al. 2014). Two groups of mice (n = 6) were treated with either 5 or 25 μg of plant-produced DIII, respectively, and were compared with mice treated with the same doses of DIII produced in *E. coli* or negative control (PBS + alum) (Fig. 2). DIII specific antibody responses were detected in the two groups of mice treated with 25 μg of DIII two weeks after the first injection. All groups immunized with DIII showed DIII specific antibody response after the third injection. The geometric mean titers (GMT) calculated from ELISA data of mouse sera indicate plant-produced DIII has equivalent potency as the DIII produced from *E. coli* in inducing DIII specific antibody responses. To evaluate which type of immune response was induced by DIII, IgG1 and IgG2a concentrations in mouse sera collected from groups immunized with 25 μg plant-produced or *E. coli*-produced DIII proteins were measured by ELISA. Apparently, IgG1 was more than 99% of the total DIII specific IgGs in both groups indicating a predominant Th2-type response. It is not surprising that this result was different from the early studies with DIII produced in *E. coli* which shows a Th1-type response (Chu et al. 2007). Previous studies with different adjuvants have shown that alum induces primarily Th2-type response (Cribbs et al. 2003) while CpG induces Th1 based responses when using the same antigen (Demento et al. 2010). Flow cytometry analysis using yeast cells that displayed WNV DIII on the surface demonstrated the anti-DIII sera showed positive binding to DIII, like that of hE16, but not the sera collected from the negative control group. This further confirms plant-produced DIII induces WNV DIII specific immune responses in mice. Competitive ELISA showed that pre-incubation with

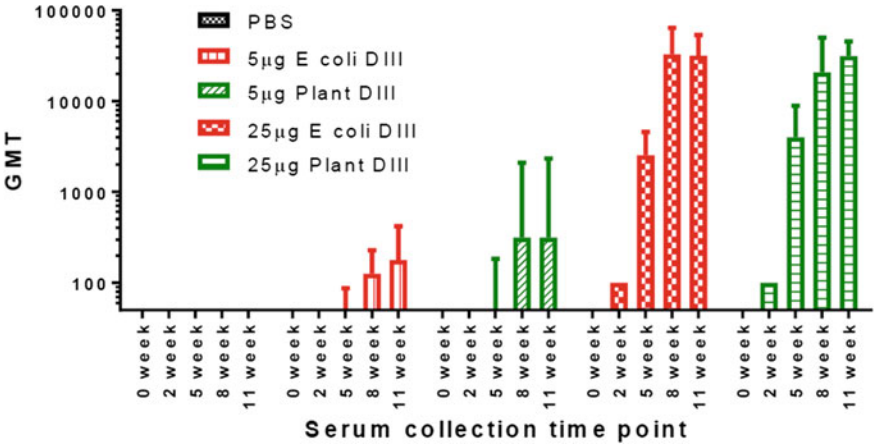

Fig. 2 Time course of WNV E DIII-specific antibody responses in mice upon subcutaneous delivery of plant-derived WNV E DIII. BALB/c mice were injected on weeks 0, 3, 6 and 9 with the indicated dosage of antigen. Blood samples were collected on the indicated weeks and serum IgG was measured by ELISA. The y axis shows the geometric means titers (GMT) and the error bars show the 95% level of confidence of the mean

anti-DIII sera inhibited the subsequent hE16 binding to the immobilized plant-produced DIII. This result indicates at least some of the antibodies in the anti-DIII sera bind to DIII in a hE16-like fashion suggesting they may have neutralizing and protective effects against WNV infection (Lai et al. 2018). These results demonstrated that *N. benthamiana* can be used to efficiently produce immunogenic WNV vaccine candidates with low cost and scalability.

3.3 Plant-Produced VLP-Based Vaccines

In recent years, VLPs have attracted great interest for vaccine development as they can resemble the native virus particles yet remain non-infectious due to deletion of the viral genetic components. Since the 1980s several VLP-based vaccines have been developed and approved for human use (Zhao et al. 2013). VLP-based vaccines have several advantages over inactivated whole virus vaccines or simple subunit vaccines. First, VLP vaccines are non-infectious which means fewer safety concerns for manufacturing or application to humans and animals. Second, they are very immunogenic and elicit both humoral and cellular immune responses. As VLPs mimic the native virus with epitopes displayed on the surface and their particulate nature, they can be easily recognized and processed by antigen presenting cells to trigger T cell activation and proliferation. In addition, VLPs can directly activate B cells, triggering epitope-specific immunoglobulin secretion. Furthermore, VLPs are more stable than subunit vaccines, allowing them to induce long-lasting antibody responses, thereby providing long term protection against viral infection. The stability of VLPs may also extend shelf life of VLP-based vaccines. These features of VLPs make them particularly useful for the development of veterinary vaccines, as VLP vaccines can be produced with lower cost and fewer doses would be needed for animal immunization, minimizing the complications from vaccine side effects.

Currently there are several expression systems being used for production of VLP-based vaccines including bacteria, yeast, insect cell, mammalian cell and plant expression systems. Bacterial expression is simple and easy to use with low cost. Several VLP based vaccines have been produced in this system including a commercialized VLP vaccine against Hepatitis E (Zhao et al. 2013). However, bacteria are prokaryotes that do not have the necessary machinery for post-translational modifications required for production of VLPs that maximally mimic the native viruses. Compared to bacteria, yeast is a better expression system for producing VLP based vaccines as yeast can perform post-translational modification. Several VLP based vaccines have been successfully produced in yeast and commercialized, such as Recombivax against Hepatitis B and Gardasil against human papillomavirus (HPV), both from Merck (Zhao et al. 2013). But expression of VLPs in yeast has its own unique challenges. For example, the HPV VLPs are not secreted by yeast cells but instead are expressed intracellularly, which increases costs for purification. Also, optimization of fermentation conditions and purification

processes were required for production of highly purified VLPs for vaccine use (Bryan et al. 2016). Furthermore, neither bacteria nor yeast can be used to produce enveloped VLPs, which means they may not be suitable for WNV VLPs as WNV is an enveloped virus. Insect cells and mammalian cells are more suitable for expressing enveloped VLPs as they can perform more complex post-translational modifications. Despite the advantages of using insect or mammalian expression systems, production of VLPs from these systems tend to be time consuming and expensive as they require large amounts of culture media and multiple steps of downstream processing.

Plant expression systems have emerged as a useful alternative for production of VLP-based vaccines, not only because of its low cost and high efficiency but also the capability to perform necessary post-translational modifications, especially the flexibility to tolerate manipulation of glycosylation patterns (Chen 2016). Our laboratory has long been interested in producing VLP-based vaccine candidates in plants which includes non-enveloped VLPs such as VLPs derived from Norwalk virus capsid protein (NVCP) or HBcAg (Santi et al. 2008; Huang et al. 2009; Lai et al. 2012), and enveloped VLPs displaying WNV prM and E protein (Chen and Lai 2013) or chimeric VLPs displaying WNV DIII of the E protein (Chen et al. 2011; Chen and Lai 2013). To explore the feasibility of producing WNV VLPs in plants, we fused the plant codon optimized WNV prM and E protein DNA sequence into the deconstructed TMV vectors and transiently expressed the construct in *N. benthamiana* (Chen and Lai 2013). Leaf proteins were extracted 7 dpi and purified for western blot analysis using an antibody against WNV E protein. The results showed that the WNV prM and E proteins were expressed at expected sizes in plants and both unprocessed prM and processed mature M proteins were detected (Fig. 3). The relative band intensity of the plant-produced prM and M protein was comparable to that of gradient-purified WN virion proteins (Chen and Lai 2013). This indicates WNV M protein was processed by the plant cell machinery similarly to the native WNV virion M protein. Assembly of prM and E protein-containing VLPs was confirmed by sucrose gradient centrifugation (Chen and Lai 2013).

HBcAg has been used as a carrier protein to display foreign antigens since the 1980s because of its excellent immunogenicity and the capability to accommodate different antigens in both prokaryotic and eukaryotic expression systems (Roose et al. 2013). To explore the feasibility of producing chimeric HBcAg VLPs displaying WNV epitopes in plants, we fused the WNV DIII coding sequence to the 3′ end of HBcAg using a BeYDV-based expression vector (Chen et al. 2011). The HBcAg-DIII fusion protein was expressed in the leaves of *N. benthamiana* and reached the highest level of accumulation (~350 μg/g LFW) at six days post infiltration (Fig. 4a). Transmission electron microscopy confirmed the assembly of HBcAg-DIII chimeric VLPs with consistent size after extraction and purification (Fig. 4b). Western blot analysis showed that the HBcAg-DIII fusion protein was detected by both anti-HBcAg and anti-WNV DIII antibodies at expected size, about 27kD (Fig. 4c, d). Display of WNV DIII on the chimeric VLP surface was confirmed by competitive ELISA that the HBcAg-DIII fusion protein can effectively compete with soluble DIII to bind the anti-DIII E16. The HBcAg-DIII chimeric VLPs also

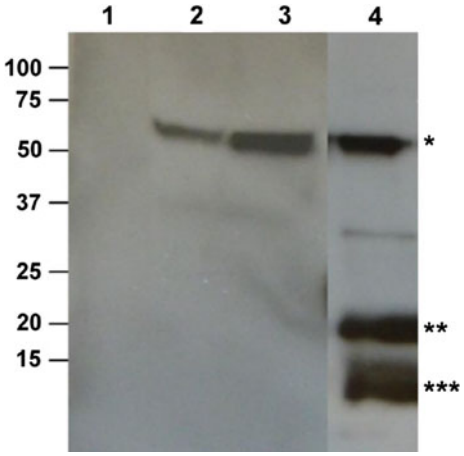

Fig. 3 Production of West Nile virus enveloped VLP based on the prM/M and the E protein in *N. benthamiana* plants. Leaf tissue was infiltrated with *Agrobacterium* harboring the WNV prM-E construct. Leaf proteins were extracted 7 DPI. PrM/M-E VLP was isolated by PEG precipitation and analyzed on 4–12% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were incubated with an anti-WNV E antibody (Lanes 1–3) or an anti-WNV M-E antibody (Lane 4). Lane 1, Protein sample from buffer-infiltrated leaves; Lane 2, Purified WNV E protein as positive control; Lanes 3–4, Samples from prM-E construct-infiltrated plants. *: E protein; **: Unprocessed prM protein; ***: Processed M protein

induced stronger DIII-specific immune responses in mice injected with a single dose (25 μg) of the chimeric VLPs than that of non-fused DIII protein (Chen 2015). Using the MagnICON vectors the WNV chimeric VLPs were expressed with an even higher level of accumulation (>1000 μg/g LFW) in *N. benthamiana* leaves. Analysis of these chimeric VLPs demonstrated that they are similar to those expressed using Geminiviral vectors, both structurally and immunologically (Chen 2015).

Our laboratory has also demonstrated the advantage of plant-made VLPs in improving the safety of vaccines against flaviviruses, including WNV and the recently emerged ZIKV. Several ZIKV vaccine candidates are being developed using inactivated whole virus and DNA or RNA that express the E protein of ZIKV. These vaccine candidates are successful in eliciting the production of ZIKV-targeted antibodies and in protecting animals against ZIKV challenges (Abbink et al. 2016; Larocca et al. 2016; Pardi and Weissman 2017). However, their use may potentially predispose vaccinated subjects to infection by related flaviviruses including DENV and WNV due to phenomenon called antibody-dependent enhancement (ADE) (Sun et al. 2017). ADE is caused by cross-reactive, but non- or sub-neutralizing antibodies, elicited by closely-related viruses or vaccines against related viruses. Instead of neutralizing the infecting virus, these cross-reactive antibodies form complexes with the infecting virus that bind to Fc gamma receptor (FcγR)-bearing cells, resulting in increased viral uptake and infection (Morens 1994). For example, previous infection or vaccination

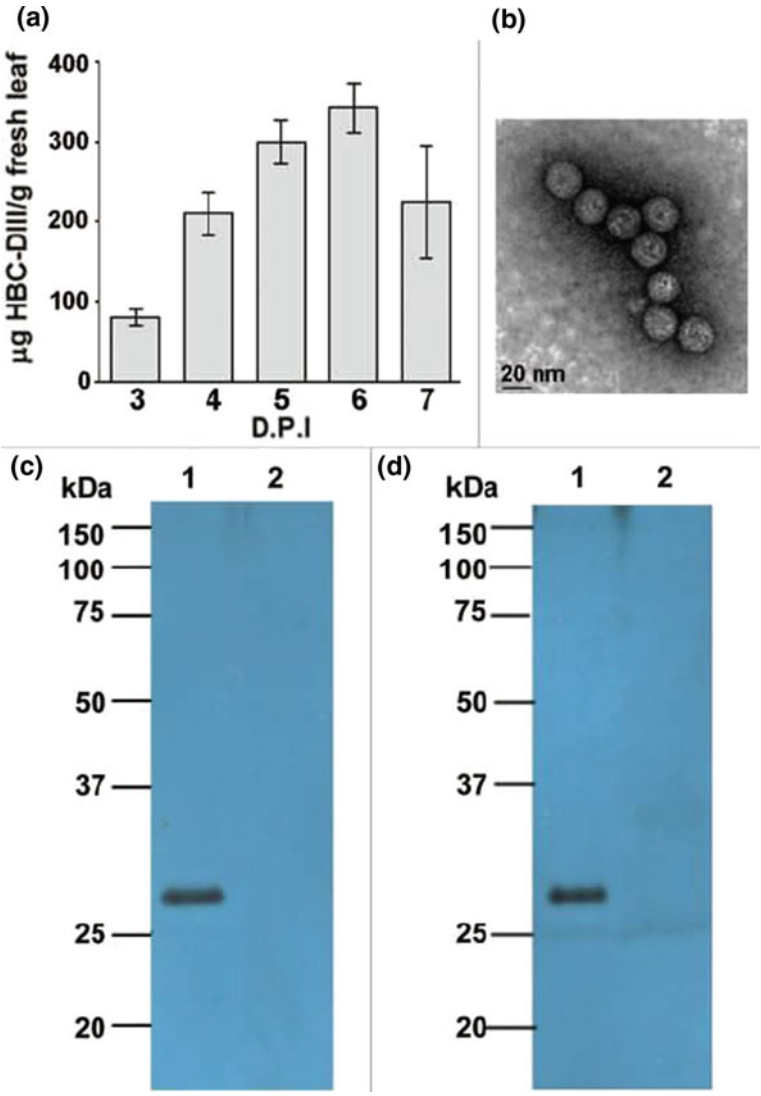

Fig. 4 West Nile virus chimeric VLP produced with BeYDV replicon in *N. benthamiana* plants. **a** Expression of HBcAg-DIII. Leaves were infiltrated with Agrobacterium transformed with the HBcAg-DIII construct. Proteins were extracted on days 3–7 DPI and were analyzed with an ELISA that detects HBcAg. Mean ± standard error (SEM) of samples from three independent infiltration experiments are presented. **b** Electron microscopy of purified HBcAg-DIII VLPs. HBcAg-DIII VLP was purified and subject to negative staining with 0.2% aqueous uranyl acetate, and transmission electron microscopy with a Philips CM-12 microscope. **c–d** Western blot analysis of HBcAg-DIII. Leaf proteins were separated on 12% SDS-PAGE gels under reducing condition and blotted onto PVDF membranes. The membranes were incubated with an anti-HBc antibody (**c**) or an anti-DIII antibody (**d**). Lane 1, Protein sample extracted from leaves infiltrated with the HBc-DIII construct; lane 2, Extract from un-infiltrated leaves

against one serotype of DENV may predispose these individuals to develop the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) through ADE if they are exposed to another serotype of DENV subsequently (Halstead 2014). The recent outbreaks of ZIKV further complicate vaccine development for flaviviruses as antibodies against DENV and ZIKV have been shown to cross-react and enhance the replication of each other in mice (Barba-Spaeth et al. 2016; Dejnirattisai et al. 2016; Stettler et al. 2016; Bardina et al. 2017). Another study also demonstrated that previous WNV infection can also enhance subsequent secondary ZIKV infection (Bardina et al. 2017), suggesting that ADE may also occur between WNV and ZIKV. This raises safety concerns for current WNV vaccine candidates in promoting heterologous flavivirus infection via ADE. In response, our research group has developed several protein subunit vaccines (Yang et al. 2017a, b, c). We demonstrated that an HBcAg VLP that displays the ZIKV E DIII (HBcAg-zDIII VLP) can be robustly produced and easily purified in large quantities from plants. When tested in mice, plant-produced HBcAg-zDIII VLPs evoked potent humoral and cellular responses against ZIKV. Notably, the neutralization potency exceeds the threshold correlated with protective immunity against multiple strains of ZIKV (Yang et al. 2017b). Remarkably, antibodies induced by HBcAg-zDIII VLPs neither cross-react with DENV, nor do they enhance the infection of DENV in FcγR-expressing cells (Fig. 5). We also demonstrated that a plant-produced WNV DIII protected mice from a lethal challenge of WNV infection but without enhancing ZIKV or DENV infectivity (Lai et al. 2018). These results highlight the potential of plant-made vaccines in offsetting the concern of WNV vaccines in sensitizing people to subsequent DENV or ZIKV infection. Overall, these results indicate plants can rapidly produce high levels of immunogenic WNV-specific VLPs.

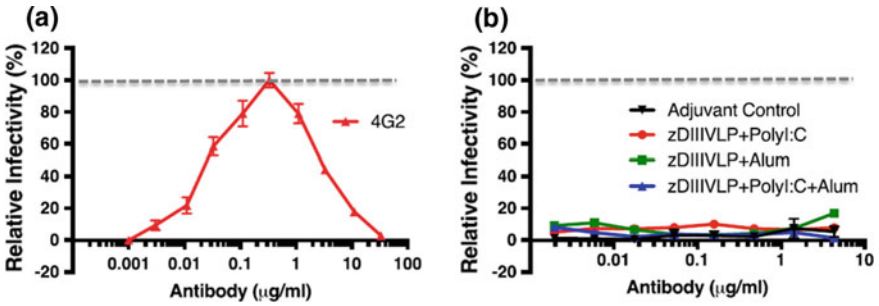

Fig. 5 Lack of enhancement of DENV infection by antibodies in serum from mice immunized with HBcAg-zEDIII VLPs. IgGs were isolated from week 5 pooled sera of mice receiving PBS + Adjuvant (adjuvant control) or HBcAg-zDIII VLP + indicated adjuvant. Serial dilutions of IgGs were mixed with DENV-2 and incubated with FcγR-expressing K562 cells. Forty-eight hour later, cells were fixed, permeabilized and analyzed by flow cytometry for DENV infection. Anti-DENV-2 E mAb 4G2 was used as an ADE positive control with its maximum infectivity defined as 100%. Enhancement by IgGs from anti-HBcAg-zEDIII sera is expressed as a % relative to that of the 4G2

3.4 *Downstream Processing of Plant-Produced WNV Vaccines*

To produce highly purified WNV vaccines, cost-effective and scalable downstream processing is required after recombinant expression in plants. Currently, downstream processing is a major barrier, not only for plant-made biologics (PMBs), but for those produced by conventional platforms as well (Sabalza et al. 2014). Downstream processing typically includes two phases: primary recovery from the plant host and purification of the recombinant protein (Wilken and Nikolov 2012). The primary recovery process usually includes homogenization of the plant tissues and extraction of recombinant protein from plant homogenate. Though the recovery phase of downstream processing varies depending on the plant host used for expression, the purification step is like that of conventional production platforms. Our laboratory has developed a scalable scheme for extraction and purification of plant-produced NVCP VLPs with the combination of a low pH precipitation step, ultrafiltration/diafiltration with a polyethersulfone tangential flow (PES TFF) membrane, and anion exchange chromatography (Chen 2008; Lai and Chen 2012). The feasibility of the NVCP VLP production and purification process was demonstrated under current Good Manufacturing Practices (cGMP) regulations to enrich the VLPs to more than 95% purity (Lai and Chen 2012). Such a downstream processing scheme was applicable to our WNV chimeric VLPs (Chen et al. 2011; Chen and Lai 2013); it yields highly purified HBcAg-DIII VLPs that are assembled with consistent size (Fig. 4b). The WNV enveloped prM and E VLPs were produced in a similar process including leaf homogenization, centrifugation and multiple chromatographic steps for purification. These results demonstrated that the downstream processing of plant-produced WNV VLP vaccines is scalable and efficient.

4 Challenges and Future Development

The development of WNV vaccines in plants has transformed the landscape of vaccine-production economics and contributed to the optimism of licensing an efficacious, safe and low-cost veterinary WNV vaccine in the future. To achieve this goal, we envision novel approaches combining the advancements in discovering more potent adjuvants with a deeper understanding of the biology of drug targeting to immune cells and stimulating systemic immune responses via oral delivery.

Currently, WNV vaccines are injected into animals through at least two doses and an annual boost to ensure protection. It would be desirable to develop oral WNV vaccines for animals. For example, vaccines produced in plant tissue can be simply fed to horses for immunization. Such oral vaccines will further reduce the production cost by eliminating the extraction and purification process and obviating costs associated with cold-storage, transportation and sterile injection.

Orally-deliverable vaccines have been elusive due to the challenges of denaturation and degradation in the digestive system, and their inability to cross the gut epithelium and be delivered to target cells. Not surprisingly, plant cells may be the best vehicle to overcome these challenges. It has been shown that plant cells can protect the vaccines they express from acids and enzymes in the stomach by bioencapsulation, due to the inability of animal digestive enzymes in hydrolyzing the glycosidic bonds in the plant cell wall. This allows the delivery of plant-made vaccines to the gut lumen where they are enzymatically released by commensal bacteria (Kwon and Daniell 2015). Recent studies also demonstrated that when tagged with a specific receptor-binding peptide, plant cell-encapsulated proteins can either be targeted to the gut immune system or cross the gut epithelium to reach circulation (Kwon and Daniell 2015; Su et al. 2015a, b). Furthermore, encapsulated protein drugs in plant cells have been found to maintain their pharmacological efficacy several years after they have been stored at room temperature (Lakshmi et al. 2013). These findings suggest that plant cell-encapsulated vaccines may present an ambient, temperature-stable product that can be delivered to animals by simply feeding, thereby, circumventing logistical costs and allowing practical implementation of vaccination programs to wild susceptible animals.

Indeed, oral immunization of a plant expressed recombinant antigen was first demonstrated in 1995 (Haq et al. 1995). Oral immunization of mice with plant-made *E. coli* heat-labile enterotoxin binding subunit (LT-B) induced production of neutralizing antibodies in serum and gut mucosal against enterotoxin. Later studies using the same foreign antigen expressed in distinct plant tissues demonstrated that the leaf-based vaccines transiently expressed in *N. benthamiana* have higher efficacy than root-based vaccines through oral immunization in mice (Pelosi et al. 2011, 2012). When LT-B was fused with a heat stable (ST) toxin and produced in transgenic tobacco plants, this fusion protein induced similar immune response to that of LT-B produced in bacteria at a much lower dose when orally administered to mice (Rosales-Mendoza et al. 2011). Furthermore, oral delivery of plant-made vaccines has shown promising results in preventing various animal diseases including plague, cholera, swine and bird flu, and porcine reproductive and respiratory syndrome (Shahid and Daniell 2016) with oral vaccines for veterinary use produced in potato, rice, maize, tobacco and other edible plants against various pathogens (Takeyama et al. 2015). These studies indicate plant-produced oral vaccines can induce protective mucosal and systemic immune responses against pathogens. Relevant to WNV vaccines, Kim et al. recently showed that a fusion protein containing the consensus DENV E DIII and M cell-targeting peptide ligand can be produced in transgenic rice calli and the fusion protein effectively binds to the target mucosal cells (Kim et al. 2013). Very recently, an oral vaccine candidate based on hepatitis C virus (HCV) E protein has been developed in lettuce plant. Feeding mice with E protein-containing lettuce powder induced both systemic and mucosal humoral responses against HCV. Since HCV is in the same *Flaviviridae* family with WNV, this study has demonstrated the feasibility of using oral vaccines to prevent WNV in horses and other animals (Liu Clarke et al. 2017).

The remaining challenge is to effectively induce strong systemic responses by oral vaccines alone, as many oral vaccines still rely on injectable priming to achieve protective potency (Sequeira and Harrison 1982). New approaches in vaccine development are required to overcome this challenge. This, in turn, needs a more thorough understanding of the biology of immune cell stimulation by orally-delivered antigens. We expect that the focus of the development of novel WNV vaccines over the next 5 years will be on not just improvements of vaccine potency and production optimization in edible plants, but also on identifying the best combination of antigen design and oral adjuvant to induce optimal protective immunity.

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