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Anderson Paul Kanagaraj

Dheeraj Verma

Henry Daniell
University of Pennsylvania

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At the time of publication, author Henry Daniell was affiliated with the University of Central Florida. Currently, he is a faculty member at the School of Dental Medicine at the University of Pennsylvania.

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Keywords

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Comments

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Expression of dengue-3 premembrane and envelope polyprotein in lettuce chloroplasts

Anderson Paul Kanagaraj, **Dheeraj Verma**, and **Henry Daniell**
Department of Molecular Biology and Microbiology, College of Medicine, University of Central Florida, 336 Biomolecular Science Building, Orlando, FL 32816-2364, USA

Abstract

Dengue is an acute febrile viral disease with >100 million infections occurring each year and more than half of the world population is at risk. Global resurgence of dengue in many urban centers of the tropics is a major concern. Therefore, development of a successful vaccine is urgently needed that is economical and provide long-lasting protection from dengue virus infections. In this manuscript, we report expression of dengue-3 serotype polyprotein (prM/E) consisting of part of capsid, complete premembrane (prM) and truncated envelope (E) protein in an edible crop lettuce. The dengue sequence was controlled by endogenous *Lactuca sativa psb*A regulatory elements. PCR and Southern blot analysis confirmed transgene integration into the lettuce chloroplast genome via homologous recombination at the *trn*I/*trn*A intergenic spacer region. Western blot analysis showed expression of polyprotein prM/E in different forms as monomers (~65 kDa) or possibly heterodimers (~130 kDa) or multimers. Multimers were solubilized into monomers using guanidine hydrochloride. Transplastomic lettuce plants expressing dengue prM/E vaccine antigens grew normally and transgenes were inherited in the T1 progeny without any segregation. Transmission electron microscopy showed the presence of virus-like particles of ~20 nm diameter in chloroplast extracts of transplastomic lettuce expressing prM/E proteins, but not in untransformed plants. The prM/E antigens expressed in lettuce chloroplasts should offer a potential source for investigating an oral Dengue vaccine.

Keywords

Chloroplast genetic engineering; Molecular farming; Plant-made biopharmaceuticals; Viral vaccine

Introduction

Dengue virus infections cause dengue fever, an acute febrile viral disease that affects infants, children and adults. Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) is a potentially lethal complication that can cause life-threatening internal bleeding, vomiting, severe abdominal pain and death. There is a global resurgence of epidemic dengue fever/dengue haemorrhagic fever (dengue/DHF) with the development of hyperendemicity in many urban centers of the tropics. A pandemic in 1998, in which 1.2 million cases of dengue fever and DHF were reported from 56 countries, was unprecedented. The World Health Organization estimates that globally 2.5 billion people live in areas where dengue viruses can be transmitted. There are around 100 million cases of dengue virus infection

Correspondence to: Henry Daniell.
daniell@mail.ucf.edu.

worldwide every year, resulting in approximately 500,000 cases of life-threatening DHF/DSS and at least 24,000 deaths each year, mainly among children. This disease occurrence is re-emerging globally and is considered a public health threat throughout the world (Gubler and Meltzer 1999; Gould and Solomon 2008; Morens and Fauci 2008; WHO 2009). Dengue is now also a leading cause of morbidity in American and European travelers and military personnel (Freedman et al. 2006). Therefore, dengue vaccines are urgently needed because the only method currently available to prevent dengue virus infections is the control of the main mosquito vector, but this approach is not very effective (Halstead 1988; Ooi et al. 2006).

There are four dengue strains genetically and serologically related but antigenically distinct serotypes named as DENV-1, DENV-2, DENV-3 and DENV-4 that cause dengue fever throughout the tropical and subtropical areas of the world. The proportion of dengue cases caused by each serotype changes dramatically between years (Guzman and Kouri 2002; Kyle and Harris 2008; Nagao and Koelle 2008). There is also substantial sequence divergence, greater than 30% among the four serotypes (Beaumier et al. 2008). Dengue virus, a category-A pathogen is unique among arboviruses because humans are the only known hosts that develop clinical symptoms after dengue virus infection and in case of lower primates the duration and magnitude of viremia is low (Brightmer and Fantato 1998). This deadly virus is transmitted from human to human through infected mosquito vectors, *Aedes aegypti* and *A. albopictus*. Dengue virus belongs to the family Flaviviridae, genus Flavivirus. It is an enveloped virus and has a single-stranded RNA genome of ~11 kb. The genome has three structural protein genes forming the capsid protein (C), premembrane protein (prM), envelope protein (E), seven non-structural protein genes that encode NS1-NS2A-NS2BNS3-NS4A-NS4B-NS5 and short non-coding regions on both the 5′ and 3′ ends (Chambers et al. 1990; Halstead 2002; Kuhn et al. 2002; Perera and Kuhn 2008). All of these proteins are expressed as a single polypeptide chain, which eventually undergoes cleavage and processing by host and viral proteases to produce individual proteins required for viral replication and packaging. Among these, domain III of envelope protein has type-specific and complex-reactive antigenic epitopes that are considered to be dominant neutralizing determinants (Roehrig 2003; Matsui et al. 2009).

Various strategies have been used to develop dengue vaccine candidates: attenuated, recombinant, subunit, chimeric and DNA vaccines (Whitehead et al. 2007; Blaney et al. 2008; Matsui et al. 2009; Wang et al. 2009; Wright et al. 2009). But, currently no licensed vaccines or anti-viral drugs against dengue are available, although vaccines for other flaviviruses such as yellow fever, Japanese encephalitis and tick-borne encephalitis have been developed (Barrett 2001; Webster et al. 2009; Noble et al. 2010). Therefore, development of a successful vaccine is urgently needed that is economical and provide protection from dengue virus infections. According to World Bank estimation, dengue vaccine's potential market value would be US $2.36 billion in the next 10 years (Callaway 2007) while pharmaceuticals giants like GenPhar and Sanofi-Aventis have estimated the market value of more than US $4 billion—21 billion in the coming years.

Dengue VLPs are considered to be very safe and effective subunit vaccine candidate antigens against all four serotypes. VLPs are non-infectious antigens and carry no infectious genetic material. Therefore, they cannot replicate on their own. The prM protein and E proteins self-assemble and form recombinant VLPs in several expression systems (Kelly et al. 2000; Konishi and Fujii 2002; Purdy and Chang 2005; Wang et al. 2009). In particular, the prM protein is important for stabilizing the functional structure of E protein during low pH exposure and also for retaining its antigenic properties (Wang et al. 2009). These structurally stable and versatile subviral particles possess excellent adjuvant properties for inducing strong cellular and humoral responses as direct immunogens (Scheerlinck and

Greenwood 2008). The prM/E protein complex mediates several important biological activities, which are essential for viral infectivity. In addition, several neutralizing epitopes which induce protective antibodies are located within the flaviviral E protein. The prM/E proteins are important antigens with high immunogenic properties and E protein is made of three distinct domains. Domain II contains cross-reactive epitopes and domain I and III contain subcomplex and type-specific epitopes. Domain III is an immunoglobulin (Ig)—like domain and it is a soluble protein when expressed separately as individual domain (Modis et al. 2004). The E protein binds to host cells through an unknown receptor and contains several serotype-specific neutralizing epitopes responsible for eliciting neutralizing antibodies. This is essential for the development of vaccination strategies against dengue virus and for use as a reagent in the rapid diagnosis of dengue virus infection (Sugrue et al. 1997; Lai et al. 2008). Also, prM is important for the processing of the E protein and is an ideal candidate for vaccines engineered by recombinant DNA technology. Dengue VLPs expressed from baculovirus, yeast, or mammalian cells are quite immunogenic, inducing both neutralizing antibody and partial or full protection in the mouse model from wild-type DENV challenge and induced neutralizing antibodies when challenged against infectious dengue virus (Kelly et al. 2000; Ocazionez Jimenez and Lopes da Fonseca 2000; Konishi and Fujii 2002; Durbin and Whitehead 2010). Therefore, in this study we investigated expression of the prM/E polyprotein in lettuce chloroplasts, which might be used for oral delivery to elicit immunogenic response against dengue virus infection. The C-terminally truncated E domain III protein of dengue serotype 2 (D2EIII) has been expressed in plant system via *Agrobacterium*-mediated transformation and TMV based vector system. In both cases, the expression level was 0.25–0.28% of total soluble protein (Saejung et al. 2007; Kim et al. 2009). The intramuscular immunization of mouse with plant-expressed D2EIII protein successfully induced the anti-dengue virus antibody production in mice. Moreover, the plant-D2EIII induced antisera have an efficient dengue serotype 2-specific neutralizing activity as determined by plaque reduction neutralization test (Saejung et al. 2007). Recently, Martínez et al. (2010) have reported expression of D2EIII envelope protein fused to hepatitis B core protein in *Nicotiana benthamiana* via nuclear genome transformation.

Oral delivery of plant-expressed vaccine antigens elicits sustained level of mucosal and systemic immune responses (Daniell et al. 2009). Foreign proteins expressed in chloroplasts are protected in the digestive tract and are efficiently delivered to the immune system (Limaye et al. 2006; Arlen et al. 2008; Davoodi-Semiromi et al. 2010; Verma et al. 2010). It is also cost effective, does not require sterile syringes and health professionals for vaccine administration and therefore, it is ideal for mass immunization programs, particularly for developing countries. High cost of therapeutic proteins and vaccines can be attributed to their production in fermentation-based system, expensive purification and processing methods, low-temperature storage, transportation, and sterile delivery using syringes through trained health professionals (Arlen et al. 2007; Verma and Daniell 2007; Chebolu and Daniell 2009; Daniell et al. 2009; Davoodi-Semiromi et al. 2009). Oral delivery of plant cells containing therapeutic proteins and antigens are protected in the stomach from acids/enzymes due to bio-encapsulation of the antigen by the plant cell wall and resulted in protective immune responses (Ruhlman et al. 2007; Arlen et al. 2008; Daniell et al. 2009; Davoodi-Semiromi et al. 2010; Verma et al. 2010). In this manuscript, we explore expression of the dengue virus 3 prM/E (DENV3prM/E) polyprotein as vaccine antigens candidate in an oral delivery system.

Experimental procedures

Chloroplast vector construction and regeneration of transplastomic plants

The primers were designed for amplification of dengue sequence (prM/E) with 96% identities to Dengue type 3 virus genome sequence (Accession No. M93130) from 365 to

2149 nucleotide encoding a polyprotein comprising of 24 amino acids of capsid protein (C), 166 amino acids of the premembrane protein (prM) and 405 amino acids of envelope protein (E). The dengue sequence was amplified with sequence specific primers (Forward 5′-GGAATTCCATATGCTGAGCATTATCAACAAA-3′ and Reverse 5′-GCTCTAGA**TCA**TCTGGCAGTGGCCTCGAACA-3′) containing flanking restriction sites (*Nde*I and *Xba*I, underlined) and stop codon in reverse primer (bold) using plasmid pDEN3 (kindly provided by Prof. Rofina Yasmin Othman and Ms. Lin Shina) as template. The amplified product was cloned into the pCR BluntII Topo vector (Invitrogen). The sequence was verified to check errors during the PCR amplification. The Den3 sequence was released by digestion with *Nde*I (complete) and *Xba*I (partial) and ligated into pDVI-1 vector (Ruhlman et al. 2010). The Den3 expression cassette was released by digestion with *Sal*I and *Not*I and ligated into the pLsDV vector (Ruhlman et al. 2010) resulting in pLsDVDENV3prM/E vector. All cloning steps to create chloroplast transformation vectors were performed in *Escherichia coli* using standard molecular biology protocols (Sambrook and Russell 2001).

Seeds of *Lactuca sativa* cv. Simpson elite (New England Seed Co.) were surface sterilized and germinated on MS media solidified with 6 g l^{-1} Phytablend® (Caisson). Fully expanded leaves from 21 day old in vitro plants were placed adaxial side up on antibiotic free modified lettuce regeneration medium (Ruhlman et al. 2010) for bombardment employing the biolistic device PDS1000/He. Gold particles (0.6 micron) coated with plasmid DNA (pLsDVDENV3prM/E) were bombarded into fully expanded leaves using 900 psi rupture disks and a target distance of 6 cm (Kumar and Daniell 2004; Ruhlman et al. 2010). Bombarded leaves were kept in the dark at 25° C for 2 days, cut into 1 cm^2 pieces and placed adaxial side down onto modified regeneration medium with 50 mg/l spectinomycin as described previously (Ruhlman et al. 2010). Spectinomycin-resistant shoots obtained after 4–6 weeks were screened by PCR for integration of selectable marker and DENV3prM/E expression cassette into the chloroplast genome. The PCR positive shoots were subjected to 2nd round of selection on the same selective medium. Regenerated shoots were transferred to half-strength MS solid medium containing 100 mg/l spectinomycin for rooting. The plantlets were then hardened in Jiffy peat pots and finally transferred to the greenhouse.

Transgene integration analysis by PCR and Southern hybridization

Genomic DNA isolated from primary transformants was analyzed by PCR using primer pairs 16SF/3M (16SF 5′-CAGCAGCCGCGGTAATACAGAGGA-3′; 3M 5′-CCGCGT TGTTTCATCAAGCCTTACG-3′) and 5P/2M (5P 5′-CTGTAGAAGTCACCATTGTTGTGC-3′; 2M 5′-TGACTGCCCAACCTGAGAGCGGACA-3′) as described earlier (Verma et al. 2008). Southern blot analysis was carried out to confirm site-specific transgene integration by homologous recombination and determine homoplasmy of transplastomic plants (Kumar and Daniell 2004). Genomic DNA was isolated from *L. sativa* leaves. Leaf material was powdered in chilled, sterile mortar and pestle with liquid N_2. Further steps of DNA isolation were carried out using a QIAGEN DNeasy® Plant mini kit following manufacturer's protocol. Five micrograms of total DNA was completely digested with *Bgl*II, the resulting fragments were separated on 0.8% (w/v) agarose gel and transferred to nylon membrane by capillary action. Chloroplast flanking sequence probe comprising the *trn*I gene, spacer region and *trn*A gene (102460–103501 nucleotide of *Lactuca sativa* chloroplast genome Accession No. NC_007578) was amplified by PCR from untransformed *L. sativa* genomic DNA. PCR products were column purified and Ready-To-Go™ DNA Labeling Beads (GE Healthcare) were used for probe labeling with α-32P-dCTP. Blots were pre-hybridized for 1 h at 68°C in QuikHyb® reagent (Stratagene, Cedar Creek, TX). Blots were hybridized for 1 h at 68°C, washed twice at room temperature in 2× SSC (0.3 M sodium chloride, 30 mM

sodium citrate, pH 7.0) and twice at 65°C in 0.1× SSC. Radiolabeled blots were exposed to autoradiography film.

Evaluation of transgene segregation

Homoplasmic shoots with well developed roots were transferred onto Jiffy-7® peat pellets for hardening. Hardened plants were transferred to the greenhouse for leaf material collection and seed harvest. T1 seeds thus collected were placed on MS medium containing 100 mg/l spectinomycin on one half of the plate and untransformed control lettuce seeds were placed on other half of the same petri-dish. Transgene segregation was studied according to Ruhlman et al. (2007). Several T1 seeds/plates were evaluated.

Western blot analysis

Lettuce transplastomic lines were grown in the greenhouse. Young (top four), mature (fully grown) and old leaves (bottom four) were collected at the same time from a single plant at 6 PM from 8 week old transplastomic plants. Leaf material was ground in liquid N_2 and stored at -80°C. Approximately 100 mg of leaf tissue was suspended in three volumes of protein extraction buffer (300 mM NaCl, 10 mM EDTA, 200 mM Tris–HCl pH 8, 0.5% Triton X-100, 100 mM DTT, 400 mM sucrose and Roche complete protease inhibitor) and vortexed vigorously for 20 min at 4°C prior to centrifugation at 10,000×*g* for 20 min. The supernatant was used for western analysis. Pellet was sonicated 3 times with 30 s pulse on ice in two volumes of 6M guanidine hydrochloride buffer with 100 mM NaH_2PO_4, 100 mM Tris–HCl, pH 8.0 and centrifuged at 10,000×*g* for 20 min at 4°C. Solubilized leaf protein from the pellet fraction was dialyzed against 100 mM NaH_2PO_4, 300 mM NaCl and 100 mM Tris–HCl, pH 8.0 at 4°C. Protein content was determined using Bio-Rad Protein Assay Reagent. Leaf proteins were separated by sodium dodecylsulphate -10% polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes for western blotting (Kumar and Daniell 2004; Verma et al. 2008). Immuno-blotting was carried out with polyclonal anti-dengue primary antibody (1:1000, catalog # ab9200, Abcam, CA) and HRP-conjugated goat anti-rabbit secondary antibody (1:3000, Southern Biotech, Birmingham, AL). A SuperSignal® West Pico HRP Substrate Kit (Pierce, Rockford, IL) was used for detection of chemiluminescence signal by exposure to autoradiography film.

Transmission electron microscopy studies of VLPs

Chloroplast isolation was carried out as described by Singh et al. (2008) with following modifications. Deveined mature leaf material (10 g) was taken from transplastomic and untransformed plants grown in large pots (10 inch) containing Miracle-Gro® Potting mix in growth chambers (16 h light/8 h dark photoperiod at 26°C). Leaf materials were briefly homogenized in a Waring blender containing 50 ml ice cold buffer (25 mM HEPES–KOH, pH 7.7, 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25% BSA, and 0.1% ascorbic acid). Homogenate was centrifuged at 1,000×*g* for 5 min after filtering it through 8 layers of muslin cloth. Isolated chloroplasts were briefly washed twice in PBS buffer (pH 7.5) containing 0.5 M NaCl, 2 mM EDTA and Roche complete protease inhibitor and vigorously homogenized in the same buffer with a Teflon homogenizer. Homogenized sample was vigorously shaken for 10 min at 4°C after 10 s of sonication and centrifuged at 10,000×*g* for 20 min at 4°C. Supernatant was filtered through 0.2 μm PES syringe filter and protein concentration was adjusted to 10 mg/ml by dilution in PBS buffer (pH 7.5) containing 0.5 M NaCl, 2 mM EDTA and Roche complete protease inhibitor.

For electron microscopy, 20 μl protein sample was placed on carbon-coated copper grids, 400 mesh (Electron Microscopy Sciences, Hatfield, PA, USA) and allowed to dry for 30 min. Grids were negatively stained with 2% uranyl acetate for 1 min and excess liquid was dried with filter paper. The samples were observed under JEOL 1011 transmission electron

microscope at 80 kV and images were captured using AMT Image Capture Engine software (Advanced Microscopy Techniques, Corp. Danvers, MA, USA).

Results

Chloroplast vector construction and regeneration of transplastomic lines

The lettuce chloroplast transformation vector pLsDVDENV3prM/E (Fig. 1a) was constructed based on pLsDV vector (Ruhlman et al. 2010) that targets the expression cassette into the spacer region between the *trn*I and *trn*A genes (Fig. 1b) of the chloroplast genome for integration via homologous recombination (Daniell, 1997). The *aad*A gene for spectinomycin resistance was driven by the constitutive endogenous plastid ribosomal operon promoter (P*rrn*) with GGAG ribosome binding site and facilitated selection of transplastomic lines. Our dengue serotype 3 sequence (DENV3prM/E) has 96% nucleotide identities to Dengue 3 reference strain H87 (Osatomi and Sumiyoshi, 1990) and encoded a polyprotein comprising of 24 amino acids of capsid protein (C), 166 amino acids of the premembrane protein (prM) and 405 amino acids of envelope protein (E). In addition, the E protein lacked carboxy-terminal hydrophobic membrane anchor region in an effort to maximize the solubility of the protein. The truncated E protein has been shown to be more immunogenic than full length E protein (Men et al. 1991). The DENV3prM/E sequence was regulated by the lettuce *psb*A promoter, 5′ untranslated region (UTR) and 3′ UTR. The use of endogenous *psb*A promoter, 5′ UTR and *psb*A 3′ UTR have been shown to enhance significant level of foreign protein expression (up to 72% tsp) in transplastomic plants (Ruhlman et al. 2010). Therefore, endogenous lettuce *psb*A regulatory elements were used to regulate expression of the prM/E genes. Seven lettuce (*Lactuca sativa* cv. Simpson Elite) shoots were obtained from forty bombardments following Ruhlman et al. (2010) protocol. The *L. sativa* cv. Simpson Elite was used for developing transplastomic plants as it showed excellent regeneration ability without any callus formation when compared to other commercial cultivars (Ruhlman et al. 2010). Spectinomycin-resistant shoots were obtained within 4–5 weeks after bombardment and were screened by PCR for site-specific transgene integration.

Determination of site-specific transgene integration and homoplasmy

Site-specific integration of the transgenes into the lettuce chloroplast genome was confirmed by PCR analysis using primer pair 16SF/3M. The 16SF primer anneal to the native chloroplast genome sequence upstream of the 16S *trn*I flanking sequence, which is absent in the lettuce transformation vector. The 3M primer anneals to the *aad*A gene, which is located within the gene cassette (Fig. 1a). PCR reaction with 16SF/3M primers generated a 2.8 kb PCR product in transplastomic lines (Fig. 2a, lanes 1–5), which could be obtained only when site-specific integration of transgenes occurred. Untransformed plants did not show any PCR product as 3M primer will not anneal (Fig. 2a, lane UT). Integration of the *aad*A and DENV3prM/E gene cassette was verified by using 5P and 2M primer pairs for PCR analysis. The 5P primer anneals to the *aad*A gene whereas the 2M primer anneals to the *trn*A coding sequence (Fig. 1a). The use of 5P/2M primer pair generated a PCR product of 3.8 kb in the transplastomic lines whereas untransformed plant did not show any product (Fig. 2b). The presence of the 2.8 and 3.8 kb PCR products confirmed site-specific integration of the transgene cassette into the chloroplast genome (Fig. 2a, b). After PCR analysis, transplastomic plants were moved to additional two rounds of selection (second and third) to achieve homoplasmy.

Southern hybridization was carried out to further confirm site-specific integration of transgenes and to determine homoplasmy, using α-32P labeled flanking sequence probe (Fig. 1b). In the lettuce chloroplast genome (GenBank Accession No. DQ383816,), *Bgl*II

restriction sites are present within the intergenic space between *rps*7 and *ycf*15 and in the *trn*A gene (Fig. 1b). Therefore, a 3.8 and 7.4 kb fragment was expected for untransformed and transplastomic plants respectively (Fig. 1) when genomic DNA was digested with *Bgl*II and hybridized with the flanking sequence probe. Hybridization of α-32P random primer labeled 1.04 kb *trn*I-*trn*A flanking probe generated a 3.8 kb fragment in untransformed plants whereas a 7.4 kb fragment was detected in transplastomic plants, as expected (Fig. 2c). Absence of a 3.8 kb fragment in transplastomic lines confirmed homoplasmy (integration into all chloroplast genomes) and stable integration of foreign genes into the chloroplast genome. Out of seven transplastomic lines obtained, five independent homoplasmic lines were fully characterized. Homoplasmic transplastomic lines with roots were transferred to Jiffy-7® peat pellets and kept under high humidity initially for 2 weeks in a growth chamber, before plants were moved to the greenhouse.

Seeds (T1) were collected from green house grown lettuce transplastomic plants expressing DENV3prM/E. Seeds were surface sterilized and spread on spectinomycin containing medium (100 mg/l) along with untransformed control seeds. DENV3prM/E expressing plants germinated on the selection medium and remained green whereas untransformed plants showed stunted root growth, started bleaching and died (Fig. 3a). The absence of Mendelian segregation of transgenes indicated that they are maternally inherited to progeny. In addition, all T1 seeds/plate placed on selection medium plates germinated well and remained green. Growth and development of transplastomic lines was similar to untransformed plants (Fig. 3b). Transplastomic plants flowered and set fertile seeds.

Immunoblot analysis of dengue prM/E vaccine antigens expression in lettuce chloroplasts

Immunoblots of leaf extract from transplastomic plants probed with anti-dengue primary polyclonal antibody revealed the presence of ~65 kDa protein which is the expected molecular mass of expressed prM/E polyprotein (Fig. 4). Untransformed leaf protein extract did not react with anti-dengue polyclonal antibody. No Coomassie-stained bands corresponding to the immunodetected prM/E protein was detected in stained gels of leaf extracts. The expression levels of prM/E vaccine antigens were examined at 6 PM, which is the time of maximal expression of several foreign proteins expressed in lettuce or tobacco chloroplasts, when controlled by *psb*A regulatory elements (Ruhlman et al. 2010; Verma et al. 2010). Maximum expression of prM/E protein depended on the developmental stage of the leaves, and was greater in mature and old leaves than in young leaves. We observed additional protein bands that cross reacted with the antibody in transplastomic leaves but not in untransformed plants. In young plants, predominantly single band was observed (Fig. 4a). In mature or old leaves, smaller immunoreactive prM/E proteins were detected. These might have been formed by cleavage of the full length protein or by premature termination of translation following a U-rich region ~158 codons upstream of the stop codon. Additional polypeptide bands that appear above 130 kDa could be due to heterodimers or multimer protein complexes of prM/E (Fig. 4a). When the pellet fraction was extracted in 6M guanidine hydrochloride buffer and dialyzed in nondenaturing conditions, most of the prM/E protein complexed into multimers in old leaves whereas in mature leaves a polypeptide corresponding to the monomer of prM/E and also an oligomeric form of prM/E appeared in western blots (Fig. 4b).

Assembly of virus-like particles

The possible assembly of the prM/E protein into higher-order structures was examined by transmission electron microscopy of negatively-stained soluble extracts of isolated chloroplasts. Structures resembling virus-like particles (VLPs) were observed in extracts of chloroplasts from transplastomic lettuce plants expressing prM/E protein (Fig. 5), but not in extracts of chloroplasts from untrans-formed lettuce leaves. Most of the VLPs formed

aggregates of three or more sub-viral particles and also they overlapped with each other in our sample preparations (Fig. 5a, b). Some individual virus-like particles formed distinct spherical structures of ~20 nm diameter (Fig. 5c).

Discussion

Although transplastomic systems were developed for few edible crops (Ruf et al. 2001; Kumar et al. 2004; Lelivelt et al. 2005; Kanamoto et al. 2006), only the lettuce system has progressed further for expression of several therapeutic proteins suitable for oral delivery of autoantigens or vaccine antigens against bacterial, viral or protozoan pathogens. Expression of therapeutic proteins and vaccine antigens in an edible leafy vegetable like lettuce should lead to development of an efficient cost-effective system for oral delivery of therapeutic proteins and vaccine antigens (Ruhlman et al. 2007; Davoodi-Semiromi et al. 2010; Ruhlman et al. 2010). In this manuscript, we report expression of the prM/E dengue vaccine candidate antigens in lettuce chloroplasts. We used endogenous *psb*A regulatory elements to attain higher levels of expression of prM/E. Because of the non-availability of protein standards, expression levels of prM/E in transplastomic lettuce plants could not be quantified. However, the use of freeze-dried leaf material, rather than fresh leaf material, would significantly increase the amount of the prM/E protein that could be administered by oral immunization. Lettuce leaves contain >95% water. Dehydration to >98% should result in 50-fold increase in antigen concentration. If dehydration to 99% is accomplished as required by FDA for prolonged storage, then there would be a 100-fold increase in antigen concentration. The exploitation of lettuce-specific regulatory elements has led to the accumulation of several gene products to high levels in transplastomic lettuce (Davoodi-Semiromi et al. 2010; Ruhlman et al. 2010).

Even though dengue soluble E protein alone is sufficient for cell binding and elicit neutralization antibodies, several other studies show that prM/M complex is necessary for formation of VLPs, proper transport, stabilization of epitope on E protein and native folding in the acidic environment (Fonseca et al. 1994; Wang et al. 1999). In tick-borne encephalitis (TBE), a virus closely related to dengue, Lorenz et al. (2002) showed that the E protein needs prM to achieve its final native conformation as long as both proteins are available in the lysate. Although complete folding of prM appeared to be possible in the absence of E protein, interaction with prM and the formation of a hetero-oligomeric complex was required for E protein to attain its final conformation. The prM and E proteins should form hetero-oligomeric complexes before they could reach their final conformations, but it was not necessary for them to be synthesized from the same polyprotein precursor (Lorenz et al. 2002). The pr propeptide has three disulfide bonds (C34–C68, C45–C80, and C53–C66) that stabilize the pr peptide structure (Li et al. 2008). The E protein is able to switch among different oligomeric states: as a trimer of prM-E hetero-dimers in immature particles, as a dimer in mature virus, and as a trimer when fused with a host cell (Modis et al. 2004). In many studies that involved inoculation of recombinant prM/E in the BALB/c mice or monkey model produced sufficient neutralizing antibodies and haemagglutination inhibition when challenged with antibodies (Fonseca et al. 1994; Blair et al. 2006; De Paula et al. 2008; Suzuki et al. 2009; Liu et al. 2010).

The presence of VLPs in soluble extracts of lettuce chloroplasts suggests that the prM/E proteins are properly folded and able to assemble into higher order structures. VLPs observed in our study structurally resembled other previously reported flavivirus VLPs. VLPs have the ability to stimulate B-cell-mediated immune responses and also have been shown to be highly effective in stimulating CD4 proliferative responses and cytotoxic T lymphocyte (CTL) responses. In addition to this, VLPs enhance uptake by dendritic cells (DC) via macropinocytosis and endocytosis that play a central role in activating innate and

adaptive immune responses. VLPs can functionally display heterologous molecules, conformational epitopes more like a native virus and thus enhance the production of neutralizing antibodies (Noad and Roy 2003; Grgacic and Anderson 2006). Immunogenicity studies in mice have shown that even low doses (0.3–2.5 μg) of purified VLPs induce significant amount of strong immune responses against many viral diseases (Noad and Roy 2003; Kang et al. 2009; D'Aoust et al. 2010). VLPs serve as building blocks of viral nanomaterials for novel nanoparticle biotechnology applications (Manchester and Steinmetz 2009). Currently there are only two licensed viral recombinant vaccines for preventing hepatitis B virus (HBV) and human papillomo virus (HPV) in the market as VLP systems (Grgacic and Anderson 2006). VLPs have been expressed via the plant nuclear genome and are shown to be effective upon delivery (Walmsley and Arntzen 2000; Huang et al. 2005; Saldana et al. 2006; Santi et al. 2006, 2008). The L1 genes with amino acid substitution at H202D of human papillomavirus type 16 (HPV16) expressed in insect cells via recombinant baculoviruses formed biologically active VLPs (Kirnbauer et al. 1993). When L1 gene was expressed in chloroplasts, it self assembled into VLPs suggesting no requirement of endoplasmic reticulum processing for VLPs formation and was highly immunogenic (Fernández-San Millán et al. 2008; Lenzi et al. 2008). Therefore, lettuce plant expressing DENV3prM/E vaccine antigens in the form of VLPs is anticipated to produce sufficient quantities of neutralizing antibodies as well as other immunogenic properties for protection against dengue virus infection.

It has been demonstrated that chloroplast-derived therapeutic proteins and vaccine antigens were effectively presented to the gut-associated lymphoid tissue (GALT) where 70% of body's immune system is present. GALT found in lining of small intestines also has dense population of immune cells such as T and B lymphocytes, intestinal Peyer's patch M cells and also gastrointestinal antigen-presenting cells. The mucosal immune system can induce secretory IgA and serum IgG responses to provide two layers of defense against mucosal pathogens. GALT also contains both the tissue-dependent and tissue-independent IgA components (Brayden et al. 2005; Takahashi et al. 2009). So it is possible to tap full power of body's primary immune response. Several labs are developing tetravalent vaccines in order to effectively neutralize all four dengue serotypes and also for overcoming cytotoxicity effect that arise due to antibody-dependent enhancement of dengue virus disease (Halstead 2003; Whitehead et al. 2007; Senior 2009; Clements et al. 2010). Therefore, expression of prM/E protein in an edible leafy vegetable would serve as a model system for investigating effectiveness of orally delivered vaccine for neutralizing dengue-3 serotype and subsequently for development of an economically affordable tetravalent vaccine for all four serotypes.

Acknowledgments

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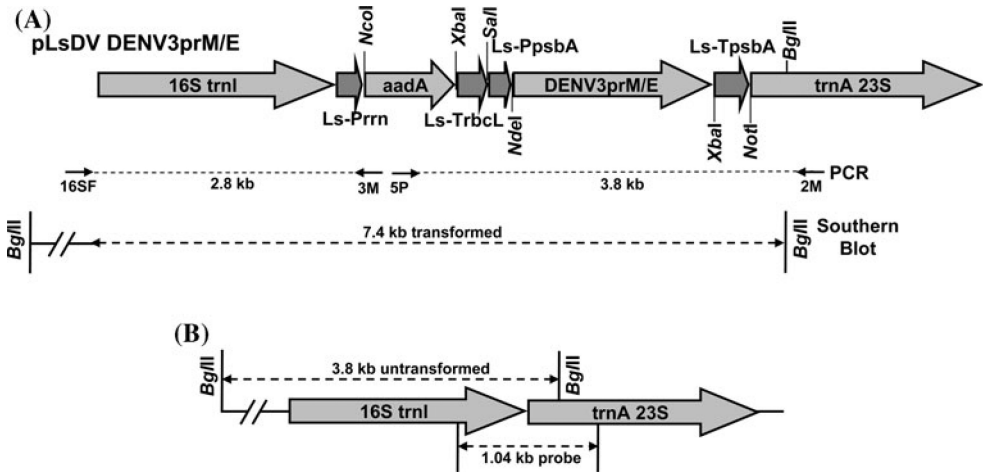

Fig. 1.

Schematic representation of chloroplast transformation vector and transgene integration. **a** Schematic representation of chloroplast transformation vector pLsDV DENV3prM, primer annealing sites (16SF/3M and 5P/2M) with expected DNA fragment size for PCR and restriction sites with expected DNA fragment size for Southern blot analysis. 16S trnI, *Lactuca sativa* flanking sequence containing 16S rRNA and *trn*I for site-specific homologous recombination; Ls-Prrn, *L. sativa* rRNA operon promoter; *aad*A, aminoglycoside 3′-adenylyltransferase gene for selection of transplastomic shoots; Ls-TrbcL, *L. sativa rbc*L 3′ untranslated region; Ls-PpsbA, *L. sativa psb*A promoter and 5′ untranslated region of the *psb*A gene; DENV3prM/E, dengue serotype 3 sequence consisting of part of capsid, complete premembrane (prM) and truncated envelope (E) gene; Ls-TpsbA, *L. sativa psb*A 3′ untranslated region; trnA 23S, *L. sativa* long flanking sequence of *trn*A and 23S rRNA for site-specific homologous recombination. (**b**) Schematic representation of the lettuce chloroplast flanking sequence used for homologous recombination. The untransformed chloroplast genome yields 3.8 kb and transplastomic plants yields 7.4 kb fragment when digested with *Bgl*II and hybridized with 1.04 kb probe

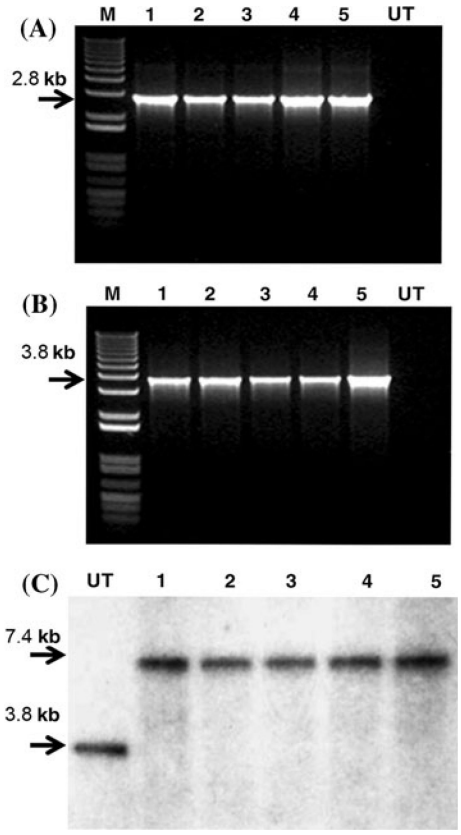

Fig. 2.

PCR analysis and Southern hybridization of transplastomic *L. sativa* plants. **a** Screening of spectinomycin-resistant shoots with 16SF (*forward*) and 3M (*reverse*) primer pair. M, 1 kb plus DNA marker; *lanes 1–5* transplastomic plants; *UT* untransformed. **b** Screening of spectinomycin-resistant shoots with 5P (*forward*) and 2M (*reverse*) primer pair. M, 1 kb plus DNA marker; *lanes 1–5* transplastomic plants; UT, untransformed. **c** Southern hybridization. For Southern hybridization, genomic DNA isolated from each individual plant was digested with *Bgl*II followed by hybridization with *trn*I-*trn*A flanking sequence probe that generated 3.8 kb fragment for untransformed and 7.4 kb fragment for transplastomic lines. *UT* untransformed; *lanes 1–5* transplastomic plants

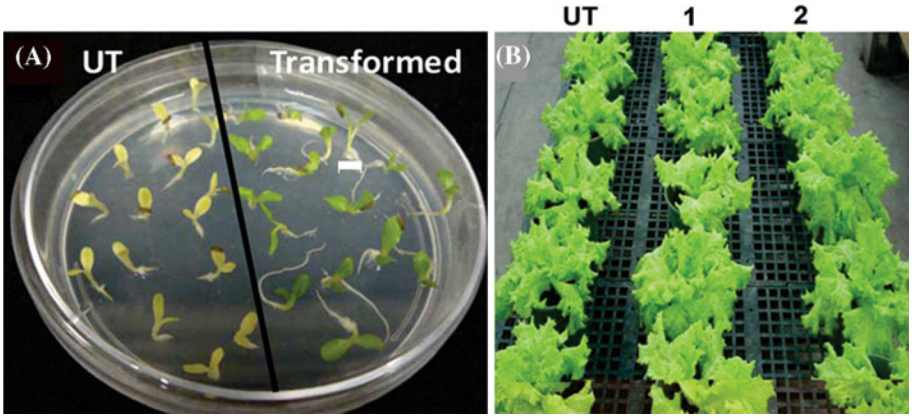

Fig. 3.
Transplastomic *L. sativa* lines expressing dengue 3 prM/E protein in the greenhouse and evaluation of transgene segregation. **a** Untransformed and T1 seeds were plated on half-strength Murashige and Skoog (*MS*) medium containing 100 mg/l spectinomycin. **b** Lettuce T1 plants expressing DENV3prM/E polyprotein and untransformed plants growing in the green house. *UT* untransformed; 1 and 2, transplastomic lines

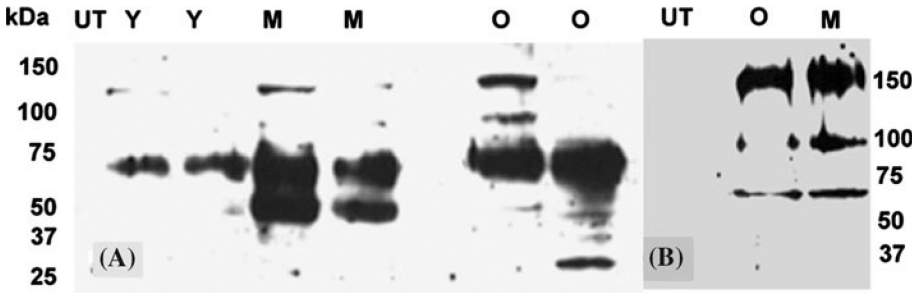

Fig. 4.

Western blot analysis of DENV3prM/E expressing plants. Total leaf protein extract (20 μg) extracted from lettuce leaves was loaded into wells for each sample and dengue-3 polyclonal antibody was used for detection. **a** Western blot analysis of young, mature and old leaves from 8 week old transplastomic plants. *UT* Untrans-formed plant extract; *Y* young; *M* mature; *O* old leaf extracts from transplastomic plants. **b** Western blot analysis of solubilized protein from the pellet fraction. Refolding of solubilized proteins extracted from pellet fraction was carried out by dialysis in the buffer containing 100 mM NaH_2PO_4, 300 mM NaCl and 100 mM Tris–HCl, pH 8.0 at 4°C for overnight and 20 μg extracted protein was loaded in each lane. *UT* untransformed; *O* old leaves; *M* mature leaves

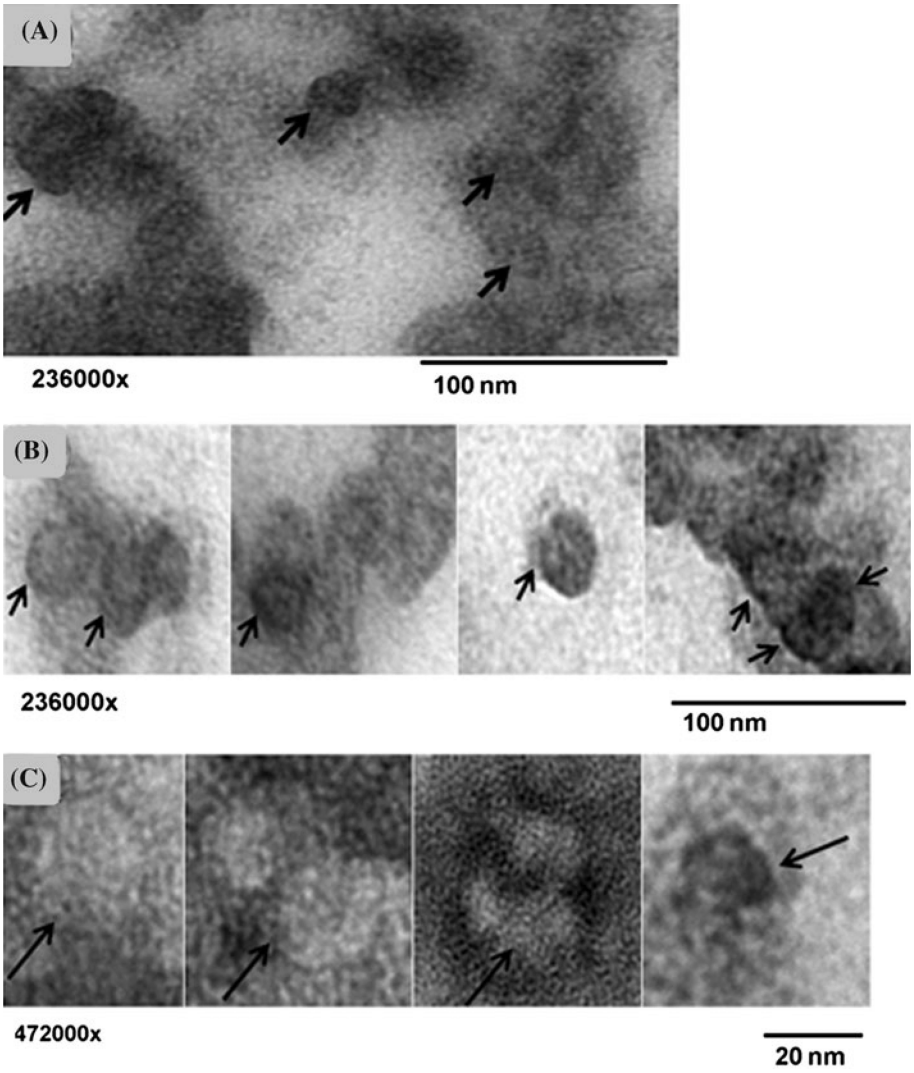

Fig. 5.

Transmission electron microscopy of negatively stained VLPs isolated from intact chloroplasts. **a**–**b** VLPs mostly aggregated and overlapped with each other (236,000×). **c** Individual VLP appeared in distinct spherical structures of ~20 nm (472,000×). Individual VLPs are indicated by *arrows*