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Research review paper

Research advances in plant-made flavivirus antigens

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ABSTRACT

Outbreaks of flaviviruses such as dengue (DV), yellow fever (YFV), Japanese encephalitis (JEV), tick-borne encephalitis (TBEV) and West Nile (WNV) affect numerous countries around the world. The fast spread of these viruses is the result of increases in the human population, rapid urbanisation and globalisation. While vector control is an important preventive measure against vector-borne diseases, it has failed to prevent the spread of these diseases, particularly in developing countries where the implementation of control measures is intermittent. As antiviral drugs against flaviviruses are not yet available, vaccination remains the most important tool for prevention. Although human vaccines for YFV, TBEV and JEV are available, on-going vaccination efforts are insufficient to prevent infection. No vaccines against DENV and WNV are available. Research advances have provided important tools for flavivirus vaccine development, such as the use of plants as a recombinant antigen production platform. This review summarises the research efforts in this area and highlights why a plant system is considered a necessary alternative production platform for high-tech subunit vaccines.

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1. Introduction

More than 25% of the total deaths per year worldwide are estimated to be caused by infectious diseases (Morens et al., 2004). Emerging and re-emerging infectious diseases affect developing countries more severely and remain a considerable public health problem.

Infectious diseases can be caused by different viruses. The genus *Flavivirus* includes more than 70 members, of which more than 50% have been associated with human illness (Gould and Solomon, 2008). Several members are important human pathogens that cause morbidity and mortality and are transmitted to their host by mosquitoes or ticks, including dengue (DV), Japanese encephalitis (JEV), yellow fever (YFV),

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West Nile (WNV) and tick-borne encephalitis (TBEV) viruses (Pugachev et al., 2003). Flaviviruses are the leading cause of arthropod-transmitted viral disease in humans and infect a significant number of patients each year worldwide. The infections mainly manifest as a mild acute febrile syndrome, but many infected patients develop severe neurological, hepatic or haemorrhagic disease that leads to death.

In previous decades, several outbreaks of different flaviviruses have affected numerous countries around the world. The fast spread is the result of a combination of different factors that are commonly associated with flaviviruses, such as increases in human population, rapid urbanisation, globalisation, which allows viraemic individuals to travel, and the reduced control of vectors, which occurs more frequently in developing countries (Hombach et al., 2005; Morens et al., 2004). While vector control is an important preventive measure for vector-borne diseases, it has failed to prevent the spread of flavivirus infections, primarily because this objective is not fully achievable in developing countries. Poor disease diagnostics and the limited funds available for surveillance in endemic countries result in a re-emergence of flavivirus diseases in areas where they already exist and an emergence in new areas where they had not been transmitted or were of only minor medical importance. Consequently, there has been a significant increase in the incidence of vector-borne diseases, making this issue a current global public health problem. In this context, the development of new technologies, such as new diagnostic tools, antiviral medicines and effective vaccines, is urgently required. Research advances have provided important tools for flavivirus vaccine development, such as the use of plants as a recombinant antigen production platform. This review summarises the research efforts made in this area and explains the necessity of a plant system as an alternative production platform for high-tech subunit vaccines.

1.1. Dengue

Currently, DV is endemic throughout tropical and subtropical areas of the world, and more than 2.5 billion people are at risk of infection (Halstead, 2007; Webster et al., 2009). The World Health Organization (WHO) considers dengue a neglected tropical disease that is associated with poverty in tropical areas (WHO, 2006). More than 6,000,000 confirmed cases of dengue were reported to the WHO from 2001 to 2009. The population of *Aedes aegypti* mosquitoes, the arthropod that transmits the virus to humans, has proliferated due to the uncontrolled urbanisation of tropical and subtropical countries. As a result of the incomplete surveillance and control of the vector, the disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific (Kyle and Harris, 2008). There are four different serotypes of DV: DV-1, DV-2, DV-3 and DV-4. The symptoms range from a febrile illness called “dengue fever” to severe dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), which typically presents as capillary permeability and can lead to death. DHF/DSS occurs more frequently in patients with second heterotypic DV infections or in infants with maternally transferred dengue immunity (Kliks et al., 1988). As DV pathogenesis is not yet completely understood, many hypotheses that are not mutually exclusive have been postulated. Based on epidemiological studies, it has been hypothesised that a severe dengue case occurs when an individual has suffered an earlier infection and possesses heterotypic DV antibodies. Through a process known as antibody-dependent enhancement (ADE), a second infection with a different serotype enhances the infection and replication of the virus in the mononuclear cell lineage (Goncalvez et al., 2007; Gubler, 1998; Guzman et al., 2007; Vaughn et al., 2000). However, severe dengue can also occur as a primary infection, which suggests that other factors are involved. The nutritional status (Kalayanarooj and Nimmannitya, 2005; Thisyakorn and Nimmannitya, 1993) and the genetic background of the patient (LaFleur et al., 2002; Nguyen et al., 2008) as well as the virulence of the infecting viral strain (White, 1999) and other epidemiological issues have been implicated in the development of a more severe disease. Despite efforts made, there are no available vaccines against DV.

1.2. Japanese encephalitis

The natural transmission cycle of JEV involves vertebrate hosts, such as domestic pigs and wading birds, and *Culex* mosquitoes. Like DV, JEV is mainly transmitted to humans through the bite of infected mosquitoes. In JEV-infected patients, the most common clinical symptom is acute encephalitis (Fischer et al., 2010), but numerous cases result in a non-specific febrile illness with acute fever, headache, and vomiting (Kumar et al., 1990; Kumar et al., 2006). More severe cases present convulsions, paralysis of extremities and respiratory and cardiac dysfunctions, apparently as a result of neuronal damage caused by virus infection (Lin et al., 2008; Pugachev et al., 2003). At least 50,000 cases of encephalitis occur each year in Asia, in which approximately 20% of patients die and almost 40% of the survivors have neurologic or psychiatric sequelae (WHO, 2006). Although JEV mostly affects children, travellers of any age that visit temporarily endemic areas can be also affected (Solomon et al., 2000). JEV has caused several outbreaks over the past few decades in Asia and parts of the Western Pacific (Ghosh and Basu, 2009; Ritchie and Rochester, 2001). An apparent increase in the geographic distribution of JEV has been observed in recent years, which seems to correlate with population shifts and changes in global climatic conditions, agricultural practices and migratory bird patterns (Fischer et al., 2010; Ghosh and Basu, 2009). Several vaccines are available for human vaccination in risk areas and for travellers.

1.3. Yellow fever

YFV is transmitted to humans by infected *Aedes* mosquitoes; wild monkeys are the primary natural hosts (Pugachev et al., 2003). Viraemias are high and prolonged in humans and are often associated with severe haemorrhagic disease and high fatality rates. Clinically, YF is characterised by the abrupt onset of high fever, headache, back and muscle aches, nausea and vomiting. The majority of infections are mild, but in more severe cases acute liver failure occurs, causing the mild jaundice by which the disease is known (Solomon and Mallewa, 2001). Approximately 15% of those infected develop moderate or severe disease with hepatic and myocardial injury and renal failure, leading to lethal haemorrhage and cardiovascular shock (Labeaud et al., 2011; Monath, 2005). YF affects at least 44 countries in tropical areas of Africa and South America and causes 200,000 cases of clinical disease and 30,000 deaths annually (Staples et al., 2010). In these countries, the disease remains a continuing public health problem as a result of the level of vaccination coverage in the human population and cyclical, ecologic and climatic factors that influence virus transmission. A YF vaccine has been available on the market for more than 70 years and is highly effective in reducing the spread of the disease.

1.4. West Nile

While DV and YFV are predominantly associated with tropical and subtropical countries, West Nile virus (WNV) is currently distributed worldwide. WNV is mostly transmitted by infected *Culex* mosquitoes to birds (Hayes et al., 2005). Humans and horses are incidental hosts (Dauphin and Zientara, 2007). Traditionally, WN disease was considered a minor risk for human and horse health, but in the past decade many outbreaks have been reported in different countries. Since 1996, WNV has become a major public health issue in different countries in Africa, Asia, Southern Europe and, more recently, the U.S. (Garmendia et al., 2001; Murgue et al., 2002; Solomon and Mallewa, 2001), Canada and México (CDC, 2003). In the U.S. alone, approximately 24,000 human cases were reported between 1999 and 2006, with almost 1000 deaths (Dauphin and Zientara, 2007). Although WNV has been historically associated with asymptomatic infections in humans, new outbreaks have

resulted in a more severe neuroinvasive disease. This re-emerging disease seems to be more pathogenic, with a higher incidence of severe neurological illness, and is associated with significant mortality and long-term morbidity (Saad et al., 2005). Once infected, approximately 1% of patients develop meningoencephalitis, a nervous form of the disease that can be lethal in the elderly (Dauphin and Zientara, 2007; Pulmanausahakul et al., 2010). The symptoms of severe neurological disease may last for a few weeks and, in several cases, result in permanent sequelae. There is no human vaccine against WNV currently available on the market.

1.5. Tick-borne encephalitis

TBEV is primarily transmitted to humans by infected ticks (*Ixodes*, *Dermacentor* and *Haemophysalis* species). The principal risk for human infection is direct contact with natural areas infested with ticks, but there are other routes of transmission, such as the ingestion of infected milk and direct contact with dead infected animals (Solomon and Mallewa, 2001). The incidence of the disease has been increasing in recent years. As a result of the increasing number of infected people in Europe, Russia and part of Asia, TBE is an international health issue because it is of concern not only for endemic areas but also for visitors and travellers from non-endemic countries (Petri et al., 2010; Wiedermann, 2010). The viruses that cause the different forms of the disease can be distinguished genetically, and three subtypes of TBE have been described: central Western subtype (European encephalitis or CEE), Siberian subtype, and Far-eastern subtype (commonly named RSSE for Russian spring–summer encephalitis) (Heinz, 2000). The disease caused by the TBEV is characterised by an initial non-specific illness that includes fever, headache and myalgia. Approximately 30% of adults who have clinical evidence of infection progress to a second phase with meningitis or meningoencephalitis (Kaiser, 1999). There is no treatment available once infected, but TBE can be effectively prevented by available vaccines (Grgic-Vitek et al., 2010).

2. Development of candidate vaccines against flaviviruses

The continuous emergence and re-emergence of different flaviviruses in different countries have led to a strong demand for new effective vaccines to prevent major epidemics. In developing countries, prevention through vaccination is one of the most cost-effective routes to public health improvement, as long as the vaccines are produced at affordable prices that benefit the population (Gould and Solomon, 2008; Peny et al., 2005). However, the budget for developing vaccines for use in developing countries is usually low compared with the total budget for the pharmaceutical market. In this context, the global agenda includes two important goals. One is to make vaccines available to poor countries in which vaccines are currently prohibitively expensive. The other is to shorten the wide time gap that exists between bringing a new vaccine to the market in the developed world versus developing countries.

Currently, commercially available vaccines only exist for YFV, JEV and TBEV. Despite research and development efforts, no licensed vaccines against DV are available. Only veterinary vaccines are available for WNV (Table 1).

For the last two decades, the World Health Organization (WHO) has encouraged the scientific community to develop a safe and effective vaccine against DV (WHO, 2008). Progress in vaccine development has been slow because of two major obstacles. One is the lack of a reliable small animal model; as most animal models are resistant to DV infection, the knowledge of the pathogenic mechanisms and host immunity and drug and vaccine research relies mainly on evidence from *in vitro* studies (Williams et al., 2009). The other obstacle is the requirement of a tetravalent formulation to prevent dengue disease. As there is no cross-protection between the four DV serotypes, efficient dengue prevention will be possible only after the development of an efficient vaccine that can confer a balanced immune response against all four serotypes. Extensive research has been performed toward the development of a vaccine against dengue using both traditional and molecular approaches (Table 1). Several groups have focused on the development of live attenuated vaccines, while others have focused on chimeric vaccines. The molecular approach includes the development of DNA, virus-vectored and recombinant subunit vaccines (Coller and Clements, 2011; Murrell et al., 2011; Pugachev et al., 2003; WHO, 2006). Live-attenuated and live-chimeric virus-based vaccines are the most advanced in their development and are in Phase II/III clinical trials (Monath, 2007). The attenuation was obtained by the repeated passage of wild-type strains of DV in primary dog kidney (PDK) and foetal rhesus monkey cell cultures. The difficulty in this approach has been to determine the correct balance in the attenuation of the candidate vaccine strains (Kanesa-thasan et al., 2001; Sun et al., 2003).

Currently, the commercially available vaccines against TBEV are based on inactivated virus and are produced in chick embryo cell cultures; these vaccines are manufactured in Austria, Germany and Russia. They are administered in multiple doses and are effective in preventing TBE in Europe (Pugachev et al., 2005). New studies are being performed to improve the existing vaccines, principally by removing stabilisers such as albumin and polygeline to reduce adverse effects and optimise vaccination schedules (Zent et al., 2004a, 2004b). Several new live-attenuated candidate vaccines are being developed against TBEV (Rendi-Wagner, 2008).

Three different vaccines for JEV are currently in use (WHO, 2006). One of them is JE-Vax, a formalin-inactivated vaccine manufactured from virus grown in mouse brain tissue. It is routinely administered to humans in Japan, South and North Korea, Taiwan, Thailand and Vietnam, and it has also been licensed in the US, Canada, Israel and Australia (Table 1). Although this vaccine has facilitated a decrease in the incidence of JE disease, it has some disadvantages, such as the high price per dose, the need for multiple doses for vaccination and allergic and neurological adverse reactions, which have an incidence of 0.1–1% (Pugachev et al., 2005). A safe and effective live-attenuated JE vaccine based on the SA14-14-2 strain has been used in China since 1989. It was obtained after passages in primary hamster kidney cells (PHKC) at the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) in China. Nowadays, it is also available in Nepal and the Republic of Korea. Efforts are on-going to meet international GMP standards and the WHO requirements for production and quality control for its international commercialisation (WHO, 2006). The third available vaccine is an inactivated, cell culture-derived vaccine based on the Beijing P-3 strain of JEV. Although widely used in China, this vaccine has been gradually replaced by the live-attenuated SA14-14-2 vaccine, which requires fewer doses. Several other promising JE vaccine candidates are in advanced stages of development (see Table 1).

The YF Asibi virus was attenuated by multiple passages in chick tissues, resulting in the development in 1937 of the 17D YF attenuated strain that is currently being used to produce the 17D live-attenuated vaccine. The vaccine has been shown to be highly successful in reducing the disease incidence. Today, over 550 million people have been vaccinated with the YF 17D vaccine, including people at risk, travellers and military personnel (Monath, 2007). New formulations of the YF and other flavivirus live-attenuated vaccines are being developed to increase their stability in the adverse climatic conditions in which vaccines are reconstituted (Wiggan et al., 2011). Although several recent works have reported viscerotropic adverse events (YEL-AVD) caused by the YF 17D vaccine (Chan et al., 2001; Martin et al., 2001; Vasconcelos et al., 2001), it has been shown to be highly effective with a single subcutaneous injection, which causes a protective antibody response in 99% of cases (Monath, 2007). As YF 17D is one of the most effective vaccines, it has been used as a backbone for the development of new live chimeric vaccines. One example is the technology termed "ChimeriVax™" in which the prM and E genes in the YF 17D 204 cDNA are exchanged

Table 1
Flavivirus vaccines available on the market, to be licensed or in development.

Vaccine type	Vaccine candidate name or trade name	Developers	Clinical trial stage or year/ place of approval	Comments	References
Dengue Virus					
Live-attenuated	–	Mahidol University/Sanofi Pasteur	Phase II completed	Not further testing	Sabchareon et al. (2002)
	TDV	WRAIR/GSK	Phase II completed	PDK cell passage, not further testing	Sun et al. (2009)
	DEN4Δ30	NIAID LID/Biological E (India), Panacea Biotech (India), Institute Butantan (Brazil) and VaBiotech (Vietnam)	Phase II	–	Durbin et al. (2006) and McArthur et al. (2008)
Live-chimeric	ChimeriVax-Dengue	Sanofi Pasteur (ex Acambis)	Phase III	YF 17D vaccine backbone with DV prM and E genes	Guy et al. (2010a), Guy et al. (2010b), and Guy et al. (2011)
	DENVax	Inviragen	Entering phase II	Chimeric viruses based on DV-2 PDK 53 background	Huang et al. (2003)
Inactivated	DENV-2 PIV	NMRC (Naval Medical Research Center)	Pre-clinical	Purified inactivated virus vaccine	Simmons et al. (2006)
Recombinant subunit	DEN-80E	Hawaii Biotech Inc./Merck	Phase I	80% E protein of DV-1, -2, 3- and -4 in *Drosophila* S2 cells	Clements et al. (2010) and Coller et al. (2011)
DNA vaccine	D1ME-VR-P	US NMRC (Naval Medical Research Center)	Phase I	prM and E genes	Beckett et al. (2011)
West Nile Virus					
Live-chimeric	ChimeriVax-West Nile (WN02)	Acambis	Phase II	YF/WN chimera, prM-E genes mutated for attenuation	Arroyo et al. (2004), Guy et al. (2010a), and Monath et al. (2006)
	WN/DEN4Delta30	NHI	Phase I	WN prM/E genes in DV-4 virus backbone	Pletnev et al. (2003)
Recombinant live-attenuated	MVschw-E(WNV)	Institut Pasteur, France	Pre-clinical	WN prM/E proteins in measles virus vector	Despres et al. (2005)
Recombinant	TRIP/sE(WNV)	Institut Pasteur, France	Pre-clinical	Lentiviral vector based vaccine	Iglesias et al. (2006)
DNA vaccine	DNA-prM-E	NIAID/Vical	Pre-clinical	WN prM/E proteins with JEV signal sequence	Davis et al. 2001)
	SRIPs	University of Queensland, Queensland Institute of Medical Research.	Pre-clinical	Single round infectious particles	Chang et al. (2008)
Recombinant subunit	WNV-LPs	NIH	Pre-clinical	prM/E and C/prM/E proteins produced in insect cells.	Qiao et al. (2004)
	80E/NS1	Hawaii Biotech Inc.	Pre-clinical	E truncated and NS1 produced in *Drosophila* S2 cells	Lieberman et al. (2007)
	rWNV-E	L2 Diagnostic	Pre-clinical	E truncated expressed in baculovirus	Bonafe et al. (2009)
	WNV E DIII	National University of Singapore	Pre-clinical	DomIII of WN-E protein	Chu et al. (2007)
Japanese Encephalitis Virus					
Live-attenuated	SA14-14-2	NICPBP (National Institute for Control of Pharmaceuticals and Biological Products)	1989/China	Strain SA14-14-2 attenuated in PHK cell passages. No international licensure	WHO (2006)
Inactivated	JE-Vax	Biken	Since 1989/Japan, India, Korea, Taiwan, Thailand, Vietnam, US, Canada, Israel, Australia	Formalin inactivated mouse brain-derived. Nakayama or Beijing-1 strain. For persons aged ≥1 years	Fischer et al. (2010)
	IC-51 (IXIARO)	Intercell Biomedical	2009/USA	Vero cell culture-derived. SA14-14-2 strain. Only for persons aged ≥17 years	Duggan and Plosker (2009)
Live-chimeric	ChimeriVAX-JE (IMOJEV)	Acambis/ Sanofi-aventis	To be licensed soon	Live-attenuated YF-JE chimeric vaccine	Appaiahgari and Vrati (2010) and Monath et al. (2003)
Yellow Fever Virus					
Live-attenuated	Depends on the manufacturer (YF-Vax, ARILVAX, etc.)	7 different manufacturers	Available on the market since 1937/ worldwide	Substrains commonly used: 17D-204, 17DD Manufactured in embryonated chicken eggs	Theiler and Smith (1937)
Recombinant live	MVA-YF, dVV-YF	Baxter Bioscience	Pre-clinical	prM/E proteins of YF 17D, non replicating vaccinia vectors	Schafer et al. (2011)
	NS1	WRAIR/ Rochester General Hospital	Pre-clinical	NS1, NS2a and NS2b in vaccinia virus	Putnak and Schlesinger (1990)
Inactivated	XRX-001	Xcellerex	Phase I	Purified and inactivated whole-virus produced in Vero cell cultures	Monath et al. (2011a) and Monath et al. (2011b)
Tick Borne Encephalitis					
Inactivated	FSME Immun	Baxter Bioscience	1976/Europe, Canada, Russia	Formalin-inactivated virus, grown in primary chick embryo cells	Loew-Baselli et al. (2011)
	Encepur	Chiron-Behring/Novartis	Europe	Adult and paediatric formulations	Leonova et al. (2007)
Live-attenuated	–	University of Vienna	Pre-clinical	Virus attenuation via specific deletions in capside protein.	Kofler et al. (2002)

Table 1 (*continued*)

Vaccine type	Vaccine candidate name or trade name	Developers	Clinical trial stage or year/ place of approval	Comments	References
Recombinant live	LGT/DEN4	Chumaskov Institute of Poliomyelitis and viral Encephalities	Pre-clinical	Langat virus/DV4-TBE	Pripuzova et al. (2009)

In this table, brand names have been used to give more detailed information to the reader. It is not the intention of the authors to express a preference of any of the products.

with the genes for DV, JEV or WNV proteins. After DNA cloning, RNA is transcribed and transfected into Vero cells, resulting in a chimeric virus with the YFV 17D replication machinery and the external coat of the heterologous flavivirus (Guy et al., 2010a). Several candidate vaccines in development are listed in Table 1.

The development of efficient vaccines and tools for WN diagnosis has been spurred by the economic impact of the recent outbreaks in the horse industry (Dauphin and Zientara, 2007). Since the epidemics in the U.S. in 1999, research has focused on the development of different types of vaccines against WNV, including live-attenuated, chimeric and recombinant subunit vaccines (Table 1). Currently, there are several commercially available veterinary vaccines, but no human vaccine has yet been licensed.

3. On-going research in flavivirus recombinant subunit vaccines

As the whole genome of the virus is not present, the advantage of subunit vaccines relies on the defined composition of the formulated vaccine. The absence of a replicative virus eliminates the possibility of unwanted reversions and inefficient attenuation or inactivation, making them safer to use and produce in comparison with live attenuated or inactivated vaccines. Moreover, subunit vaccines allow the immune response to different serotypes to be manipulated more easily, which is particularly important for the development of a vaccine against dengue virus (Clements et al., 2010).

As was mentioned previously, commercially available flavivirus vaccines are live-attenuated or inactivated. In addition to economic challenges, these vaccines could present several risks associated with the use and production of live-attenuated or inactivated virus and have several disadvantages. For instance, for live-attenuated flavivirus vaccines, it has been hypothesised that recombination between the vaccine virus and *wild-type* circulating flaviviruses may occur, resulting in new, unexpected recombinant viruses. This recombination is facilitated by the increasing geographic dispersal of flaviviruses, which increases the probability of natural co-infections (Seligman and Gould, 2004). As a consequence, research efforts are focussed on developing new platforms for producing recombinant subunit vaccines against flaviviruses.

3.1. Molecular components

Flaviviruses are enveloped plus-stranded RNA viruses. The virus particle contains a nucleocapsid (genomic RNA within a viral capsid C protein) that is surrounded by a lipid bilayer in which the viral proteins M (membrane) and E (envelope) are embedded (Rice et al., 1985). Flaviviruses enter the host cell by receptor-mediated endocytosis, and after disassembly, the viral genome is released and translated (Fig. 1). Its translation results in a single polyprotein precursor that contains three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A/NS2B, NS3, NS4A/NS4B, and NS5). The structural proteins are structural components of the virion, and the NS proteins are required for genome replication and expression (Lindenbach et al., 2007). Both viral and host proteases are involved in the processing of the polyprotein, which occurs co- and post-translationally to form the final mature viral proteins (Pugachev et al., 2003).

The M protein is the mature form of the pre-membrane (prM) protein and is important for virus infectivity and pathogenicity. The E protein is responsible for viral attachment to cellular receptors and specific membrane fusion and is the major target for virus-specific neutralising and haemagglutination-inhibiting antibodies (Pugachev et al., 2003; Zhang et al., 2004). The elucidation of the three-dimensional structure of the flavivirus E protein showed that it has three domains, DomI, DomII and DomIII (Rey et al., 1995).

3.2. Immunology of flavivirus infections

Although considerable research has been carried out to elucidate the immunology of flavivirus infections, further *in vitro* and *in vivo* studies are needed to completely understand the entire process. Certainly, flavivirus human infection triggers both innate and adaptive immunity in naïve individuals and this immune response is important in reducing viraemia and viral clearance (King et al., 2007). Available data show that the humoral response, especially IgM and IgG, and cellular immune response jointly prevent dissemination and persistence of flavivirus infection (Diamond et al., 2003b). This immune response initiated to eliminate the virus becomes pathological to the host. While adaptive immune responses have been shown to play an important role in controlling primary WNV infection in mice, this role is not fully known for all flaviviruses (Koraka et al., 2010).

Interferon (IFN)-dependent innate immune response is essential for protection against flavivirus infections. The production of several proinflammatory cytokines, such as IFN and tumour necrosis factor (TNF), inhibits flavivirus infection in cell culture and in animal models by preventing translation and replication of infectious viral RNA (Diamond, 2003; Monath, 2008). For DV, these cytokines might affect directly or indirectly the vascular permeability, leading to the more severe disease DHF/DSS (Stephenson, 2005). For YF, it is named 'cytokine storm' and may contribute to terminal events and death (Monath, 2008). However, as it was proposed before (Diamond, 2003), flaviviruses may have developed mechanisms to attenuate some of the IFN-dependent antiviral effector mechanisms.

The cellular immune response is important for the eradication of virus-infected cells from the host. Infection induces cytotoxic lymphocytes that recognise flavivirus-infected cells (Diamond, 2003). T-helper and T-cytotoxic cells have been shown to participate in controlling the infection of mice infected with WNV and DV (Koraka et al., 2010). The involvement of T cells in the immunopathology of the diseases varies among the different flaviviruses (King et al., 2007).

In terms of vaccine-induced protection, humoral response is generally considered to be more relevant.

The antibody response is different for each viral protein. Neutralising antibodies, which recognise epitopes located predominantly in the structural E protein, are necessary for the induction of the humoral immune response (Crill and Chang, 2004). However, the potent, type-specific, neutralising epitopes are located in the upper lateral surface of DomIII (Beasley and Barrett, 2002; Guzman et al., 2010). For this reason, DomIII peptides are potential candidates for flavivirus vaccines, as immunisation with these peptides can induce the production of neutralising antibodies and cellular immune responses as well (Chavez et al., 2010). A subset of neutralising antibodies also recognises prM protein (Diamond, 2003).

In addition to immunity against the E protein, NS1 and NS3 proteins are targets of antibodies in flavivirus infections. Both anti-E and anti-NS1 immunities provide more effective immune responses against flavivirus infection (Diamond, 2003; Ishikawa et al., 2011;

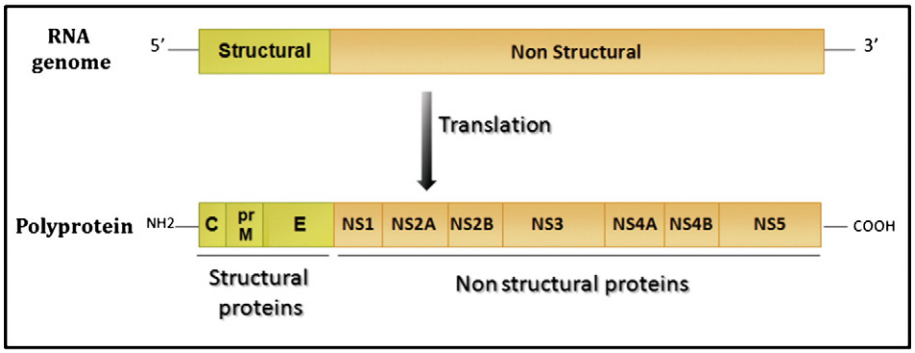


Fig. 1. Schematic representation of the flavivirus genomic RNA translation and the resulting polyprotein. Genomic RNA is translated into a unique polyprotein precursor that is processed co- and post-translationally resulting in structural and non structural proteins (C: capside; E: envelope; prM: pre-membrane precursor; NS: non-structural).

Sun et al., 2012). Although the mechanism through which the protection is induced by anti-NS1 antibodies remains unclear, it has been postulated that these antibodies can trigger complement-mediated lysis of the infected cell (Ishikawa et al., 2011). NS3 protein stimulates T-cell response that produces high levels of IFN-γ as well as TNF-α, TNF-β, and chemokines in DV infections (Chaturvedi et al., 2005).

3.3. Design of recombinant flavivirus subunit vaccines

The study of the molecular structures of the neutralising epitopes in different flavivirus proteins has provided useful information for the design of new drugs and vaccines. Using molecular biology and bioinformatics, this new information has contributed to the identification of many new target antigens for the rational design of subunit vaccines (Koraka et al., 2010). Based on this information, different expression strategies have focused mainly on the heterologous production of the C, prM and E structural flavivirus proteins and the non-structural protein NS1. In particular, several reports have focused on the expression of the whole recombinant E protein, the truncated version of it (Et) or the co-expression of E with the C and prM proteins (CprME). In these reports, the E protein has been shown to be the most effective candidate for the development of a subunit vaccine and a promising antigen for diagnostic kits when expressed in heterologous systems, as it induces a high titre of neutralising antibodies in mice, rabbits or monkeys. The different expression systems used and the antigens expressed are listed in Table 2.

DomIII of the flavivirus E-protein is an important target for the development of new recombinant flavivirus subunit vaccines and antigens for diagnostic kits. The major neutralisation sites of WNV, YFV, DV, TBEV and JEV have all been mapped in DomIII, whereas DomI and DomII contain cross-reactive epitopes that generate cross-neutralisation between different flaviviruses (Chavez et al., 2010). This is a central issue for the research and development of recombinant flavivirus subunit vaccines, as the cross-reactivity of non-neutralising anti-E antibodies is a non-desirable effect. For DV vaccines, this is of critical concern due to the high risk of developing DHF or DSS in a second infection with a different serotype (Murrell et al., 2011). In addition, the serological diagnosis of flavivirus infections is commonly difficult due to the antigenic cross-reactivity among the different members of the genus (Chavez et al., 2010). To overcome this problem, different research projects have aimed to produce recombinant DomIII (Chavez et al., 2010; Holbrook et al., 2004). For instance, DomIII of the DV-E protein is responsible for receptor binding and induces a type-specific neutralising response that potentially avoids ADE. Among other virus factors, ADE has been suggested to contribute to the progression and severity of dengue disease, such as DHF and DSS (Guzman et al., 2010). For JEV, antibodies raised against DomIII of the E-protein neutralise the JE virus and inhibit JEV infection in stable porcine kidney cells (Verma et al., 2009). As for DV, DomIII of the E-protein of the WN and TBE viruses has been proposed as the receptor-binding domain with neutralising epitopes. The passive transfer of WN DomIII-specific antibodies protects mice from WNV challenge. WN DomIII produced in recombinant systems has been tested in animal models and has proven to be effective in protecting against WNV infection (Chu et al., 2007; Martina et al., 2008). For the YFV 17D vaccine strain, the protection mechanism is not completely clear, but the loss of virulence was attributed to mutations in the original virus, mostly in DomIII of the E protein (Lee and Lobigs, 2008; Volk et al., 2009).

4. Why is a plant system required for producing subunit vaccines?

The adverse effects and high cost associated with inactivated or live-attenuated vaccines have led to an exploration of alternatives for the development of new candidates, with the final objective of obtaining an economical, safe and effective vaccine. Among the alternative expression systems, subunit vaccines have experimented two decades of research and refinement. Although subunit vaccines against flavivirus are in the early stages of development, plant expression system has shown that it can potentially serve as a practical, cost-effective and safe platform for the development of new subunit candidates, with many advantages over conventional systems (Faye and Gomord, 2010).

After twenty years of research in this area, the first plant-made pharmaceuticals are close to market or on the market (Davies, 2010; Kaiser, 2008). For instance, the human glucocerebrosidase (GCD) recombinant enzyme for Gaucher's disease therapy is being produced in suspension-cultured carrot cells (Protalix Biotherapeutics) in replacement of the recombinant protein produced in Chinese hamster ovary (CHO) cells (Kaiser, 2008). The plant-derived enzyme is safer, cheaper, stays in the bloodstream longer, and can be injected less often. At present, plant-produced GCD is undergoing Phase III clinical trials (Aviezer et al., 2009) and is awarded with a temporary authorization for its use in France and the U.S.

As in the example given above, plant expression systems can produce biopharmaceuticals cost-effectively, which represents an important advantage over other prokaryotic and eukaryotic expression systems. Reduced manufacturing costs can be achieved by replacing fermenters with greenhouses or fields, where large biomasses can easily be obtained using the same infrastructure used for common agricultural procedures (Daniell et al., 2009; Tiwari et al., 2009). Plant cells can produce high-quality proteins with the proper post-translational modifications, an intrinsic characteristic of eukaryotic systems. In comparison with mammalian cells, transgenic animals and bacteria, plant-produced proteins are not subject to potential viral, prion or bacterial endotoxin contamination (Ma et al., 2003). Moreover, the traditional systems used for recombinant protein production are sometimes technologically complex and expensive, making them economically prohibitive for developing countries (Tiwari et al., 2009). Thus, plant systems have emerged as a viable alternative to fermentation technology and promise to overcome several problems

Table 2
Several recombinant subunit candidate flavivirus vaccines. Different expression systems have been used for the production of DV, WNV, JEV, TBEV and YFV antigens. (Et: truncated version of E protein; DomIII: domain III of E protein; NS1: non structural protein. HBcore: hepatitis B core antigen; HBsAg: hepatitis B surface antigen; VLP: virus-like particles).

Virus	Expression system	Antigen	References
Dengue virus	Yeast	Et	Mune et al. (2003)
		CprME	Sugrue et al. (1997b)
		E-HBsAg	Bisht et al. (2002)
		prME	Liu et al. (2010)
	Bacteria	DomIII	Jaiswal et al. (2004), Saejung et al. (2006), Sim et al. (2008), and Zhang et al. (2007b)
		E	Sugrue et al. (1997a)
	Insect cells	E	Eckels et al. (1994), Kelly et al. (2000), and Staropoli et al. (1997)
		Et	Clements et al. (2010) and Putnak et al. (1991)
		NS1	Feighny et al. (1992)
	Mammalian cells	prME	Kochel et al. (1997) and Konishi and Fujii (2002)
		prMEt	Kochel et al. (1997)
	Plant cells	DomIII	Kim et al. (2010) and Saejung et al. (2007)
		E	Martinez et al. (2011)
		Et, CprMEt, DomIII	Martinez et al. (2010)
		prME-VLP	Kanagaraj et al. (2011)
West Nile	Bacteria	DomIII	Holbrook et al. (2004) and Spohn et al. (2010)
	Insect cell	Et	Bonafe et al. (2009) and Ledizet et al. (2005)
		DomIII	Alonso-Padilla et al. (2011) and Chu et al. (2007)
		prME/CprME	Qiao et al. (2004)
Japanese encephalitis	Bacteria	E	Wu et al. (2004)
	Yeast	E	Fujita et al. (1987)
	Insect cells	E	Zhang et al. (2007a)
	Mammalian cells	E-VLP	Mutoh et al. (2004)
		E fragments	Konishi et al. (2001)
	Plant cells	prME, E	Appaiahgari et al. (2009)
		E	Wang et al. (2009)
Tick-borne encephalitis	Bacteria	DomIII	Holbrook et al. (2004)
	Mammalian cells	prME, prMEt	Lorenz et al. (2003)
Yellow fever	Insect cells	E	Barros et al. (2011)
		E/NS1	Despres et al. (1991)

faced by conventional expression technology (Davoodi-Semiromi et al., 2009).

5. Plant-based flavivirus candidate antigens

Plant-made vaccine production can be achieved using the whole plant or *in vitro* plant cell cultures. Whole plants can produce the recombinant protein at agronomic levels. In some special applications, the produced protein accumulates in certain plant organs to produce 'edible vaccines'. However, the term 'edible vaccines' has been replaced by 'plant-derived vaccine antigens' because it has been difficult to obtain the same expression levels in fruits of the same plant, complicating the calculation of an exact vaccine dose, and because of the difficulty of separating fruit intended for human consumption from that for pharmaceutical use (Kaiser, 2008; Rybicki, 2010).

In vitro culture of plant cells, tissues and organs (reviewed in Hellwig et al., 2004; Huang and McDonald, 2009) is an alternative technology for foreign protein production because these materials can be grown under controlled and optimised conditions in confined bioreactors using media with simple nutritional requirements (de Sousa Sampaio et al., 2010). Plant cells cultured in bioreactors allow recombinant proteins to be secreted into the culture medium for easier purification and to avoid transgene dissemination and food chain contamination. Therefore, reproducible product yields and biosafety conditions are guaranteed, and regulatory compliance is easier to achieve. These characteristics are particularly relevant for the production of human therapeutic proteins (Huang and McDonald, 2009).

5.1. Plant expression approaches for the production of flavivirus subunit vaccine candidates

Saejung et al. (2007) first reported the successful expression of a flavivirus protein in a plant expression system, in which the DomIII of DV was expressed in a TMV-based transient expression system in *Nicotiana benthamiana* plants (Saejung et al., 2007). Previously, flavivirus antigens had been expressed mainly in *Escherichia coli*, baculovirus and yeast. Based on data from those expression systems, various research groups began developing different plant systems for the production of DV, JEV and WNV antigens. Saejung et al. found that the plant-produced recombinant DomIII was reactive with an anti-DomIII polyclonal antibody and that mouse intramuscular immunisation induced the production of anti-dengue antibodies. The induced antibodies had neutralising activity against DV-2. These results indicated for the first time that plants can produce dengue virus antigen that possesses the appropriate antigenicity and immunogenicity in the assayed animal model. However, the level of induction in the immunised mice was low, even though the TiterMax Gold adjuvant was used in the experiments. No antibody induction was detected when the mice were immunised with plant-produced DomIII protein without adjuvant. The authors hypothesised that the low level of anti-dengue virus antibody induction in mice may have been due to the small size of the antigenic fragment used.

This report opened a new research area for the use of plants to produce flavivirus antigens. In 2009, Teoh et al. reported the development of a recombinant plant virus vector-based expression system in which foreign peptides could be fused with the viral coat protein (Teoh et al., 2009). This expression system has been widely studied as an antigen presentation system as well as for the production of pharmaceutically important peptides. In the initial report, a truncated version of the DV E-protein (EB4) was fused to the coat protein of the *Cucumber green mottle mosaic virus* (CGMMV). The clone, which contained a read-through signal from the wild-type CGMMV genome, was infectious and produced chimeric CGMMV/EB4.

At the same time, research groups from India (Appaiahgari et al., 2009) and China (Wang et al., 2009) independently reported the production of JEV antigens in plants. Previous studies showed that the E protein of JEV did not fold correctly in *E. coli* and, therefore, failed to induce JEV-neutralising antibodies, which are mostly directed against conformational epitopes. In addition, *E. coli*-expressed recombinant proteins are non-glycosylated, and E-protein glycosylation may be critical for its immunogenicity (Rauthan et al., 2004). Based on their own results in *E. coli* and the previous results of others in mammalian expression systems, Appaiahgari et al. carried out a detailed study of the expression of JEV antigens in plants (Appaiahgari et al., 2009). They expressed the E protein and its truncated version (Es) alone and co-expressed the prM and E viral proteins in mammalian cells (HEK 293A) and *Nicotiana tabacum* var. *Xanthi* plants. In contrast with mammalian cells, in which the prM protein of JEV has been shown to be necessary for the proper expression and folding of the E protein, the JEV E protein could only be expressed in transgenic tobacco plants in the absence of the prM protein. The co-expression of prM drastically reduced the expression levels of the E protein, possibly due to the poor translation of the prM sequence in the plant system.

Simultaneously, Wang et al. reported the successful expression of the JEV E protein in transgenic rice plants with the aim of obtaining a plant-derived vaccine antigen. The E protein was expressed in the leaves at levels of 1.1–1.9 μg/mg of total soluble protein (TSP) (Wang et al., 2009). The immunogenicity was evaluated in mice injected intraperitoneally and fed with the protein extract from the transgenic rice plants. The authors found that mice that were immunised intraperitoneally

with the plant-derived JEV E-protein presented a strong IgG neutralising antibody response and had almost the same IgG antibody titre as mice immunised with the *E. coli*-derived JEV E protein. When immunised orally, the IgG levels obtained with the plant-derived protein extract at day 36 were similar to those obtained with the *E. coli*-derived protein, indicating that it was possible to induce both systemic and mucosal immunity through this vaccination method. In the intestinal washes of the sacrificed animals, the IgA levels were higher in the group immunised with the plant-derived JEV E protein than in the group immunised with the *E. coli*-derived protein. Although these results are very promising, the antibody titres obtained in the immunised mice were significantly lower than those in mice that received the JEV SA14-14-2 oil adjuvant inactivated vaccine.

Our laboratory has long been interested in developing plant cells to express DV proteins as a future vaccine and diagnostic kit component. As part of a project to develop a plant-made DV antigen, we explored different expression strategies using transiently transformed *N. benthamiana* plants. Transient plant expression is an alternative system for producing recombinant proteins quickly and with high yields, permitting a rapid production and harvesting that cannot be achieved with stable genomic integration (Regnard et al., 2010). Transient expression using viral vectors can be limited by the low infectivity of the viral vector that carries the gene of the recombinant protein. To address this issue, Icon Genetics (Bayer) developed 'Magnifection', an efficient whole-plant infiltration technique in which deconstructed RNA viral vectors (or modules) are efficiently transfected by *Agrobacterium*-mediated systemic delivery as DNA precursors to the plant cell. Once inside the plant cell, the DNA is transcribed, and the modules are assembled with the help of a site-specific recombinase, generating a RNA replicon that produces a high expression yield of the recombinant protein (Gleba et al., 2005; Marillonnet et al., 2004; Marillonnet et al., 2005). Magnifection enables rapid production and high recombinant protein expression levels, and it is a versatile process that has been used successfully with several recombinant proteins (Gleba et al., 2005).

Using this technology, the truncated version of the DV E protein (Et), which lacks the membrane anchor domain, was expressed alone or co-expressed with the C and prM structural proteins (CprMEt) (Martinez et al., 2010). It was previously unclear whether the anchor domain was necessary for expressing the E protein in plant systems. As deleting the carboxy-terminal end of the DV-1 E protein minimises its proteolysis during expression in *Pichia pastoris* (Bisht et al., 2002), we designed a plant-adapted expression cassette to express the truncated version of the E-protein in plants. Without the anchor domain, the recombinant protein is soluble and may be easier to purify. The co-expression of the DV structural proteins (C, prM and E) has several advantages. As mentioned previously, numerous studies of different flaviviruses have suggested that the prM protein is required for the proper formation of recombinant E protein antigenic structure (Kelly et al., 2000), and DV structural proteins co-expressed in *P. pastoris* protect the E protein from degradation (Sugrue et al., 1997b). The C-terminal region of the C protein has a hydrophobic signal sequence. This signal sequence is needed to anchor the C protein into the ER membrane and consequently allows the translocation of prM and E into the lumen of the ER. In addition, prM and E proteins contain transmembrane domains, stop transfer and signal sequences that are involved in the orientation of the polyprotein in the ER membrane. Transmembrane regions of C, prM and E proteins contribute to the accurate orientation of the polyprotein in the ER membrane. This particular arrangement of the polyprotein precursor has been shown to permit its correct co- and post-translational proteolytic processing (Fig. 2).

Our results showed that the Et and CprMEt recombinant proteins were produced in *N. benthamiana* plants using Magnifection technology at 0.6 and 0.5 mg/g leaf fresh weight, respectively, at 7–10 days post-infection (dpi). Both proteins were reactive with anti-E antibodies. However, based on our results, we assume that the polyprotein CprMEt was not fully processed in the plant cell as the fused proteins prM-Et co-migrated in the western blot analysis. These results evidenced the uncompleted processing of the polyprotein CprMEt by the plant cell machinery. These results are opposite to those for the JEV proteins described above (Appaiahgari et al., 2009).

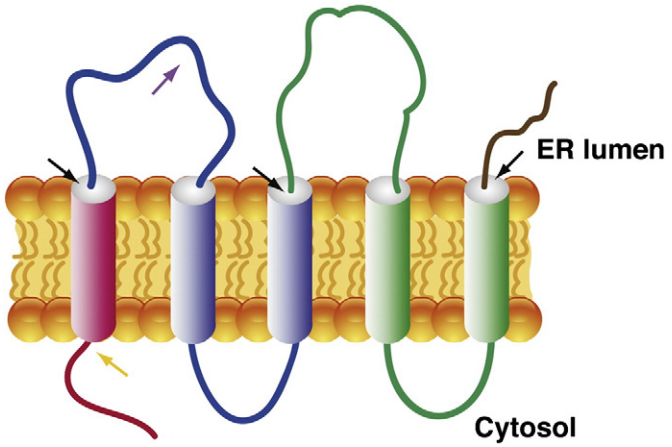


Fig. 2. Schematic representation of the spatial accommodation of flavivirus polyprotein in the host-derived ER membrane. Red: C protein; Blue: prM protein; Green: E protein. Arrows indicate post-translational cleavage sites that occur during the polyprotein processing: viral cleavage site (yellow), host cell cleavage site (black), furin protease cleavage site (violet). Cylinders represent transmembrane helices.

The same expression system was used by He et al. (2012) to produce the WNV DomIII protein. The authors produced a plant-derived antigen using Magnifection technology and obtained 0.1 mg/g leaf fresh weight of the recombinant protein in *N. benthamiana* plants.

As plant-produced DomIII did not elicit immune responses in mice without adjuvant (Saejung et al., 2007), different alternatives have been developed to overcome this obstacle. One involves the development of virus-like particles, which will be discussed in the next section. Another is the fusion of flavivirus antigens with carrier molecules such as the cholera toxin B subunit (CTB) or the *E. coli* heat-labile enterotoxin B subunit (LTB) to increase the adjuvant properties of the flavivirus antigens and induce strong cellular and humoural responses in animal models. Kim et al. expressed the fusion protein of DV DomIII and CTB in edible tobacco plants with low or no nicotine content (Kim et al., 2010). CTB was used as a vaccine adjuvant to effectively improve the immune response by increasing antigen flavivirus uptake into intestinal mucosal immune systems. The expression level of the fusion protein reached 0.019% TSP when a stable transformation was performed. No further mice immunisation studies have been performed yet.

5.2. In vitro plant cell culture for the production of flavivirus antigens

As mentioned previously, *in vitro* plant cell culture offers a controlled environment with improved safety and has a number of potential advantages over transgenic plants for antigen production. A large number of recombinant proteins, enzymes and antigens are produced in confined plant systems; the recombinant human GCD enzyme is not the only *in vitro* plant cell-cultured protein close to reaching the market. Antigens of the Newcastle virus are being produced for a poultry vaccine in tobacco cell suspension cultures by Dow AgroScience (www.dowagro.com). The recombinant protein received the first regulatory approval for a plant-made vaccine from the USDA in 2006 and is close to market.

The choice of this production platform for plant cell-produced antigens obviates concerns about the whole plant or food crops, provides a safe and bio-contained environment for the final recombinant product and offers GMP-compatible production environments that are more

acceptable to the pharmaceutical industry (Xu et al., 2012). In addition, *in vitro* plant cell cultures can be scaled-up in fermenters for industrial production. If the recombinant protein is secreted into the culture medium, downstream processing is less expensive because fewer separation and purification steps are required (Su, 2006). Unfortunately, the major bottleneck in exploiting *in vitro* plant cell culture for the stable production of recombinant proteins has been low productivity. Studies have attempted to improve transgene expression (Streatfield, 2007) and prevent recombinant protein degradation in *in vitro* plant cell cultures (Shih and Doran, 2009), with the ultimate goal of improving the yield and quality of the recombinant antigens obtained from these expression systems. We have recently studied the subcellular targeting of DV antigens to increase their accumulation levels in *N. tabacum* and *Morinda citrifolia* cell suspension cultures (Martinez et al., 2011). The DV-E protein was targeted to the ER by adding the ER retention/retrieval sequence KDEL (Lys–Asp–Glu–Leu) to the C-terminal end. In addition to increasing accumulation levels, the addition of the KDEL sequence avoids unwanted complex N-glycans in the trans-Golgi apparatus, which could reduce the immunogenicity of antigens expressed in plant cell cultures (Twyman et al., 2003). In our work, the highest levels of DV-E protein accumulation were observed in BY-2 tobacco cells when the KDEL sequence was present (0.71 ± 0.06 mg DV-E/L or 0.3% TSP), and the recombinant protein obtained was reactive with anti-E antibodies. To the best of our knowledge, this is the first report in which an *in vitro* plant culture was used to successfully produce a flaviviral protein.

5.3. Virus-like particles produced in plant systems as delivery system for flavivirus vaccination

The use of virus-like particles (VLPs) for the development of vaccines has long been studied. VLPs are bionanoparticles that self-assemble into highly organised 'nanosystems' with different shapes and sizes (Lee and Wang, 2006). In addition to uses in bio-imaging, drug delivery and gene therapy, bionanoparticles possess distinctive characteristics that make them suitable for the development of new vaccines for humans. Recently, the U.S. Food and Drug Administration approved a quadrivalent vaccine against papillomavirus (HPV) that consists of recombinant human HPV VLPs produced in yeast (GARDASIL®, Merck Research Laboratories), including the L1 major capsid protein of the HPV 6, 11, 16 and 18 virus serotypes (Villa et al., 2006). In addition, a recombinant subunit vaccine against hepatitis B virus produced in yeast and based on VLP production has been licensed (RECOMBIVAX HB®, Merck Research Laboratories and ENGERIX-B®, GlaxoSmithKline). These events exemplify and support the great potential of VLPs for vaccine development and should promote research and development in this area.

Recombinantly expressed viral capsid or envelope proteins assemble into highly immunogenic structures that lack the viral genetic material. These structures morphologically mimic the authentic viral particle and produce potent immune responses, with the advantage that they are completely non-infective because the viral nucleic acid is absent (Huang et al., 2009; Noad and Roy, 2003; Yoo et al., 2011). VLPs can be produced on a large scale using different recombinant expression systems, and their production in plants has been extensively studied (reviewed in Lai et al., 2011; Santi et al., 2006). As in yeast, insect and mammalian cells, plant cells have been shown to effectively produce VLPs of different viruses, such as Norovirus (Herbst-Kralovetz et al., 2011), influenza (D'Aoust et al., 2010), HPV (Biemelt et al., 2003; Warzecha et al., 2003), hepatitis B surface antigen (Huang et al., 2005; Huang et al., 2006; Smith et al., 2002), hepatitis B core antigen (Huang et al., 2006) and, more recently, dengue virus (Kanagaraj et al., 2011). VLPs are a good strategy for developing new subunit vaccines with increased immunogenicity. In particular, flaviviral VLPs have been shown to be excellent immunogens because they produce a similar antibody response and protection against viral challenge compared to inactivated viruses and therefore represent good candidates for the development of subunit vaccines against flavivirus infections (Goto et al., 2005). Recombinant VLPs of different flaviviruses, such as DV, WNV, TBEV and JEV, have been produced efficiently by co-expressing the prM and E proteins, which self-assemble and form VLPs in different expression systems. Using various expression systems, including mammalian cells, vaccinia virus, baculovirus and yeast, the recombinant expression of prM/E proteins has led to the production of VLPs for DV (Kanagaraj et al., 2011; Purdy and Chang, 2005; Qing et al., 2011; Sugrue et al., 1997b; Zhang et al., 2011), JEV (Konishi et al., 2001; Mutoh et al., 2004), TBE (Goncharova et al., 2002; Goto et al., 2005; Schalich et al., 1996) and WNV (Ohtaki et al., 2010; Scholle et al., 2004). Kanagaraj et al. (2011) recently reported the first production of DV VLPs in plants, with the aim of developing a subunit vaccine candidate for oral delivery. As DV prM/E proteins produced in different expression systems have been shown to possess good adjuvant properties as direct immunogens for inducing strong cellular and humoural responses, the authors transformed lettuce chloroplasts with genes encoding 24 amino acids of the capsid protein (C), 166 amino acids of prM and 405 amino acids of the E protein of DV-3 (DENV3prM/E) (Kanagaraj et al., 2011). Structures resembling VLPs of ~20 nm diameter were observed in chloroplast extracts from transplastomic plants by transmission electron microscopy. The presence of VLPs in these soluble extracts suggests that the prM/E proteins are properly folded in plant cells and are able to form higher-order structures (Kanagaraj et al., 2011).

Another interesting approach for developing VLP-based flavivirus subunit vaccines in plants is the co-expression of DV proteins with the hepatitis B *core* antigen (HBcore). HBcore is a highly immunogenic subviral particle that has been used as a particulate carrier protein for heterologous epitopes. It can incorporate foreign DNA and still undergo correct folding and self-assembly (Pumpens and Grens, 1999; Schodel et al., 1996). One of the most important advantages of HBcore particles is their ability to induce B cell and T cell responses and the good immunological properties exhibited by the inserted foreign epitopes. Moreover, the chimeric HBcore retains its ability to form particles in high yield in prokaryotic and eukaryotic recombinant expression systems and is easy to purify due to its particulate nature (Pumpens and Grens, 1999).

As mentioned at the beginning of this section, DV DomIII produced in plants and intramuscularly injected in mice did not yield the expected level of induction in the antisera of immunised mice, and no antibody induction was detected in the absence of an adjuvant (Saejung et al., 2007). A potential approach for improving the immunological properties of plant-produced DV DomIII is the fusion of the flavivirus antigen with the HBcore protein. In collaboration with Dr. Hugh S. Mason (Center for Infectious Diseases and Vaccinology, Biodesign Institute, Arizona State University, U.S.), we have expressed DomIII of the DV-E protein fused to the HBcore protein (HBcore-DV2d3) (Martinez et al., 2010). The fused proteins were linked by a flexible glycine-rich linker to permit correct folding. *N. benthamiana* plants were transiently infiltrated using Magnifection technology. The expected fusion protein was observed at 7 dpi and was reactive with anti-DV and anti-HBcore antibodies. No lower bands were detected in the immunoblot, indicating that no cleavage of the recombinant fusion protein occurred. The recombinant protein yield was 0.4 mg/g fresh weight leaf at 7 dpi. While electron microscopy studies are currently in progress, preliminary sucrose gradient sedimentation analysis showed that the plant-produced fusion protein had a particulate nature (data not published). Recently, Chen and coworkers published results using a geminiviral replicon system based on the bean yellow dwarf virus (BeYDV) that permits the high-level expression of the fusion of WNV DomIII with HBcore protein in tobacco plants. The chimeric protein can be readily purified from plant leaves at high level of accumulation with scalable processing methods (Chen et al., 2011).

6. Concluding remarks

The current situation of flavivirus vaccines briefly outlined above discloses the existing need of new candidate vaccines, either by improving the existing ones or by providing new platforms for the production of safe and cost-effective vaccines. Among them, plant-made antigens have emerged as an alternative for vaccine production, especially in developing countries, where the infectious diseases caused by flaviviruses have been spread. One of the main advantages of this platform is the possibility of using low cost facilities since they do not require the building of a classical industrial fermentation plant. Concerning plant-made flavivirus antigens, the production of most of them has been focused on DV E protein or in its main immunogenic epitope, Domain III. Different strategies have been explored to produce flavivirus antigens, which include stable expression *via* nuclear and plastid genome as well as transient expression systems. Chloroplast-derived and transient expression systems reached higher levels of antigen accumulation than nuclear-derived ones. Besides, VLPs were successfully produced in plant cells, making them an interesting strategy for developing new flavivirus subunit vaccines.

Although plant-made flavivirus antigens are in the first stages of development, the results that we reviewed here represent good evidence about the possibilities of using plant systems as bioreactors for flavivirus antigen production. New challenges are not only improving expression levels but also developing integrated processes for the production and purification of recombinant antigens.

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References

Alonso-Padilla J, de Oya NJ, Blazquez AB, Escribano-Romero E, Escribano JM, Saiz JC. Recombinant West Nile virus envelope protein E and domain III expressed in insect larvae protects mice against West Nile disease. Vaccine 2011;29(9):1830–5.

Appaiahgari MB, Vrati S. IMOJEV: a Yellow fever virus-based novel Japanese encephalitis vaccine. Expert Rev Vaccines 2010;9(12):1371–84.

Appaiahgari MB, Abdin MZ, Bansal KC, Vrati S. Expression of Japanese encephalitis virus envelope protein in transgenic tobacco plants. J Virol Methods 2009;162(1–2):22–9.

Arroyo J, Miller C, Catalan J, Myers GA, Ratterree MS, Trent DW, et al. ChimeriVax-West Nile virus live-attenuated vaccine: preclinical evaluation of safety, immunogenicity, and efficacy. J Virol 2004;78(22):12497–507.

Aviezer D, Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, Mizrachi S, et al. A plant-derived recombinant human glucocerebrosidase enzyme—a preclinical and phase I investigation. PLoS One 2009;4(3):e4792.

Barros MC, Galasso TG, Chaib AJ, Degallier N, Nagata T, Ribeiro BM. Yellow fever virus envelope protein expressed in insect cells is capable of syncytium formation in lepidopteran cells and could be used for immunodetection of YFV in human sera. Virol J 2011;8:261.

Beasley DW, Barrett AD. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol 2002;76(24):13097–100.

Beckett CG, Tjaden J, Burgess T, Danko JR, Tamminga C, Simmons M, et al. Evaluation of a prototype dengue-1 DNA vaccine in a Phase 1 clinical trial. Vaccine 2011;29(5):960–8.

Biemelt S, Sonnewald U, Galmbacher P, Willmitzer L, Muller M. Production of human papillomavirus type 16 virus-like particles in transgenic plants. J Virol 2003;77(17):9211–20.

Bisht H, Chugh DA, Raje M, Swaminathan SS, Khanna N. Recombinant dengue virus type 2 envelope/hepatitis B surface antigen hybrid protein expressed in *Pichia pastoris* can function as a bivalent immunogen. J Biotechnol 2002;99(2):97-110.

Bonafe N, Rininger JA, Chubet RG, Foellmer HG, Fader S, Anderson JF, et al. A recombinant West Nile virus envelope protein vaccine candidate produced in *Spodoptera frugiperda* expresSF+ cells. Vaccine 2009;27(2):213–22.

CDC. Epidemic/Epizootic West Nile Virus in the United States: Guidelines for Surveillance, Prevention, and Control. 3rd Revision 2003;1–78.

Chan RC, Penney DJ, Little D, Carter IW, Roberts JA, Rawlinson WD. Hepatitis and death following vaccination with 17D-204 yellow fever vaccine. Lancet 2001;358(9276):121–2.

Chang DC, Liu WJ, Anraku I, Clark DC, Pollitt CC, Suhrbier A, et al. Single-round infectious particles enhance immunogenicity of a DNA vaccine against West Nile virus. Nat Biotechnol 2008;26(5):571–7.

Chaturvedi UC, Shrivastava R, Nagar R. Dengue vaccines: problems and prospects. Indian J Med Res 2005;121(5):639–52.

Chavez JH, Silva JR, Amarilla AA, Moraes Figueiredo LT. Domain III peptides from flavivirus envelope protein are useful antigens for serologic diagnosis and targets for immunization. Biologicals 2010;38(6):613–8.

Chen Q, He J, Phoolcharoen W, Mason HS. Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. Hum Vaccin 2011;7(3):331–8.

Chu JH, Chiang CC, Ng ML. Immunization of flavivirus West Nile recombinant envelope domain III protein induced specific immune response and protection against West Nile virus infection. J Immunol 2007;178(5):2699–705.

Clements DE, Coller BA, Lieberman MM, Ogata S, Wang G, Harada KE, et al. Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. Vaccine 2010;28(15):2705–15.

Coller BA, Clements DE. Dengue vaccines: progress and challenges. Curr Opin Immunol 2011;23(3):391–8.

Coller BA, Clements DE, Bett AJ, Sagar SL, Ter Meulen JH. The development of recombinant subunit envelope-based vaccines to protect against dengue virus induced disease. Vaccine 2011;29(42):7267–75.

Crill WD, Chang GJ. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J Virol 2004;78(24):13975–86.

Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 2009;14(12):669–79.

D'Aoust MA, Couture MM, Charland N, Trepanier S, Landry N, Ors F, et al. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol J 2010;8(5):607–19.

Dauphin G, Zientara S. West Nile virus: recent trends in diagnosis and vaccine development. Vaccine 2007;25(30):5563–76.

Davies HM. Review article: commercialization of whole-plant systems for biomanufacturing of protein products: evolution and prospects. Plant Biotechnol J 2010;8(8):845–61.

Davis BS, Chang GJ, Cropp B, Roehrig JT, Martin DA, Mitchell CJ, et al. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol 2001;75(9):4040–7.

Davoodi-Semiromi A, Samson N, Daniell H. The green vaccine: a global strategy to combat infectious and autoimmune diseases. Hum Vaccin 2009;5(7):488–93.

de Sousa Sampaio PN, Neto H, Poejo P, Serrazina SMT, Soares Pais MS. Overexpression and characterization of cyprosin B in transformed suspension cells of *Cynara cardunculus*. Plant Cell Tissue Organ Cult 2010;101:311–21.

Despres P, Dietrich J, Girard M, Bouloy M. Recombinant baculoviruses expressing yellow fever virus E and NS1 proteins elicit protective immunity in mice. J Gen Virol 1991;72(Pt 11):2811–6.

Despres P, Combredet C, Frenkiel MP, Lorin C, Brahic M, Tangy F. Live measles vaccine expressing the secreted form of the West Nile virus envelope glycoprotein protects against West Nile virus encephalitis. J Infect Dis 2005;191(2):207–14.

Diamond MS. Evasion of innate and adaptive immunity by flaviviruses. Immunol Cell Biol 2003;81(3):196–206.

Diamond MS, Shrestha B, Marri A, Mahan D, Engle M. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. J Virol 2003;77(4):2578–86.

Duggan ST, Plosker GL. Japanese encephalitis vaccine (inactivated, adsorbed) [IXIARO]. Drugs 2009;69(1):115–22.

Durbin AP, McArthur JH, Marron JA, Blaney JE, Thumar B, Wanionek K, et al. rDEN2/4Delta30(ME), a live attenuated chimeric dengue serotype 2 vaccine is safe and highly immunogenic in healthy dengue-naive adults. Hum Vaccin 2006;2(6):255–60.

Eckels KH, Dubois DR, Summers PL, Schlesinger JJ, Shelly M, Cohen S, et al. Immunization of monkeys with baculovirus-dengue type-4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection. Am J Trop Med Hyg 1994;50(4):472–8.

Faye L, Gomord V. Success stories in molecular farming—a brief overview. Plant Biotechnol J 2010;8(5):525–8.

Feighny R, Burrous J, McCown J, Hoke C, Putnak R. Purification of native dengue-2 viral proteins and the ability of purified proteins to protect mice. Am J Trop Med Hyg 1992;47(4):405–12.

Fischer M, Lindsey N, Staples JE, Hills S. Japanese encephalitis vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2010;59(RR-1):1-27.

Fujita H, Sumiyoshi H, Mori C, Manabe S, Takagi M, Yoshida I, et al. Studies in the development of Japanese encephalitis vaccine: expression of virus envelope glycoprotein V3 (E) gene in yeast. Bull World Health Organ 1987;65(3):303–8.

Garmendia AE, Van Kruiningen HJ, French RA. The West Nile virus: its recent emergence in North America. Microbes Infect 2001;3(3):223–9.

Ghosh D, Basu A. Japanese encephalitis—a pathological and clinical perspective. PLoS Negl Trop Dis 2009;3(9):e437.

Gleba Y, Klimyuk V, Marillonnet S. Magnifection—a new platform for expressing recombinant vaccines in plants. Vaccine 2005;23(17–18):2042–8.

Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ. Monoclonal antibody-mediated enhancement of dengue virus infection *in vitro* and *in vivo* and strategies for prevention. Proc Natl Acad Sci U S A 2007;104(22):9422–7.

Goncharova EP, Ryzhikov AB, Bulychev LE, Sizov AA, Lebedev LR, Poryvaev VD, et al. A study of systems for delivering antigens and plasmid DNA for intranasal immunization against tick-borne encephalitis virus. Wien Klin Wochenschr 2002;114(13–14):630–5.

Goto A, Yoshii K, Obara M, Ueki T, Mizutani T, Kariwa H, et al. Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion. Vaccine 2005;23(23):3043–52.
Gould EA, Solomon T. Pathogenic flaviviruses. Lancet 2008;371(9611):500–9.
Grgic-Vitek M, Avsic-Zupanc T, Klavs I. Tick-borne encephalitis after vaccination: vaccine failure or misdiagnosis. Vaccine 2010;28(46):7396–400.
Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 1998;11(3):480–96.
Guy B, Guirakhoo F, Barban V, Higgs S, Monath TP, Lang J. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. Vaccine 2010a;28(3):632–49.
Guy B, Saville M, Lang J. Development of Sanofi Pasteur tetravalent dengue vaccine. Hum Vaccin 2010b;6(9).
Guy B, Barrere B, Malinowski C, Saville M, Teyssou R, Lang J. From research to phase III: Preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. Vaccine 2011;29(42):7229–41.
Guzman MG, Alvarez M, Rodriguez-Roche R, Bernardo L, Montes T, Vazquez S, et al. Neutralizing antibodies after infection with dengue 1 virus. Emerg Infect Dis 2007;13(2):282–6.
Guzman MG, Hermida L, Bernardo L, Ramirez R, Guillen G. Domain III of the envelope protein as a dengue vaccine target. Expert Rev Vaccines 2010;9(2):137–47.
Halstead SB. Dengue. Lancet 2007;370(9599):1644–52.
Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis 2005;11(8): 1167–73.
He J, Lai H, Brock C, Chen Q. A novel system for rapid and cost-effective production of detection and diagnostic reagents of West Nile virus in plants. J Biomed Biotechnol 2012;2012:106783.
Heinz FX, Collett MS, Purcell RH, Gould EA, Howard CR, Houghton M, Moorman RJM, Rice CM, Thiel HJ. Family flaviviridae. In: Regenmortel HV, Fauquet CM, Bishop DHL, Carstens E, Estes MK, Lemon S, Maniloff J, Mayo MA, McGeogch D, Pringle CR, Wickner RB, editors. Virus taxonomy. 7th International Committee for the Taxonomy of Viruses. San Diego, California: Academic Press; 2000.
Hellwig S, Drossard J, Twyman RM, Fischer R. Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 2004;22(11):1415–22.
Herbst-Kralovetz M, Mason HS, Chen Q. Norwalk virus-like particles as vaccines. Expert Rev Vaccines 2011;9(3):299–307.
Holbrook MR, Shope RE, Barrett AD. Use of recombinant E protein domain III-based enzyme-linked immunosorbent assays for differentiation of tick-borne encephalitis serocomplex flaviviruses from mosquito-borne flaviviruses. J Clin Microbiol 2004;42(9):4101–10.
Hombach J, Barrett AD, Cardosa MJ, Deubel V, Guzman M, Kurane I, et al. Review on flavivirus vaccine development. Proceedings of a meeting jointly organised by the World Health Organization and the Thai Ministry of Public Health, 26–27 April 2004, Bangkok, Thailand. Vaccine 2005;23(21):2689–95.
Huang T-K, McDonald KA. Bioreactor engineering for recombinant protein production in plant cell suspension cultures. Biochem Eng J 2009;45:168–84.
Huang CY, Butrapet S, Tsuchiya KR, Bhamarapravati N, Gubler DJ, Kinney RM. Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development. J Virol 2003;77(21):11436–47.
Huang Z, Elkin G, Maloney BJ, Beuhner N, Arntzen CJ, Thanavala Y, et al. Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. Vaccine 2005;23(15):1851–8.
Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ, Mason HS. Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. Vaccine 2006;24(14):2506–13.
Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H. A DNA replicon system for rapid high-level production of virus-like particles in plants. Biotechnol Bioeng 2009;103(4):706–14.
Iglesias MC, Frenkiel MP, Mollier K, Souque P, Despres P, Charneau P. A single immunization with a minute dose of a lentiviral vector-based vaccine is highly effective at eliciting protective humoral immunity against West Nile virus. J Gene Med 2006;8(3):265–74.
Ishikawa T, Wang G, Widman DG, Infante E, Winkelmann ER, Bourne N, et al. Enhancing the utility of a prM/E-expressing chimeric vaccine for Japanese encephalitis by addition of the JEV NS1 gene. Vaccine 2011;29(43):7444–55.
Jaiswal S, Khanna N, Swaminathan S. High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in Escherichia coli. Protein Expr Purif 2004;33(1):80–91.
Kaiser R. The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994–98: a prospective study of 656 patients. Brain 1999;122(Pt 11): 2067–78.
Kaiser J. Is the drought over for pharming? Science 2008;320(5875):473–5.
Kalayanarooj S, Nimmannitya S. Is dengue severity related to nutritional status? Southeast Asian J Trop Med Public Health 2005;36(2):378–84.
Kanagaraj AP, Verma D, Daniell H. Expression of dengue-3 premembrane and envelope polyprotein in lettuce chloroplasts. Plant Mol Biol 2011;76(3–5):323–33.
Kanesa-thasan N, Sun W, Kim-Ahn G, Van Albert S, Putnak JR, King A, et al. Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. Vaccine 2001;19(23–24):3179–88.
Kelly EP, Greene JJ, King AD, Innis BL. Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. Vaccine 2000;18(23): 2549–59.
Kim TG, Kim MY, Yang MS. Cholera toxin B subunit-domain III of dengue virus envelope glycoprotein E fusion protein production in transgenic plants. Protein Expr Purif 2010;74(2):236–41.
King NJ, Getts DR, Getts MT, Rana S, Shrestha B, Kesson AM. Immunopathology of flavivirus infections. Immunol Cell Biol 2007;85(1):33–42.
Kliks SC, Nimmanitya S, Nisalak A, Burke DS. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 1988;38(2):411–9.
Kochel T, Wu SJ, Raviprakash K, Hobart P, Hoffman S, Porter K, et al. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. Vaccine 1997;15(5):547–52.
Kofler RM, Heinz FX, Mandl CW. Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence. J Virol 2002;76(7):3534–43.
Konishi E, Fujii A. Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. Vaccine 2002;20(7–8):1058–67.
Konishi E, Fujii A, Mason PW. Generation and characterization of a mammalian cell line continuously expressing Japanese encephalitis virus subviral particles. J Virol 2001;75(5):2204–12.
Koraka P, Martina BE, Osterhaus AD. Bioinformatics in new generation flavivirus vaccines. J Biomed Biotechnol 2010;2010:864029.
Kumar R, Mathur A, Kumar A, Sharma S, Chakraborty S, Chaturvedi UC. Clinical features & prognostic indicators of Japanese encephalitis in children in Lucknow (India). Indian J Med Res 1990;91:321–7.
Kumar R, Tripathi P, Singh S, Bannerji G. Clinical features in children hospitalized during the 2005 epidemic of Japanese encephalitis in Uttar Pradesh, India. Clin Infect Dis 2006;43(2):123–31.
Kyle JL, Harris E. Global spread and persistence of dengue. Annu Rev Microbiol 2008;62:71–92.
Labeaud AD, Bashir F, King CH. Measuring the burden of arboviral diseases: the spectrum of morbidity and mortality from four prevalent infections. Popul Health Metr 2011;9(1):1.
LaFleur C, Granados J, Vargas-Alarcon G, Ruiz-Morales J, Villarreal-Garza C, Higuera L, et al. HLA-DR antigen frequencies in Mexican patients with dengue virus infection: HLA-DR4 as a possible genetic resistance factor for dengue hemorrhagic fever. Hum Immunol 2002;63(11):1039–44.
Lai H, He J, Engle M, Diamond MS, Chen Q. Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. Plant Biotechnol J 2011;10(1):95-104.
Ledizet M, Kar K, Foellmer HG, Wang T, Bushmich SL, Anderson JF, et al. A recombinant envelope protein vaccine against West Nile virus. Vaccine 2005;23(30):3915–24.
Lee E, Lobigs M. E protein domain III determinants of yellow fever virus 17D vaccine strain enhance binding to glycosaminoglycans, impede virus spread, and attenuate virulence. J Virol 2008;82(12):6024–33.
Lee LA, Wang Q. Adaptations of nanoscale viruses and other protein cages for medical applications. Nanomedicine 2006;2(3):137–49.
Leonova GN, Ternovoi VA, Pavlenko EV, Maistrovskaya OS, Protopopova EV, Loktev VB. Evaluation of vaccine Encepur Adult for induction of human neutralizing antibodies against recent Far Eastern subtype strains of tick-borne encephalitis virus. Vaccine 2007;25(5):895–901.
Lieberman MM, Clements DE, Ogata S, Wang G, Corpuz G, Wong T, et al. Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. Vaccine 2007;25(3):414–23.
Lin CW, Liu KT, Huang HD, Chen WJ. Protective immunity of *E. coli*-synthesized NS1 protein of Japanese encephalitis virus. Biotechnol Lett 2008;30(2):205–14.
Lindenbach BD, Thiel HJ, Rice CM. Flaviviridae: the viruses and their replication. In: Knipe DM, Howley PM, editors. Fields Virology. Philadelphia: Lippincott-Raven; 2007, pp. 1101–1152.
Liu W, Jiang H, Zhou J, Yang X, Tang Y, Fang D, et al. Recombinant dengue virus-like particles from *Pichia pastoris*: efficient production and immunological properties. Virus Genes 2010;40(1):53–9.
Loew-Baselli A, Poellabauer EM, Pavlova BG, Fritsch S, Firth C, Petermann R, et al. Prevention of tick-borne encephalitis by FSME-IMMUN((R)) vaccines: review of a clinical development programme. Vaccine 2011;29(43):7307–19.
Lorenz IC, Kartenbeck J, Mezzacasa A, Allison SL, Heinz FX, Helenius A. Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. J Virol 2003;77(7):4370–82.
Ma JK, Drake PM, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet 2003;4(10):794–805.
Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y. In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. Proc Natl Acad Sci U S A 2004;101(18):6852–7.
Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y. Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 2005;23(6):718–23.
Martin M, Tsai TF, Cropp B, Chang GJ, Holmes DA, Tseng J, et al. Fever and multisystem organ failure associated with 17D-204 yellow fever vaccination: a report of four cases. Lancet 2001;358(9276):98-104.
Martina BE, Koraka P, van den Doel P, van Amerongen G, Rimmelzwaan GF, Osterhaus AD. Immunization with West Nile virus envelope domain III protects mice against lethal infection with homologous and heterologous virus. Vaccine 2008;26(2): 153–7.
Martinez CA, Topal E, Giulietti AM, Talou JR, Mason H. Exploring different strategies to express Dengue virus envelope protein in a plant system. Biotechnol Lett 2010;32(6): 867–75.
Martinez CA, Giulietti AM, Rodriguez Talou J. Expression of a KDEL-tagged dengue virus protein in cell suspension cultures of *Nicotiana tabacum* and *Morinda citrifolia*. Plant Cell Tissue Organ Cult 2011;107(1):91-100.
McArthur JH, Durbin AP, Marron JA, Wanionek KA, Thumar B, Pierro DJ, et al. Phase I clinical evaluation of rDEN4Delta30-200,201: a live attenuated dengue 4 vaccine

candidate designed for decreased hepatotoxicity. Am J Trop Med Hyg 2008;79(5):678–84.
Monath TP. Yellow fever vaccine. Expert Rev Vaccines 2005;4(4):553–74.
Monath TP. Dengue and yellow fever—challenges for the development and use of vaccines. N Engl J Med 2007;357(22):2222–5.
Monath TP. Treatment of yellow fever. Antiviral Res 2008;78(1):116–24.
Monath TP, Guirakhoo F, Nichols R, Yoksan S, Schrader R, Murphy C, et al. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis 2003;188(8):1213–30.
Monath TP, Liu J, Kanesa-Thasan N, Myers GA, Nichols R, Deary A, et al. A live, attenuated recombinant West Nile virus vaccine. Proc Natl Acad Sci U S A 2006;103(17):6694–9.
Monath TP, Fowler E, Johnson CT, Balser J, Morin MJ, Sisti M, et al. An inactivated cell-culture vaccine against yellow fever. N Engl J Med 2011a;364(14):1326–33.
Monath TP, Lee CK, Julander JG, Brown A, Beasley DW, Watts DM, et al. Inactivated yellow fever 17D vaccine: development and nonclinical safety, immunogenicity and protective activity. Vaccine 2011b;28(22):3827–40.
Morens DM, Folkers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. Nature 2004;430(6996):242–9.
Mune M, Rodriguez R, Ramirez R, Soto Y, Sierra B, Rodriguez Roche R, et al. Carboxy-terminally truncated Dengue 4 virus envelope glycoprotein expressed in *Pichia pastoris* induced neutralizing antibodies and resistance to Dengue 4 virus challenge in mice. Arch Virol 2003;148(11):2267–73.
Murgue B, Zeller H, Deubel V. The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. Curr Top Microbiol Immunol 2002;267:195–221.
Murrell S, Wu SC, Butler M. Review of dengue virus and the development of a vaccine. Biotechnol Adv 2011;29(2):239–47.
Mutoh E, Ishikawa T, Takamizawa A, Kurata T, Sata T, Kojima A. Japanese encephalitis subunit vaccine composed of virus-like envelope antigen particles purified from serum-free medium of a high-producer J12#26 cell clone. Vaccine 2004;22(20):2599–608.
Nguyen TP, Kikuchi M, Vu TQ, Do QH, Tran TT, Vo DT, et al. Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. PLoS Negl Trop Dis 2008;2(10):e304.
Noad R, Roy P. Virus-like particles as immunogens. Trends Microbiol 2003;11(9):438–44.
Ohtaki N, Takahashi H, Kaneko K, Gomi Y, Ishikawa T, Higashi Y, et al. Immunogenicity and efficacy of two types of West Nile virus-like particles different in size and maturation as a second-generation vaccine candidate. Vaccine 2010;28(40):6588–96.
Peny JM, Gleizes O, Covilard JP. Financial requirements of immunisation programmes in developing countries: a 2004–2014 perspective. Vaccine 2005;23(37):4610–8.
Petri E, Gniel D, Zent O. Tick-borne encephalitis (TBE) trends in epidemiology and current and future management. Travel Med Infect Dis 2010;8(4):233–45.
Pletnev AG, Claire MS, Elkins R, Speicher J, Murphy BR, Chanock RM. Molecularly engineered live-attenuated chimeric West Nile/dengue virus vaccines protect rhesus monkeys from West Nile virus. Virology 2003;314(1):190–5.
Pripuzova NS, Tereshkina NV, Gmyl LV, Dzhivanyan TI, Rumyantsev AA, Romanova L, et al. Safety evaluation of chimeric Langat/Dengue 4 flavivirus, a live vaccine candidate against tick-borne encephalitis. J Med Virol 2009;81(10):1777–85.
Pugachev KV, Guirakhoo F, Trent DW, Monath TP. Traditional and novel approaches to flavivirus vaccines. Int J Parasitol 2003;33(5–6):567–82.
Pugachev KV, Guirakhoo F, Monath TP. New developments in flavivirus vaccines with special attention to yellow fever. Curr Opin Infect Dis 2005;18(5):387–94.
Pulmanausahakul R, Khakpoor A, Smith DR. The development of flavivirus vaccines. Afr J Biotechnol 2010;9(4):409–15.
Pumpens P, Grens E. Hepatitis B core particles as a universal display model: a structure-function basis for development. FEBS Lett 1999;442(1):1–6.
Purdy DE, Chang GJ. Secretion of noninfectious dengue virus-like particles and identification of amino acids in the stem region involved in intracellular retention of envelope protein. Virology 2005;333(2):239–50.
Putnak JR, Schlesinger JJ. Protection of mice against yellow fever virus encephalitis by immunization with a vaccinia virus recombinant encoding the yellow fever virus non-structural proteins, NS1, NS2a and NS2b. J Gen Virol 1990;71(Pt 8):1697–702.
Putnak R, Feighny R, Burrous J, Cochran M, Hackett C, Smith G, et al. Dengue-1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicits virus-neutralizing antibody in mice and protects them from virus challenge. Am J Trop Med Hyg 1991;45(2):159–67.
Qiao M, Ashok M, Bernard KA, Palacios G, Zhou ZH, Lipkin WI, et al. Induction of sterilizing immunity against West Nile Virus (WNV), by immunization with WNV-like particles produced in insect cells. J Infect Dis 2004;190(12):2104–8.
Qing M, Liu W, Yuan Z, Gu F, Shi PY. A high-throughput assay using dengue-1 virus-like particles for drug discovery. Antiviral Res 2011;86(2):163–71.
Rauthan M, Kaur R, Appaiahgari MB, Vrati S. Oral immunization of mice with Japanese encephalitis virus envelope protein synthesized in *Escherichia coli* induces anti-viral antibodies. Microbes Infect 2004;6(14):1305–11.
Regnard GL, Halley-Stott RP, Tanzer FL, Hitzeroth II, Rybicki EP. High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. Plant Biotechnol J 2010;8(1):38–46.
Rendi-Wagner P. Advances in vaccination against tick-borne encephalitis. Expert Rev Vaccines 2008;7(5):589–96.
Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 1995;375(6529):291–8.
Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, Strauss JH. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 1985;229(4715):726–33.
Ritchie SA, Rochester W. Wind-blown mosquitoes and introduction of Japanese encephalitis into Australia. Emerg Infect Dis 2001;7(5):900–3.
Rybicki EP. Plant-made vaccines for humans and animals. Plant Biotechnol J 2010;8(5):620–37.
Saad M, Youssef S, Kirschke D, Shubair M, Haddadin D, Myers J, et al. Acute flaccid paralysis: the spectrum of a newly recognized complication of West Nile virus infection. J Infect 2005;51(2):120–7.
Sabchareon A, Lang J, Chanthavanich P, Yoksan S, Forrat R, Attanath P, et al. Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. Am J Trop Med Hyg 2002;66(3):264–72.
Saejung W, Puttikhunt C, Prommool T, Sojikul P, Tanaka R, Fujiyama K, et al. Enhancement of recombinant soluble dengue virus 2 envelope domain III protein production in *Escherichia coli* trxB and gor double mutant. J Biosci Bioeng 2006;102(4):333–9.
Saejung W, Fujiyama K, Takasaki T, Ito M, Hori K, Malasit P, et al. Production of dengue 2 envelope domain III in plant using TMV-based vector system. Vaccine 2007;25(36):6646–54.
Santi L, Huang Z, Mason H. Virus-like particles production in green plants. Methods 2006;40(1):66–76.
Schafer B, Holzer GW, Joachimsthaler A, Coulibaly S, Schwendinger M, Crowe BA, et al. Pre-clinical efficacy and safety of experimental vaccines based on non-replicating vaccinia vectors against yellow fever. PLoS One 2011;6(9):e24505.
Schalich J, Allison SL, Stiasny K, Mandl CW, Kunz C, Heinz FX. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J Virol 1996;70(7):4549–57.
Schodel F, Peterson D, Hughes J, Wirtz R, Milich D. Hybrid hepatitis B virus core antigen as a vaccine carrier moiety: I. Presentation of foreign epitopes. J Biotechnol 1996;44(1–3):91–6.
Scholle F, Girard YA, Zhao Q, Higgs S, Mason PW. Trans-packaged West Nile virus-like particles: infectious properties *in vitro* and in infected mosquito vectors. J Virol 2004;78(21):11605–14.
Seligman SJ, Gould EA. Live flavivirus vaccines: reasons for caution. Lancet 2004;363(9426):2073–5.
Shih SM, Doran PM. Foreign protein production using plant cell and organ cultures: advantages and limitations. Biotechnol Adv 2009;27(6):1036–42.
Sim AC, Lin W, Tan GK, Sim MS, Chow VT, Alonso S. Induction of neutralizing antibodies against dengue virus type 2 upon mucosal administration of a recombinant *Lactococcus lactis* strain expressing envelope domain III antigen. Vaccine 2008;26(9):1145–54.
Simmons M, Porter KR, Hayes CG, Vaughn DW, Putnak R. Characterization of antibody responses to combinations of a dengue virus type 2 DNA vaccine and two dengue virus type 2 protein vaccines in rhesus macaques. J Virol 2006;80(19):9577–85.
Smith ML, Mason HS, Shuler ML. Hepatitis B surface antigen (HBsAg) expression in plant cell culture: kinetics of antigen accumulation in batch culture and its intracellular form. Biotechnol Bioeng 2002;80(7):812–22.
Solomon T, Mallewa M. Dengue and other emerging flaviviruses. J Infect 2001;42(2):104–15.
Solomon T, Dung NM, Kneen R, Gainsborough M, Vaughn DW, Khanh VT. Japanese encephalitis. J Neurol Neurosurg Psychiatry 2000;68(4):405–15.
Spohn G, Jennings GT, Martina BE, Keller I, Beck M, Pumpens P, et al. A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. Virol J 2010;7:146.
Staples JE, Gershman M, Fischer M. Yellow fever vaccine. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 2010;59(RR-7):1-27.
Staropoli I, Frenkiel MP, Megret F, Deubel V. Affinity-purified dengue-2 virus envelope glycoprotein induces neutralizing antibodies and protective immunity in mice. Vaccine 1997;15(17–18):1946–54.
Stephenson JR. Understanding dengue pathogenesis: implications for vaccine design. Bull World Health Organ 2005;83(4):308–14.
Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 2007;5(1):2-15.
Su W. Bioreactor engineering for recombinant protein production using plant cell suspension culture. Plant Tissue Culture Engineering 2006;6:135–59.
Sugrue RJ, Cui T, Xu Q, Fu J, Chan YC. The production of recombinant dengue virus E protein using *Escherichia coli* and *Pichia pastoris*. J Virol Methods 1997a;69(1–2):159–69.
Sugrue RJ, Fu J, Howe J, Chan YC. Expression of the dengue virus structural proteins in *Pichia pastoris* leads to the generation of virus-like particles. J Gen Virol 1997b;78(Pt 8):1861–6.
Sun W, Edelman R, Kanesa-Thasan N, Eckels KH, Putnak JR, King AD, et al. Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. Am J Trop Med Hyg 2003;69(6 Suppl):24–31.
Sun W, Cunningham D, Wasserman SS, Perry J, Putnak JR, Eckels KH, et al. Phase 2 clinical trial of three formulations of tetravalent live-attenuated dengue vaccine in flavivirus-naive adults. Hum Vaccin 2009;5(1):33–40.
Sun E, Zhao J, Liu N, Yang T, Xu Q, Qin Y, et al. Comprehensive mapping of common immunodominant epitopes in the west Nile virus nonstructural protein 1 recognized by avian antibody responses. PLoS One 2012;7(2):e31434.
Teoh PG, Ooi AS, AbuBakar S, Othman RY. Virus-specific read-through codon preference affects infectivity of chimeric cucumber green mottle mosaic viruses displaying a dengue virus epitope. J Biomed Biotechnol 2009;2009:781712.
Theiler M, Smith HH. The use of Yellow Fever Virus modified by *in vitro* cultivation for human immunization. J Exp Med 1937;65(6):787–800.

Thisyakorn U, Nimmannitya S. Nutritional status of children with dengue hemorrhagic fever. Clin Infect Dis 1993;16(2):295–7.
Tiwari S, Verma PC, Singh PK, Tuli R. Plants as bioreactors for the production of vaccine antigens. Biotechnol Adv 2009;27(4):449–67.
Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: host systems and expression technology. Trends Biotechnol 2003;21(12): 570–8.
Vasconcelos PF, Luna EJ, Galler R, Silva LJ, Coimbra TL, Barros VL, et al. Serious adverse events associated with yellow fever 17DD vaccine in Brazil: a report of two cases. Lancet 2001;358(9276):91–7.
Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000;181(1):2–9.
Verma SK, Gupta N, Pattnaik P, Babu JP, Rao PV, Kumar S. Antibodies against refolded recombinant envelope protein (domain III) of Japanese encephalitis virus inhibit the JEV infection to Porcine Stable Kidney cells. Protein Pept Lett 2009;16(11): 1334–41.
Villa LL, Ault KA, Giuliano AR, Costa RL, Petta CA, Andrade RP, et al. Immunologic responses following administration of a vaccine targeting human papillomavirus Types 6, 11, 16, and 18. Vaccine 2006;24(27–28):5571–83.
Volk DE, May FJ, Gandham SH, Anderson A, Von Lindern JJ, Beasley DW, et al. Structure of yellow fever virus envelope protein domain III. Virology 2009;394(1):12–8.
Wang Y, Deng H, Zhang X, Xiao H, Jiang Y, Song Y, et al. Generation and immunogenicity of Japanese encephalitis virus envelope protein expressed in transgenic rice. Biochem Biophys Res Commun 2009;380(2):292–7.
Warzecha H, Mason HS, Lane C, Tryggvesson A, Rybicki E, Williamson AL, et al. Oral immunogenicity of human papillomavirus-like particles expressed in potato. J Virol 2003;77(16):8702–11.
Webster DP, Farrar J, Rowland-Jones S. Progress towards a dengue vaccine. Lancet Infect Dis 2009;9(11):678–87.
White NJ. Variation in virulence of dengue virus. Lancet 1999;354(9188):1401–2.
WHO. Japanese encephalitis virus vaccines. Wkly Epidemiol Rec 2006;81(34/35):325–40.
WHO. Guidelines for the clinical evaluation of dengue vaccines in endemic areas. Immunization, vaccines and biological, 2008, pp. 1–55.
Wiedermann U. Tick borne encephalitis TBE—vaccination in non-endemic countries. Travel Med Infect Dis 2010;8(4):251–6.
Wiggan O, Livengood JA, Silengo SJ, Kinney RM, Osorio JE, Huang CY, et al. Novel formulations enhance the thermal stability of live-attenuated flavivirus vaccines. Vaccine 2011;29(43):7456–62.
Williams KL, Zompi S, Beatty PR, Harris E. A mouse model for studying dengue virus pathogenesis and immune response. Ann N Y Acad Sci 2009;1171(Suppl. 1):E12–23.
Wu HH, Chen CT, Lin YL, Lee ST. Sub-fragments of the envelope gene are highly protective against the Japanese encephalitis virus lethal infection in DNA priming-protein boosting immunization strategies. Vaccine 2004;22(5–6):793–800.
Xu J, Dolan MC, Medrano G, Cramer CL, Weathers PJ. Green factory: Plants as bioproduction platforms for recombinant proteins. Biotechnol Adv 2012;30(5):1171–84.
Yoo JW, Irvine DJ, Discher DE, Mitragotri S. Bio-inspired, bioengineered and biomimetic drug delivery carriers. Nat Rev Drug Discov 2011;10(7):521–35.
Zent O, Schwarz TF, Plentz A, Banzhoff A, Jilg W. TBE booster immunization in adults—first experience with a new tick-borne encephalitis (TBE) vaccine, free of protein-derived stabilizer. Int J Med Microbiol 2004a;293(Suppl. 37):134–8.
Zent O, Wittermann C, Banzhoff A, Plentz A, Jilg W. New TBE vaccine formulations—free of proteineous stabilizer and preservatives. Overview of clinical studies and first year post-marketing experience. MMW Fortschr Med 2004b;146(3–4):43.
Zhang Y, Zhang W, Ogata S, Clements D, Strauss JH, Baker TS, et al. Conformational changes of the flavivirus E glycoprotein. Structure 2004;12(9):1607–18.
Zhang F, Ma W, Zhang L, Aasa-Chapman M, Zhang H. Expression of particulate-form of Japanese encephalitis virus envelope protein in a stably transfected Drosophila cell line. Virol J 2007a;4:17.
Zhang ZS, Yan YS, Weng YW, Huang HL, Li SQ, He S, et al. High-level expression of recombinant dengue virus type 2 envelope domain III protein and induction of neutralizing antibodies in BALB/C mice. J Virol Methods 2007b;143(2):125–31.
Zhang S, Liang M, Gu W, Li C, Miao F, Wang X, et al. Vaccination with dengue virus-like particles induces humoral and cellular immune responses in mice. Virol J 2011;8:333.