





# Hepatitis C virus E2 envelope glycoprotein produced in *Nicotiana benthamiana* triggers humoral response with virus-neutralizing activity in vaccinated mice

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Received 23 October 2020;

revised 27 April 2021;

accepted 13 May 2021.

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## Summary

Chronic infection with hepatitis C virus (HCV) remains a leading cause of liver-related pathologies and a global health problem, currently affecting more than 71 million people worldwide. The development of a prophylactic vaccine is much needed to complement the effective antiviral treatment available and achieve HCV eradication. Current strategies focus on increasing the immunogenicity of the HCV envelope glycoprotein E2, the major target of virus-neutralizing antibodies, by testing various expression systems or manipulating the protein conformation and the N-glycosylation pattern. Here we report the first evidence of successful production of the full-length HCV E2 glycoprotein in *Nicotiana benthamiana*, by using the *Agrobacterium*-mediated transient expression technology. Molecular and functional analysis showed that the viral protein was correctly processed in plant cells and achieved the native folding required for binding to CD81, one of the HCV receptors. N-glycan analysis of HCV-E2 produced in *N. benthamiana* and mammalian cells indicated host-specific trimming of mannose residues and possibly, protein trafficking. Notably, the plant-derived viral antigen triggered a significant immune response in vaccinated mice, characterized by the presence of antibodies with HCV-neutralizing activity. Together, our study demonstrates that *N. benthamiana* is a viable alternative to costly mammalian cell cultures for the expression of complex viral antigens and supports the use of plants as cost-effective production platforms for the development of HCV vaccines.

**Keywords:** plant biopharmaceuticals, HCV vaccine, E2 glycoprotein, N-glycosylation, HCV-neutralizing antibodies.

## Introduction

Hepatitis C Virus (HCV), a Hepacivirus member of the *Flaviviridae* family of enveloped RNA viruses, is a major human pathogen, currently affecting more than 71 million individuals worldwide (WHO, 2019). Chronic infection leads to severe liver damage, cirrhosis, and hepatocellular carcinoma (HCC) and eventually death (Levrero, 2006). The new curative, all-oral direct-acting antivirals (DAAs) treatment for HCV have been met with enthusiasm due to their simplicity of administration, efficiency in more than 90% of treated patients and cross-genotypic effect (Gonzalez-Grande et al., 2016; Pawlotsky, 2014). Despite these remarkable results, the DAAs therapy is still costly, which makes it less accessible in low-income countries confronted with highest HCV infection rates, and does not confer immunity to patients who have cleared the pathogen. The development of a prophylactic vaccine would not only fill these gaps, but would clearly complement current treatments towards the ultimate goal of HCV eradication. Due to the high mutation rate of the viral RNA genome, the production of an efficient HCV vaccine remains

challenging. Nevertheless, accumulating evidence indicates that a strong humoral immune response inducing virus-neutralizing antibodies (nAbs) early in infection, contributes significantly to HCV clearance (Osburn et al., 2014; Walker et al., 2019). Most of these nAbs are directed against the E2 glycoprotein, one of the two HCV surface proteins spanning the lipid bilayer of the viral envelope (Pierce et al., 2016), making this protein a promising B cell-based vaccine candidate. Therefore, many experimental strategies have focussed on increasing the E2 glycoprotein immunogenicity and its ability to elicit cross-genotype nAbs. These approaches include recombinant expression in various hosts, removal of hypervariable domains, truncations, and manipulation of the redox status or of the N-glycan pattern (Fenouillet et al., 2008; Heile et al., 2000; Urbanowicz et al., 2019).

We have recently reported the successful production of an immunogenic HCV E1E2 heterodimer in *Lactuca sativa*, demonstrating the feasibility of using plants as expression systems for complex transmembrane glycoproteins, whose synthesis requires extensive co- and post-translational maturation steps (Clarke et al., 2017). While plant cells possess a similar protein folding

machinery and have the ability to carry out similar post-translational modifications as mammalian cells, the production of recombinant proteins in plants is more cost-effective, easier to scale up and not prone to contamination with potentially harmful animal pathogens (Bock, 2015; Chan and Daniell, 2015; Chan et al., 2016; Clarke and Daniell, 2011). Successful production of human therapeutics such as the ZMapp™ cocktail of anti-Ebola antibodies, which proved its clinical utility during the recent Ebola outbreak, has illustrated the potential of plant molecular farming (Qiu et al., 2014).

In this study, we have explored the potential of *N. benthamiana* as a suitable host for the production of the HCV E2 glycoprotein, the current HCV vaccine candidate, by using our established agroinfiltration facility (Clarke et al., 2017; van Eerde et al., 2020). Production of biopharmaceuticals in *Nicotiana* (tobacco) species has several key advantages over other plant-based expression systems. As tobacco is a non-food, non-feed crop, the regulatory requirements for the production of recombinant proteins are less cumbersome (Schillberg et al., 2019). In addition, the vacuum infiltration-based transient expression system permits recombinant protein accumulation exclusively in the leaves (thus preventing expression in reproductive tissues). Plants are safely discarded after harvest of the leafy biomass, and in this way, unwanted seed-based transgene dispersal can be excluded (Clarke et al., 2017).

Our work provides the first evidence of efficient production of the HCV E2 glycoprotein in *N. benthamiana*. We show that the viral protein is correctly processed in plant cells and acquires the native conformation required for specific binding to CD81, a critical receptor involved in HCV entry into hepatocytes (Feneant et al., 2014; Pileri et al., 1998). Interestingly, sequencing of the E2 N-linked glycans revealed subtle differences in mannose trimming between mammalian and plant-expressed protein. Mice vaccination with the E2 antigen purified from *N. benthamiana* leaves triggered a robust systemic immune response characterized by the early presence of anti-HCV IgM antibodies followed by isotype maturation to IgG. Most importantly, the presence of nAbs against the homologous HCV isolate was demonstrated, supporting the deployment of plants as a cost-effective production platform for HCV vaccines.

## Results

### Production of the HCV-E2 antigen in *N. benthamiana* and HEK 293T

To investigate whether *N. benthamiana* is a suitable host for the production of the HCV E2 glycoprotein, a vector containing the E2 antigen for use in transient *Agrobacterium*-mediated expression was designed (Figure 1a). As a reference system for the plant-produced E2, the viral glycoprotein was also produced in HEK293T cells. During infection, the viral genome is translated into a single polyprotein that is processed by cellular and viral proteases into individual structural and non-structural proteins (Penin et al., 2004; Reed and Rice, 2000). The E1 and E2 envelope proteins are translocated across the endoplasmic reticulum (ER), where the C-terminal part of E1 forms a transmembrane helix enabling initiation of E2 translocation. Both E1 and E2 are then released from the polyprotein following cleavage by the ER-resident signal peptidase (Cocquerel et al., 2000). Therefore, to ensure proper ER localization of HCV E2 when expressed alone, the coding sequence of E2 was preceded by the last 32 codons of E1 in our

expression vector. This preceding sequence was designed to act as a signal sequence and is expected to be removed by processing by the host signal peptidase, to produce mature full-length E2.

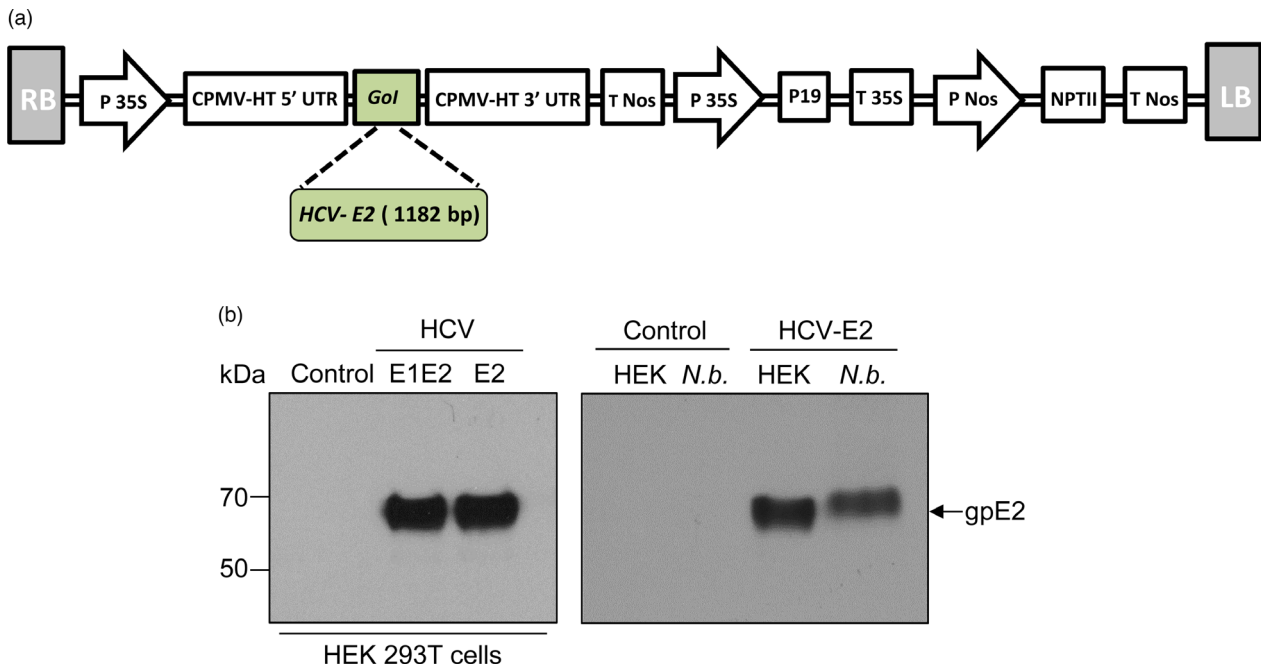
Indeed, HEK 293T cell transfection with plasmids encoding either the E1E2 polyprotein (pHCMVdeltaC-E1E2) or the N-terminally extended E2 (pcDNA3-E2), followed by immunoblotting showed a band at the predicted molecular weight of the fully-glycosylated, mature E2 protein of about 70 kDa (Shimotohno et al., 1995) (Figure 1b, left panel). The sequence coding for the N-terminally extended E2 was therefore introduced into a plant expression vector of the pEAQ series (Figure 1a), and used in agroinfiltration of *N. benthamiana* leaves. A side-by-side immunoblot analysis of agroinfiltrated *N. benthamiana* leaves and transfected HEK293T cell extracts revealed a slightly lower electrophoretic mobility of HCV-E2 produced in the former system (Figure 1b, right panel). The strong signal detected in the tobacco system suggests that the E2 protein alone can be successfully produced in *N. benthamiana* using this expression strategy.

### Molecular characterization of the HCV-E2 antigen produced in *N. benthamiana* leaves

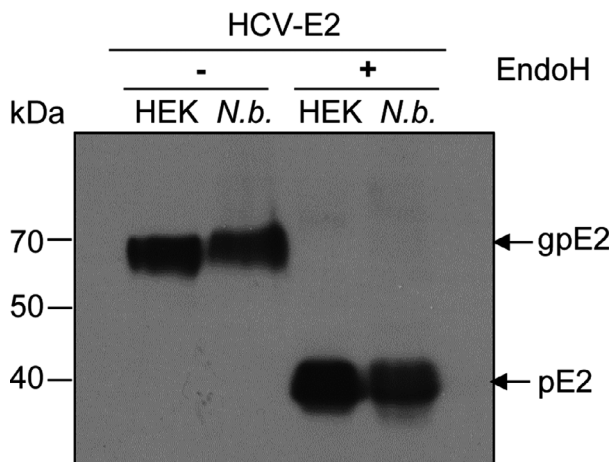
The observed difference in the electrophoretic mobility of the E2 protein between the two expression systems (Figure 1b, right panel) could be the result of different N-glycosylation/N-glycan processing or, alternatively, impaired processing of the E2 signal sequence in plant cells. To distinguish between these possibilities, mammalian and plant samples were subjected to Endo H digestion prior to western blot analysis (Figure 2). This enzyme cleaves between the first two N-acetylglucosamine residues of N-linked high-mannose type oligosaccharides and is often used to monitor protein trafficking along the secretory pathway (Freeze and Kranz, 2010). Endo H digestion generated a band of ~40 kDa, the expected molecular weight of the E2 polypeptide, in both samples. This finding indicates faithful removal of the ER signal sequence from E2 also in the tobacco expression system (Figure 2, + samples). Susceptibility to Endo H hydrolysis also points to an ER-specific, high-mannose glycosylation pattern, as this endoglycosidase does not remove mammalian or plant-specific complex N-linked sugars following their transformation in Golgi. This pattern is the result of sequential trimming of the Man<sub>9</sub>GlcNAc<sub>2</sub> glycan precursor attached to the proteins by the ER-resident  $\alpha$ 1,2-mannosidases, generating smaller N-glycans that contain five to eight mannose residues. Upon leaving the ER, these glycans are further trimmed to complex structures by the ER-Golgi Intermediate Compartment (ERGIC) and Golgi glycosyltransferases (Schwarz and Aebi, 2011). HCV E2 is a highly glycosylated protein, with 11 N-linked glycans accounting for about half of its molecular weight (Goffard et al., 2005). Therefore, the E2 mobility observed upon SDS-PAGE is likely the consequence of mannose residues being trimmed with different efficiency in mammalian as compared to plant cells, a hypothesis that we investigated further by performing N-glycan sequencing.

### Purification of the HCV-E2 antigen from *N. benthamiana*

The E2 protein was purified from plant leaf extracts by GNA affinity chromatography (Figure 3a), followed by size-exclusion chromatography (SEC). The electrophoretically separated proteins in the plant extract are also shown by Coomassie staining in Figure 3a (left panel). Fractions containing the antigen were eluted from SEC as a major peak, as shown by SDS-PAGE under



**Figure 1** Production of the HCV-E2 antigen in HEK392T and *Nicotiana benthamiana*. (a) Schematic representation of the T-DNA of the pEAQ-HT-DEST1-derived vector for HCV-E2 expression in *N. benthamiana*. Gol, gene of interest (i.e. the E2 coding region preceded by the last 32 E1 codons). RB/LB, right and left borders; 5' UTR and 3' UTR, 5' and 3' untranslated regions derived from Cowpea mosaic virus (CPMV); P 35S, Cauliflower mosaic virus (CaMV) 35S promoter; T 35S, CaMV 35S terminator; P Nos, nopaline synthase promoter; T Nos, nopaline synthase terminator; P19, suppressor of gene silencing; NPT II, neomycin phosphotransferase II gene conferring kanamycin resistance. (b) Lysates from HEK293T cells expressing HCV-E2 resulted from processing of the E1E2 polypeptide or the HCV-E2 protein (left panel), and lysates from HEK293T cells (HEK) or *N. benthamiana* leaves (*N.b.*) expressing HCV-E2 or the control vector (right panel), harvested at 7 dpi were subjected to immunoblotting and detection with anti-E2 3/11 Abs.

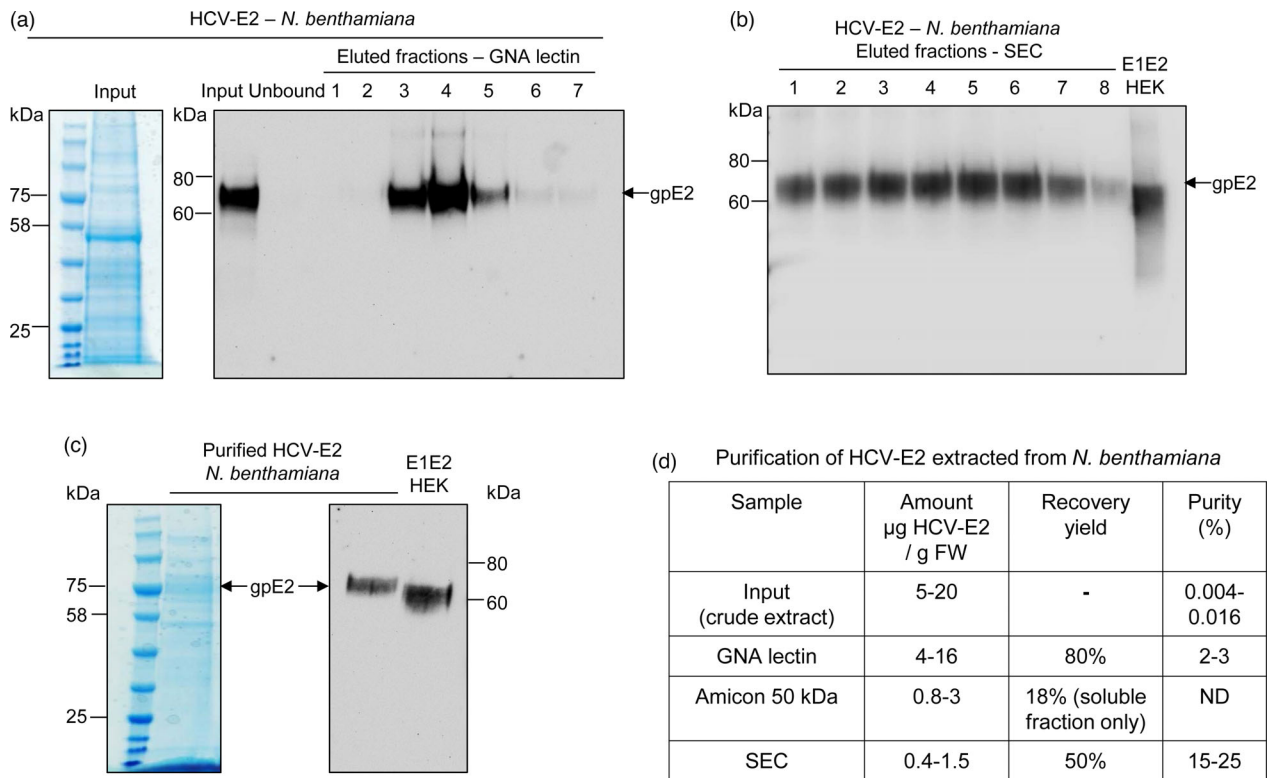


**Figure 2** Glycosylation characteristics of the HCV-E2 antigen produced in *Nicotiana benthamiana*. Lysates from HEK293T cells (HEK) and *N. benthamiana* leaves (*N.b.*) expressing HCV-E2 were subjected to Endo H digestion (+) or left untreated (-) followed by western blotting. Glycosylated (gp) and deglycosylated (p) polypeptides were detected using anti-E2 3/11 Abs.

denaturing conditions and western blot analysis with anti-E2 antibodies (Figure 3b). The total elution volume corresponded to that of the 158 kDa calibration marker, suggesting antigen association into dimers. Samples were concentrated by

ultrafiltration and tested again by western blot analysis to verify the stability of the purified protein. This analysis revealed the presence of a major band at ~75 kDa for the E2 purified from *N. benthamiana* and confirmed the higher apparent molecular weight as compared to the mammalian cell-produced counterpart (Figure 3c). Importantly, there was no evidence of E2 proteolysis during purification from tobacco leaves and mammalian cells, indicating similar stability of the viral antigen, regardless of the expression system.

HCV-E2 quantification in *N. benthamiana* extracts indicated expression levels varying between 5 and 20 µg/g FW, most likely reflecting variable efficiency of agroinfiltration in different leaf areas (Figure 3d). Total protein and viral antigen quantification revealed good recovery yields after affinity chromatography and SEC, respectively, and a final antigen purity reaching ~25% (Figure 3d), which was further confirmed by Coomassie staining of gel electrophoretically separated samples (Figure 3c, left panel). However, protein concentration by ultrafiltration (on Amicon-50) resulted in a significant loss of the soluble antigen fraction. This behaviour is not unusual for transmembrane proteins like HCV-E2, which often require the presence of detergents for maintenance of a soluble, properly folded state, once the proteins are removed from their native membrane environment. Although this loss of material has not impeded the immunology studies, future large-scale application of this purification protocol will require optimization of storage buffers with regard to detergent content, ionic strength and temperature exposure of samples to prevent antigen precipitation.



**Figure 3** Purification of the HCV-E2 antigen produced in *Nicotiana benthamiana*. (a) Lysates from *N. benthamiana* leaves expressing the HCV-E2 antigen were resolved by SDS-PAGE and stained with Coomassie Instant Blue (left panel) before loading onto a GNA lectin column. Samples from the input lysate, the unbound flow-through and the methyl- $\alpha$ -D-mannopyranoside-eluted fractions were subjected to immunoblotting and detection with anti-E2 3/11 Abs (right panel). (b) The positive GNA fractions were concentrated and loaded onto a HiLoad 16/600 Superdex 200 pg column. The HCV-E2 protein was detected in the new collected fractions by western blotting using anti-E2 3/11 Abs. (c) The HCV-E2 antigen eluted from the HiLoad 16/600 Superdex 200 pg column was concentrated on a Amicon-50 ultra filter, subjected to SDS-PAGE and either stained with Coomassie Instant Blue (left panel) or detected by western blot with anti-E2 3/11 antibodies, alongside the HEK293T-E1E2 control purified on GNA lectin (right panel). (d) Amount, recovery yield and purity of the HCV-E2 antigen in the *N. benthamiana* crude extract (input) and at each purification step were determined by using ELISA. The recovery yields are shown as percentages of HCV-E2 antigen from the amount recovered at the previous purification step. FW, Fresh Weight; ND, Not Determined.

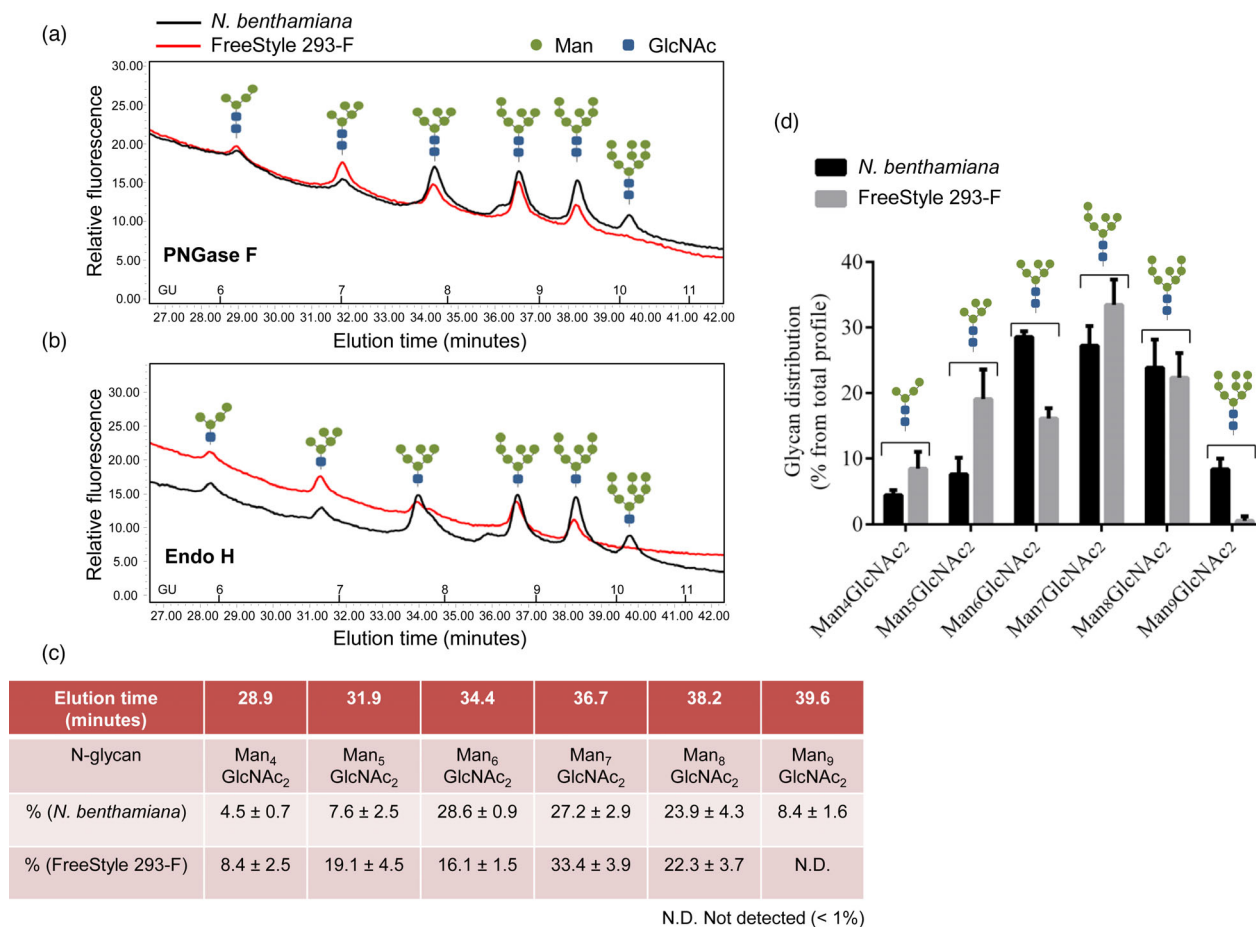
### HCV-E2 produced in *N. benthamiana* contains less processed N-linked glycans

To understand whether N-glycosylation bears host-specific features that contribute to the different apparent molecular weights of the HCV-E2 proteins produced in plant versus mammalian cells, we carried out side-by-side N-glycosylation analysis of the purified viral antigens obtained in the two expression systems. The composition of N-linked glycans released from E2 by enzymatic digestions with PNGase F and Endo H was determined by NP-HPLC (Figure 4a, b). As expected for ER-resident proteins, only oligomannose glycoforms were identified on HCV-E2. However, whereas Man<sub>9</sub> species were virtually undetectable in HEK293-F-derived samples, a significant proportion of unprocessed glycans were found in the case of the *N. benthamiana*-expressed antigen, as indicated by quantification of the corresponding peaks in extracted chromatograms (Figure 4c, d). Conversely, highly processed Man<sub>4</sub>-Man<sub>5</sub> oligosaccharides are ~3-fold more abundant on E2 produced in mammalian cells as compared to plants. Therefore, the shift observed in the electrophoretic mobility of E2 produced in the two expression hosts (Figures 1b and 2) is likely due to the different extent of N-glycan trimming, and, in particular, by the presence of higher

amounts of underprocessed glycans on the *N. benthamiana*-derived antigen.

### HCV-E2 is expressed in native conformation in *N. benthamiana*

HCV attachment to CD81 is mediated by a highly conserved E2 region, spanning amino acid residues 412 to 423 (Owsianka et al., 2001). This sequence is the main target of antibodies neutralizing HCV upon infection, of which AP33 and 3/11 have been extensively characterized both *in vitro* and in animal studies (Desombere et al., 2016; Flint et al., 1999; Owsianka et al., 2005). Acquisition of the E2 native conformation is crucial for exposure of the CD81-binding domain and efficient interaction with the large extracellular loop (LEL) of the HCV receptor. Therefore, to assess the folding state of the tobacco-produced HCV-E2, CD81 (LEL) -E2 binding assays were carried out (Clarke et al., 2017; Pileri et al., 1998). The plant-purified E2 strongly interacted with both CD81 and 3/11 anti-E2 antibodies, as revealed by capture ELISA (Figure 5). Moreover, E2 binding to CD81 was significantly inhibited by 3/11 antibodies, confirming the specificity of the interaction. The lack of 3/11 antibody reactivity with the CD81-unbound E2 further verified the quantitative E2 binding to CD81. The results were confirmed in independent CD81 pull-down



**Figure 4** N-glycan profile of HCV-E2 produced in *Nicotiana benthamiana* and mammalian cells. GNA-purified HCV-E2 protein from *N. benthamiana* leaves (black) or FreeStyle 293-F cells (red) was subjected to in-gel-digestion with either PNGase F (a) or Endo H (b). The released N-linked glycans were fluorescently labelled with 2-AA, purified and analysed by NP-HPLC. Representative chromatograms are shown. Glucose units (GU) corresponding to a labelled glucose oligomer ladder are also indicated. GU were calculated for each peak and glycan structures were assigned. The percentages of glycan distribution were calculated for each *N. benthamiana*- and FreeStyle293-F-produced E2 glycoprotein (c), and plotted (d). The data represent means ± SD from two independent experiments for each of the PNGase F and Endo H digestions.

experiments performed at different E2 purification steps, followed by western blot analysis. Notably, while CD81 efficiently bound the GNA-eluted E2 (i.e. prior to protein concentration), sample dialysis against diluted PBS resulted in complete loss of the ability of E2 to interact with CD81, suggesting conformational changes of the viral antigen (Figure S1). Moreover, the vacuum concentration of dialyzed samples had a significant impact on E2 solubility in that only a small protein fraction was recovered in a soluble state, even after heat-treatment with SDS-PAGE sample buffer under reducing conditions. Altogether, the results demonstrate that HCV E2 is produced in a functional conformation in *N. benthamiana* and highlight the importance of external factors during processing influencing its native folding and solubility.

#### *N. benthamiana*-derived HCV-E2 protein triggers a specific humoral response in vaccinated mice

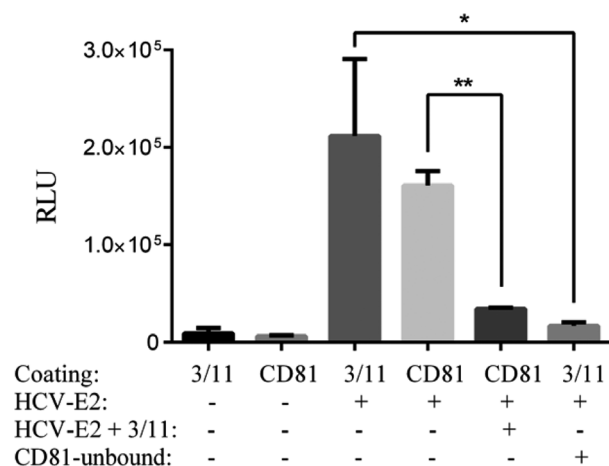
To determine the humoral immune responses elicited in mice vaccinated with *N. benthamiana*-derived HCV-E2, serum samples collected prior immunization (day 0) and at days 27 and 49 post-immunization were evaluated for the presence of E2-specific IgM and IgG by ELISA. Serum samples collected at day 27 (after two antigen administrations; see Experimental Procedures) showed a

significant increase of the IgM titre, which slowly declined by day 49 (Figure 6a). Conversely, the small IgG titre at day 27 increased significantly by day 49 (i.e. after completion of the immunization schedule by the third antigen administration), indicating B-cell activation and maturation (Figure 6b). IgG is the main Ab isotype associated with HCV neutralization. Virus clearance appears to depend on the envelope-specific IgG subclasses developing early in HCV-infected patients (Walker et al., 2020). Therefore, to gain insight into the characteristics of the IgG-based immune response triggered by vaccination with the plant-derived antigen, mice sera were further tested for the presence of E2-specific IgG subclasses. The analysis revealed significant titres of the IgG1 subclass as detected by ELISA (Figure 6c).

#### *N. benthamiana*-derived HCV-E2 induces antibodies with HCV-neutralizing activity

Sera from mice vaccinated with *N. benthamiana*-derived E2 (1:25 dilution) were further tested for the presence of nAbs against the homologous HCV isolate by using a luciferase-based HCVpp neutralization assay. The values obtained were normalized to those of the pooled pre-immune sera (day 0) corresponding to the groups of animals immunized with the HCV antigen or





**Figure 5** Analysis of binding of the plant-produced HCV-E2 antigen to the CD81 receptor. HCV-E2 binding to the CD81 receptor was evaluated by ELISA. 96-well plates were coated with either the recombinant large extracellular loop of human CD81 (CD81-LEL) or anti-E2 3/11 Abs (rat). The CD81-coated wells were incubated with purified samples from *Nicotiana benthamiana* control plants (CD81 - HCV-E2) or from plants expressing HCV-E2 antigen (CD81 + HCV-E2). The purified HCV-E2 was pre-incubated with anti-E2 3/11 Abs (CD81 + HCV-E2 + 3/11), as indicated. The anti-E2 3/11-coated wells were incubated with purified samples from *N. benthamiana* control plants (3/11 - HCV-E2) or from plants expressing HCV-E2 antigen (3/11 + HCV-E2). HCV-E2 protein left after binding on CD81-coated wells was also added onto the 3/11-coated wells (3/11 + HCV-E2 + CD81-unbound). Anti-E2 H53 (mouse) and anti-mouse-HRP Abs were used for detection. Mean RLU values and standard deviations are represented. Statistical significance was calculated using Student's *t*-test (\*) \*,  $P < 0.05$ ; \*\*,  $P < 0.01$

*N. benthamiana* background proteins (control), respectively. Remarkably, sera from all mice in the E2-vaccinated group displayed inhibition of HCVpp infection (Figure 7a), with the inhibition being statistically significant when compared to the control group. The HCV nAb AP33 was included in the experiment to control for the specificity of HCVpp neutralization. The results indicated efficient inhibition of HCVpp infection by AP33, as demonstrated before (Desombere et al., 2016). To investigate the specificity of the response against HCV, we have also tested the same serum dilution (1:25) against Vesicular Stomatitis Virus (VSV), an unrelated virus with broad cell tropism (Bartosch et al., 2003), using the same approach. The results indicate no inhibition of VSV-Gpp infection in the presence of the immune sera (Figure 7b).

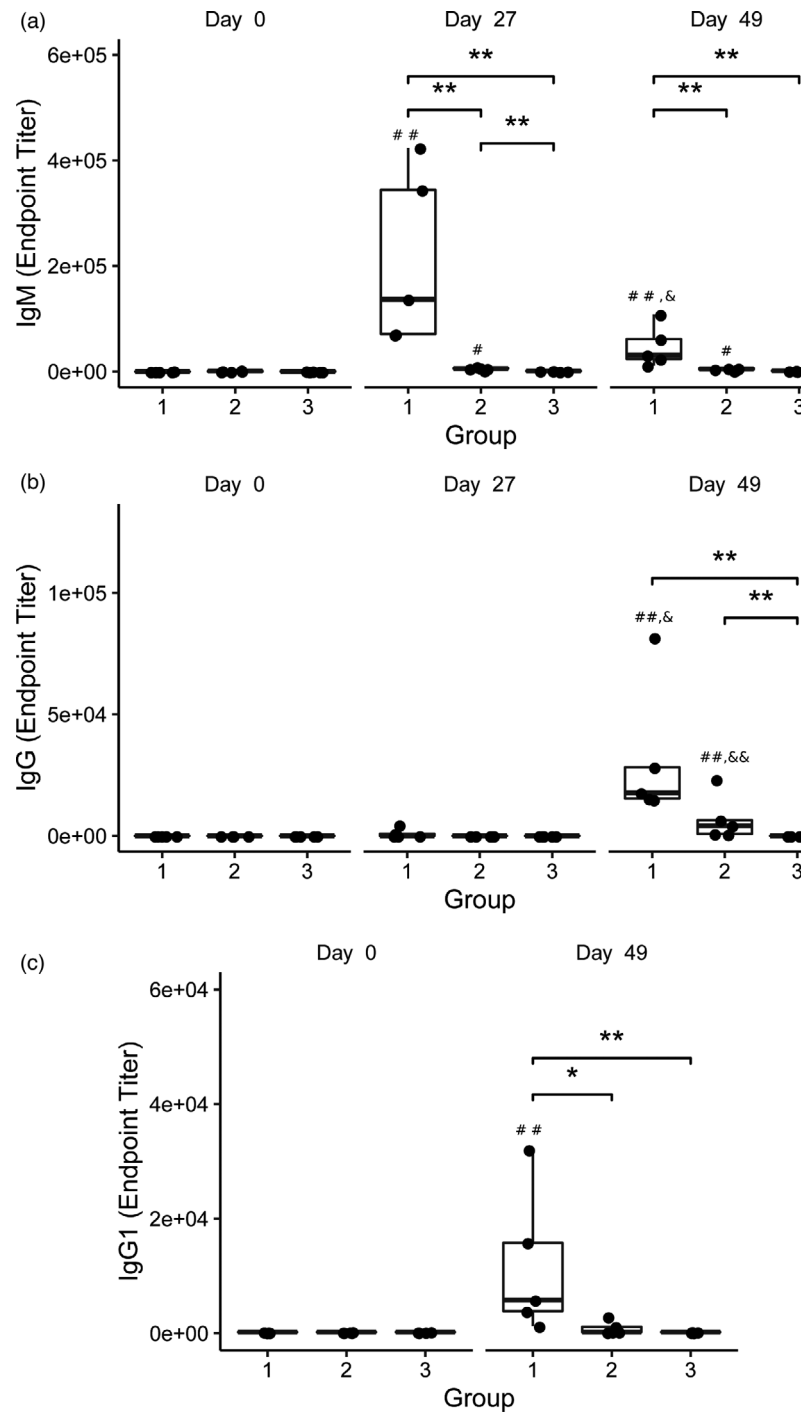
## Discussion

The development of a B cell-based vaccine has remained an unmet goal of HCV preventive therapies. Apart from the genetic diversity inherent to RNA viruses, the intrinsic structural features and low immunogenicity of the viral envelope proteins are additional complicating factors that contribute to the limited success of all efforts to produce such a vaccine. Previous studies have indicated that manipulating E2 N-glycosylation by removing N-glycans that shield key epitopes (Falkowska et al., 2007; Helle et al., 2007), or producing truncated (soluble) forms of the envelope glycoproteins in hosts with distinct N-glycosylation pathways (Grzyb et al., 2016; Li et al., 2016; Urbanowicz et al., 2019) improves protein antigenicity. However, whether or not

these changes significantly increase the production of broadly neutralizing Abs is still a matter of debate (Urbanowicz et al., 2019).

In this work, we have successfully expressed the full-length HCV-E2 in *N. benthamiana*. As cost-effective, carbon-neutral factories, plants are attractive platforms for the production of biopharmaceuticals, vaccines, and other high-value bioproducts using advanced biotechnological tools. During the past decades, considerable efforts have been made towards using plants as production hosts for vaccines against diseases such as Alzheimer's disease (Ishiura and Yoshida, 2019), cancer (Wong-Arce et al., 2017), dengue fever (van Eerde et al., 2019), hepatitis B (Dobrica et al., 2017; Fedorowicz-Stronska et al., 2016; Pniewski et al., 2018) or poliomyelitis (Chan et al., 2016). However, the immunogenicity of plant-made antigens varies considerably and can be influenced substantially by the plant-specific protein glycosylation.

The HCV-E2 protein expressed in *N. benthamiana* displayed Endo H-sensitive, high-mannose glycans, suggesting that the transmembrane domain functioning as an ER-retention signal in mammalian cells (Cocquerel et al., 1998) may work similarly in plants. Comparison of the N-glycans attached to E2 proteins produced in plant leaves and mammalian cells revealed interesting features that may indicate host-dependent mannose trimming occurring in the ER (Figure 4). This is not surprising, since the removal of the terminal  $\alpha$ 1,2-linked mannose residue on the internal (B) branch of the  $\text{Man}_9\text{GlcNAc}_2$  precursor by the ER-resident  $\alpha$ -mannosidase I is the last conserved step of the N-glycosylation pathway in yeast, plants, and mammals (Liebminger et al., 2009; Montero-Morales and Steinkellner, 2018). In plants, MNS3 is the only  $\alpha$ -mannosidase that has been shown to efficiently perform this enzymatic activity and generate the  $\text{Man}_8\text{GlcNAc}_2$  oligosaccharide (Liebminger et al., 2009; Strasser, 2016). This glycan undergoes further mannose trimming by the Golgi-resident  $\alpha$ -mannosidases MNS1 and MNS2 on proteins destined for secretion (Liebminger et al., 2009; Strasser, 2016). Notably, ERMan1, the mammalian MNS3 orthologue, is mainly localized in ER quality control vesicles (ERQCVs), consistent with its function in early N-glycan processing and the central role it plays in glycoprotein folding and ER-associated protein degradation (ERAD) (Benyair et al., 2015). By contrast, MNS3 is not involved in the ERAD of plant glycoproteins (Huttner et al., 2014) and instead, appears to reside in cis-Golgi compartments, as shown in a recent study performed in *Arabidopsis thaliana* (Schoberer et al., 2019). Therefore, in order to undergo mannose processing by MNS3, ER-resident proteins must cycle between the ER and the cis-Golgi (Schoberer et al., 2019). In mammalian cells, the transmembrane domain of HCV E2 determines the localization of the protein in the ER by retention rather than recycling, as cis- and medial-Golgi specific  $\text{Man}_4$ - $\text{Man}_5$  glycans were not identified for HCV E1 and E2 proteins when expressed in hepatic cells (Cocquerel et al., 1999; Duvet et al., 1998). It appears likely that the advanced mannose trimming observed for E2 when expressed in HEK FreeStyle 293 cells is the result of protein overexpression that pushes a fraction of the protein down the secretory pathway and exposes it to processing by Golgi mannosidases. Interestingly, plant ER-resident glycoproteins are characterized by the presence of large amounts of  $\text{Man}_8\text{GlcNAc}_2$  glycans and the lack of downstream mannose processing (Pagny et al., 2000). However, HCV-E2 produced in *N. benthamiana* contains a significant pool of  $\text{Man}_6$ - $\text{Man}_7$  oligosaccharides, possibly suggesting that the intrinsic structural properties of the



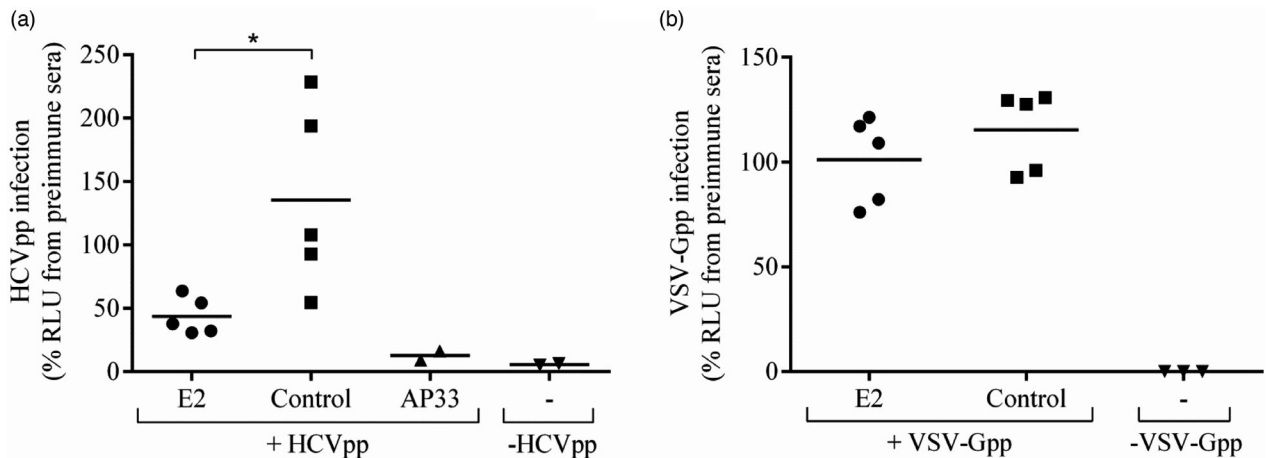
**Figure 6** Humoral immune response to administered tobacco-based HCV-E2 antigen as analysed in sera of immunized mice. Groups of five mice were immunized with adjuvanted *Nicotiana benthamiana*-produced HCV-E2 protein (1) adjuvanted *N. benthamiana* control protein (2) or Al(OH)<sub>3</sub> adjuvant only (3) three times, at 14-day intervals. IgM (a), IgG (b), and IgG1 (c) endpoint titres at days 0, 27 and 49 were calculated based on a 4-parameter logistic regression curve fitted to a pool of immune sera, as the reciprocal sample dilution that would result in three times baseline + standard error as derived from the internal standard curve by multiplication. Individual titres are presented and data are summarized as Tukey plots. Ab titres were compared by using the Wilcoxon rank-sum test. \*, comparison between groups at the same time point (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ), #, time points at days 27 and 49 compared to day 0 (#,  $P < 0.05$ ; ##,  $P < 0.01$ ), &, time point at day 49 compared to day 27, (&,  $P < 0.05$ ; &&,  $P < 0.01$ ).

viral protein favour longer retention in this compartment. Alternatively, the E2 transmembrane domain may allow for protein recycling between cis-Golgi and the ER in plant cells. More detailed investigation of the intracellular localization of the HCV-E2 protein in plants is needed to clarify the exact N-glycan composition in relation to antigen protein trafficking along the secretory pathway.

Whether or not the tobacco-specific N-glycan processing imparts the HCV-E2 antigen with improved immunogenic properties, as compared to other expression systems investigated so far, remains to be determined. Nevertheless, mice vaccination with the plant-derived E2 has triggered a vigorous early humoral

immune response characterized by the presence of high IgM titres, followed by isotype maturation to IgG, mostly of the IgG1 subclass.

Accumulating evidence indicates that the pattern of the immune responses during natural viral infections, particularly the induction of IgG subclasses, strongly influence the outcome of the disease. In HIV-infected patients, an IgG3-predominant response against the envelope glycoproteins was associated with reduced virus titres and significant virus neutralization activity (Cavacini et al., 2003). Similarly, sera of patients recovering from hepatitis B infections contain free IgG Abs targeting the envelope proteins, which belong to the IgG1 and IgG3 subclasses (Rath and



**Figure 7** Neutralization of HCVpp infection. Huh7 cells were infected with pseudo-particles (pp) containing a luciferase gene and the HCV-1a E1E2 envelope (+ HCVpp), the VSV-G envelope (+VSV-G) or no envelope (-HCVpp/-VSV-Gpp), produced in HEK293T cells. HCVpp (a) and VSV-Gpp (b) infections were performed in the presence of day 56-serum from individual mice immunized with *Nicotiana benthamiana*-produced HCV-E2, control protein (five mice/group) or pooled pre-immune sera (dilution 1:25). Anti-E2 AP33 Abs (10  $\mu$ g/mL) were used as positive control for inhibition of HCVpp infection. The luciferase activity was determined in each sample. Black dots and squares represent RLU values for individual mice calculated as average percentages from the pooled pre-immune serum-treated HCVpp/VSV-Gpp corresponding to each animal group. A representative experiment is shown. Black triangles represent control RLU values as percentages from untreated HCVpp/VSV-Gpp. Black bars indicate the means of the shown values for each condition. The P value between the HCV-E2-immunized and the control-immunized groups was determined using the Mann–Whitney test. \*,  $P < 0.05$ .

Devey, 1988). In the case of HCV infection, characterization of the early immune response has been hampered by the absence of patients seeking medical care at the initial, asymptomatic stages of the disease. One of the few prospective cohort studies performed in newly infected HCV patients indicated that individuals who clear the viral infection have significant anti-E2 IgG1 and IgG3 titres and negligible amounts of IgG2 and IgG4 Abs (Walker et al., 2020). Remarkably, the coexistence of IgM and IgG responses, correlating with increased virus-neutralizing activity was observed in sera of HCV clearers (Walker et al., 2020).

The IgM and IgG pattern induced by vaccination with the tobacco-derived E2 may thus explain the robust HCV-neutralizing activity, despite much lower amounts of antigen and fewer boosts being used in this study, as compared to other reports investigating E2 antigens produced in different expression hosts (Urbanowicz et al., 2019).

Taken together, our results provide the first proof that *N. benthamiana* is a suitable host for the expression of functional HCV-E2. So far, the production of HCV envelope glycoproteins in plants has only been reported for lettuce (*L. sativa*). However, the rather modest protein yields obtained for the E1E2 heterodimer of only ~90 ng/g leaf fresh weight has prevented further purification and parenteral administration in vaccination studies (Clarke et al., 2017). By contrast, using a similar *Agrobacterium*-mediated, transient expression approach as in *L. sativa*, we achieved a 100-fold increase of HCV-E2 production in *N. benthamiana*. Notably, these amounts are similar to those reported for the hepatitis B surface antigens (HBsAg) obtained in this plant by applying the same methodology (Dobrica et al., 2017). Unlike HBsAg, the HCV E2 protein is not able to form virus-like particles (VLPs) in any expression system used to date (Masavuli et al., 2017). Together, these observations suggest that *N. benthamiana* might possess a higher capacity for recombinant protein synthesis and/or provide a more appropriate milieu for folding and assembly of complex viral proteins, regardless of their

assembly state. Together with the fast growth rate and the high biomass produced by tobacco plants, this argues for tobacco as a preferred host in molecular farming for vaccine proteins. There is clearly potential for further improvement of HCV antigen expression in plants. In the present study, we have used non-replicating vectors for transient expression by agroinfiltration. Previous work has shown that agroinfection of plants with viral replicons can substantially boost transient protein expression levels (Gleba et al., 2007; Marillonnet et al., 2005). Similarly, geminivirus-derived DNA replicons can be tested for their potential to enhance transient expression levels through transgene amplification (Huang et al., 2009). Future studies will also address the optimization potential of the purification process, by fusion of the viral antigens with plant-specific signal sequences for more efficient ER translocation, the addition of tags to increase solubility and aid purification.

Recently, the first RNA-based vaccines have been commercialized and proved to be highly effective in combating the COVID-19 pandemic. However, conventional vaccines based on recombinant proteins remain of high interest due to their ability to elicit high levels of virus-neutralizing antibodies, as recently shown for the CoV-RBD219N1 vaccine candidate in preclinical trials (Hotez et al., 2020). In this respect, plants have already proved their potential as flexible platforms for rapid production of the SARS-CoV-2 receptor, the angiotensin-converting enzyme 2 (ACE2) that can be used both for therapeutic purposes or vaccine development (Siriwattananon et al., 2020). Also, an oral administration of ACE2 produced in edible plants is expected to trigger a mucosal immune response that is likely needed to prevent SARS-CoV-2 infection at the viral entry sites (Daniell et al., 2021). In addition, it is worth mentioning that, in their chloroplasts, plants can accumulate large amounts of foreign RNA, including long double-stranded RNAs (He et al., 2020), suggesting that plants could also be used as factories for large-scale RNA production for the synthesis of RNA-based vaccines.



## Experimental procedures

### Mammalian cell lines and plasmids

HEK293T cells (European Collection of Animal Cell Culture, Porton Down, UK) were grown as described (Lazar et al., 2017). FreeStyle 293-F cells (Thermo Scientific) were grown in FreeStyle 293 Expression Medium (Thermo Scientific), using 500 mL Erlenmeyer flasks with 0.2 µm vent caps (Corning, NY), in an incubator at 37 °C, 8% CO<sub>2</sub> and shaking at 135 rpm. Huh7 cells (Bartosch et al., 2003) were maintained in DMEM (Dulbecco's Modified Eagle Medium) (1X) with GlutamaxTM-I (Life Technologies) supplemented with 10% FBS (Foetal Bovine Serum) (Life Technologies) and 1% Non-essential Amino Acids 100X (Life Technologies).

The plasmid pcDNA3-E2 encoding the full-length HCV-E2 preceded by a 32 codon sequence corresponding to the C-terminal part of E1, and the plasmid pHCMVdeltaC-E1E2 encoding the wild-type E1E2 polypeptide have been described elsewhere (Bartosch et al., 2003). Both HCV sequences are genotype 1a, isolate H77.

### Expression of HCV-E2 antigens in mammalian cells

HEK293T cells were transiently transfected with plasmids encoding HCV-E2 and E1E2, by using polyethylenimine, as previously described (Clarke et al., 2017). The HCV-E2 protein was also produced in FreeStyle 293-F cells, by using the FreeStyle 293 Expression System (Thermo Scientific), according to the manufacturer's protocol. A volume of 200 mL suspension culture of FreeStyle 293-F cells containing  $1.2 \times 10^6$  cells/mL was transfected with 200 µg pcDNA3-E2 and 200 µL FreeStyle MAX transfection reagent. The cells were harvested 4 days post-transfection and pelleted by centrifugation at 10,000× g, for 10 min, at 4°C.

### Construction of a HCV E2 plant expression vector

The coding region corresponding to the last 32 codons of E1 and the E2 sequence was excised from the pcDNA3-E2 vector and inserted into the plant transient expression vector pEAQ-HT-DEST1 (Sainsbury et al., 2009), using Gateway cloning, essentially as described previously (Clarke et al., 2017). Briefly, attB1 and attB2 recombination sites flanking the coding region were generated by PCR using primers 5'-GGGACAAAGTTGTACAAAAAAGCAGGCTATGTGGGGAGTCTCGCGGG-3' (forward) and 5'-GGGACCACCTTTGTACAAGAAAGCTGGGTTACTCCGCTTGGGATATG-3' (reverse), followed by insertion of the amplified fragment into donor vector pDONR/Zeo. The coding region was then transferred to the destination vector pEAQ-HT-DEST1, generating the pEAQ-HT-DEST1/E2 expression vector.

### Transient expression of HCV-E2 antigens in *N. benthamiana* leaves

The plant expression vector pEAQ-HT-DEST1/E2 was introduced into ElectroMAX *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen) by electroporation, as described before (Clarke et al., 2017). Subsequently, the pEAQ-HT-DEST1/E2-containing *Agrobacterium* cells were cultured and prepared for infiltration as published (Sainsbury et al., 2009; Saxena et al., 2016). For agroinfiltration, six-week-old *N. benthamiana* plants grown in a growth chamber under a photoperiod of 16 h light and 8 h dark at 22 °C were used. Agroinfiltration was performed with a 2 mL syringe without a needle on the underside of the leaf. The

agroinfiltrated plants were further grown until harvest at 7 days post-infiltration (dpi) in a confined S2 safety level growth chamber under the same light and temperature conditions. *N. benthamiana* plants transformed with the empty pEAQ-HT-DEST1 vector were used as a control in characterization and immunization experiments.

### Protein extraction from HEK293T and FreeStyle 293-F cells

Transfected cells were lysed in buffer (10 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, pH 7.5) containing 0.5% Triton X-100 and a mixture of protease inhibitors (Santa Cruz Biotechnology, Dallas, TX), for 30 min, on ice. Cell lysates were clarified by centrifugation at 10 000× g, for 10 min, at 4 °C, and supernatants were used for further analysis.

### Protein extraction from *N. benthamiana* leaves

Plant leaves were ground in liquid nitrogen and further homogenized in five volumes of extraction buffer (0.15 M NaCl, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM sodium ascorbate, pH 7) containing 0.5% Triton X-100 and a protease inhibitor cocktail (Santa Cruz Biotechnology) for 30 min, on ice. Lysates were centrifuged at 1000× g for 5 min and then clarified by ultracentrifugation at 27 000× g for 15 min. All centrifugation steps were performed at 4 °C. The supernatants were collected and used for subsequent analysis.

### Endoglycosidase H (Endo H) treatment

*Nicotiana benthamiana* and HEK293T cell lysates were treated with Endo H (New England Biolabs, Hitchin, UK) to investigate the N-glycosylation pattern of the HCV antigen, according to the protocol provided by the manufacturer, or left untreated, as control. Protein samples were subjected to SDS-PAGE and immunoblotting, as described below.

### SDS-PAGE and western blot analyses

Plant and mammalian cell lysates were heat-denatured, separated by SDS-PAGE under reducing conditions followed by electroblotting onto PVDF membranes, as described (Clarke et al., 2017). The HCV E2 was detected by overnight incubation of blots with anti-E2 3/11 antibodies (raised in rats, kindly provided by J.A. McKeating, Oxford University, UK) (diluted 1:2,000 in PBS containing 5% nonfat milk and 0.1% Tween) followed by 1 h incubation with goat anti-rat-HRP (Santa Cruz Biotechnology, diluted 1:10,000 in PBS containing 1% nonfat milk and 0.1% Tween). Protein signals were visualized by using a chemiluminescence kit (ECL, Thermo Scientific).

### Purification of the HCV-E2 antigen by affinity and size-exclusion chromatography

FreeStyle 293-F cell lysates and *N. benthamiana* leaf extracts expressing the E2 protein were applied to a column packed with *Galanthus nivalis* lectin-agarose (GNA) conjugate (Sigma-Aldrich) (flow rate 3 mL/min), using either the ÄKTA prime or the ÄKTA start protein purification systems (GE Healthcare). The column was washed three times with PBS, then the bound high-mannose, glycosylated proteins were eluted using 0.5 M methyl- $\alpha$ -D-mannopyranoside (Sigma-Aldrich) in PBS (3 mL/min flow rate). Fractions containing E2, as determined by immunoblot analysis with anti-E2 3/11 antibodies, were pooled and concentrated by ultrafiltration using an Amicon MWCO 50 kDa device (Millipore).

E2 samples were then loaded onto a SEC HiLoad 16/600 Superdex 200 pg column (GE Healthcare), using the ÄKTA prime or ÄKTA pure protein purification systems. The column was equilibrated in PBS. Following elution with PBS, fractions were checked for the presence of E2 by western blot, pooled, and concentrated as above. The total protein concentration was monitored at various purification steps by using the bicinchoninic acid (BCA) assay (Thermo Scientific) and by staining of SDS-PAGE gels with Coomassie Instant Blue (Expediton).

#### Quantification of HCV-E2 protein by ELISA

Serial dilutions of a commercial HCV-E2 fragment corresponding to amino acid positions 383-663, genotype 1b (My BioSource, MBS43003) were adsorbed onto 96-well plates (MaxiSorp, Thermo Scientific; 50  $\mu$ L/well, overnight, at 4°C). The plates were washed three times with PBS + 0.05% Tween, followed by blocking with 5% nonfat milk in PBS, for 1 h, at room temperature (RT). *N. benthamiana* and mammalian cell extracts and HCV-E2 samples at different purification steps were first adsorbed onto 96-well plates (1 h, RT) previously coated with GNA (250 ng/well, overnight, at 4°C) to remove non-glycosylated background proteins that could interfere with antigen binding, washed, and blocked as described above. Plates were washed two times and were further incubated with rat anti-E2 3/11 antibodies at 1:1000 dilution, for 1 h, at RT. After five washes, plates were incubated with goat anti-rat-HRP antibody for 1 h, at RT, and washed again before the addition of the TMB substrate (Bio Rad). The enzymatic reaction was developed for 30 min, stopped by the addition of 1 N sulphuric acid and the absorbance at 450 nm was recorded by using a Mithras LB940 plate reader (Berthold Technologies, Bad Wildbad, Germany).

#### Analysis of HCV-E2 binding to CD81 by capture ELISA

A 20  $\mu$ g/mL PBS solution of the large extracellular loop of the human CD81 (CD81-LEL) fused to glutathione S-transferase (GST) (Bartosch et al., 2003) was adsorbed onto 96-wells white plates (Corning Costar, NY), at 50  $\mu$ L/well, overnight, at 4°C. The plates were washed three times with PBS with 0.05% Tween 20 and non-specific binding sites were blocked with 5% nonfat milk in PBS, for 1 h, at RT. Plates were further incubated with a PBS solution of the HCV-E2 antigen purified from *N. benthamiana* leaves (1  $\mu$ g/mL, 50  $\mu$ L/well), pre-incubated or not with anti-E2 3/11 antibody (diluted 1:50), for 1 h, at RT, to control for the specificity of HCV-E2 binding to CD81. The plates were washed five times with PBS with 0.05% Tween 20, then incubated with mouse anti-E2 H53 antibody for 1 h (Op De Beeck et al., 2004) (diluted 1:1000). The plates were washed again as above, incubated for 1 h with secondary anti-mouse-HRP antibody (Santa Cruz Biotechnology, diluted 1:10 000), followed by the addition of SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Luminescence signals were determined as Relative light units (RLU) by using a Mithras LB940 plate reader (Berthold Technologies). In a control experiment, 96-well white plates were coated with 60  $\mu$ g/mL of anti-E2 3/11 antibody (50  $\mu$ L/well), overnight at 4°C. The plates were washed with PBS and blocked with 5% nonfat milk as above, before incubation with either 1  $\mu$ g/mL (50  $\mu$ L/well) of HCV-E2 antigen purified from *N. benthamiana* leaves or the unbound HCV-E2 antigen after adsorption onto CD81-coated wells. A luminescence assay was performed using the same protocol as above. Samples from non-transformed *N. benthamiana* leaves were used as the negative control in both CD81 and anti-E2 3/11 antibody binding assays.

#### In-gel deglycosylation of HCV-E2

E2 samples purified from either *N. benthamiana* or FreeStyle 293F cells were separated by SDS-PAGE followed by protein staining with InstantBlue (Expediton). The protein bands at ~70 kDa were excised from the gel, cut into smaller pieces and dehydrated in acetonitrile by vigorous vortexing followed by washing with H<sub>2</sub>O. These steps were repeated three times, and the dehydrated gel fragments were then incubated with either PNGase F or Endo H (New England Biolabs) overnight, at 37 °C. The reaction solutions were collected and the gel fragments were washed three times with H<sub>2</sub>O. The new washing solutions were added to the reaction mixture, which was subsequently subjected to speed-vacuum drying, at 35°C. The dried samples were resuspended in 30  $\mu$ L H<sub>2</sub>O. Proteins remaining in the gel fragments were eluted in SDS-PAGE sample buffer and analysed by western blot with anti-E2 3/11 antibodies.

#### Analysis of HCV-E2 N-glycans by 2-aminobenzoic acid (2-AA) labelling and normal phase-high performance liquid chromatography (NP-HPLC)

N-glycans released by in-gel treatment with PNGase F or Endo H were fluorescently labelled by reductive amination with 80  $\mu$ L of a mixture containing 30 mg/mL 2-AA, 45 mg/mL sodium cyanoborohydride, 4% sodium acetate-tri-hydrate and 2% boric acid in methanol, for 1 h at 80 °C. Labelled glycans were purified essentially as described (Caputo et al., 2016), with minor changes. Briefly, the 2-AA-labelled glycans were mixed with 1 mL 97% acetonitrile, flowed by gravity through a Speed Amide-2 SPE column (Applied Separations) and eluted with 1.5 mL H<sub>2</sub>O. The recovered samples were then loaded onto a Concanavalin A (ConA)-Sepharose 4B (Sigma-Aldrich) packed column and eluted with 1.5 mL 0.5 M methyl- $\alpha$ -D-mannopyranoside. To remove the methyl- $\alpha$ -D-mannopyranoside, the glycans were further separated on graphitized carbon (PGC) SPE columns and eluted with 1 mL 50% acetonitrile containing 0.1% trifluoroacetic acid. Speed-vacuum-dried samples were resuspended in H<sub>2</sub>O, mixed with one volume acetonitrile and analysed by NP-HPLC using a TSK gel-amide 80 column on a Waters Alliance 2695 separation system. The in-line 474 fluorescence detector was set at 360 nm ( $\lambda$ excitation) and 425 nm ( $\lambda$ emission). Data were processed using the Empower 3 Waters software. Glucose unit (GU) values were determined using a 2-AA-labeled glucose oligomer ladder consisting of partially hydrolyzed dextran (Sigma-Aldrich), and glycan structures were assigned based on GUs as a function of elution time.

#### Animals and immunization

Groups of five, 6-8-week-old female BALB/c mice, were immunized by intra-muscular (i.m) administration, three times, at 14-day interval. On day 0, animal vaccination was performed with a 50  $\mu$ L PBS suspension containing 5  $\mu$ g HCV-E2 and 0.25 mg/mL Al(OH)<sub>3</sub> (Alhydrogel vac-alu-250 InVivoGen trademark Brenntag Biosector A/S) adjuvant, followed by boosting with 2  $\mu$ g of antigen on days 14 and 28. The control groups received the same concentrations of *N. benthamiana* background protein from control leaves (19.2 and 8  $\mu$ g for prime and boost injections, respectively), formulated with 0.25 mg/mL Al(OH)<sub>3</sub> adjuvant or the adjuvant only. Prior to immunization, and on days 27 and 49, serum samples were collected by orbital puncture, centrifuged at 14 000 $\times$  g for 10 min and stored at -80° C until further use. Animal experiments were conducted according to standards set

forth in the Council Directive 86/609/EEC and national legislation, and approved by the national designated authority ANSVSA, registration number 488/22.01.2020.

### Quantification of the immune response

Flat bottom 384-well MediSorp plates (Sigma-Aldrich) were coated with 0.4 µg/mL (20 µL/well) of HEK293T-produced E1E2 antigen in PBS and incubated overnight at 4 °C. After extensive washing with PBS containing 0.05% Tween 20 (Sigma-Aldrich), plates were blocked for 1 h, at RT with PBS containing 5% nonfat dry milk (BioRad) and then washed as before. Six binary dilutions, starting from 1:200, of serum in PBS containing 5% nonfat dry milk were added to the antigen-coated plates (40 µL/well) and incubated for 2 h, at RT. The plates were washed, incubated with HRP-conjugated goat anti-mouse IgM/IgG (Southern Biotech, AL), diluted 6000-fold or with HRP-conjugated goat anti-mouse IgG1/IgG2a (Southern Biotech) diluted 1000-fold in PBS containing 5% nonfat dry milk, for 2 h, at RT. Enzymatic reactions were developed following the addition of the TMB substrate (R&D Systems), for 20 min at RT then stopped with 20 µL/well of 1 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was measured at 450 nm using a Tecan Infinite M1000 microplate reader. Normalization across ELISA plates was performed by pooling all the immune sera from day 49 which served as an internal standard. Serum pool dilution starting from 1:100 was used as an internal standard on each plate and a 4-parameter logistic regression model was fitted to the measured values.

### HCV pseudo-particles (HCVpp) neutralization assay

HCVpp were obtained by co-transfection of HEK293T cells with murine leukaemia virus (MLV) Gag-Pol packaging vector (pCMV-5349), a luciferase-encoding plasmid (pTG126) and pCMVdeltaC-E1E2 (Op De Beeck et al., 2004) in a 1:1:1 ratio, using polyethylenimine and Opti-MEM. To control the specificity of the neutralization, plasmid pCMV-G encoding the Vesicular Stomatitis Virus glycoprotein G (VSV-G) (Bartosch et al., 2003) was included. Owing to the broad VSV tropism, the resulting pseudo-particles (VSV-Gpp) also served as a positive control for successful pseudotyping and infection, while plasmid pcDNA3.1 (Invitrogen) was used as a negative control. Opti-MEM medium was replaced with DMEM 6 h after transfection. The extracellular medium was collected at 48 h post-transfection, cleared by centrifugation at 1500 ×g and further filtered through 0.45 µm filters. The supernatants were stored at 4 °C for maximum of 2 weeks before use. Mice sera collected at 0 and 56 days post-vaccination were heat-inactivated at 56 °C, for 30 min, prior to incubation with HCVpp/ VSV-Gpp for 1 h, at 37 °C, at 1:25 dilution. Pre-immune mice sera (day 0) were pooled for each group of animals. Huh7 cells seeded in 48-well plates were inoculated with 250 µL/well HCVpp or VSV-Gpp treated or not with vaccinated mice sera, pooled pre-immune mice sera or anti-E2 AP33 antibodies (10 µg/mL), as a positive control for inhibition of HCVpp infection. Cells were harvested at 72 h post-infection and luciferase activity was determined by using the Luciferase Assay System kit (Promega), following the manufacturer's protocol. The cell lysates were transferred in 96-well white plates and luminescence was measured immediately after the addition of the luciferase substrate, using a plate reader Mithras LB940 (Berthold Technologies). Relative light units (RLU) recorded for vaccinated mice sera were normalized to the pre-immune sera corresponding to each group. All experimental conditions were performed twice, in quadruplicates.

### Statistics

Intergroup comparisons for the humoral immune response were performed by Wilcoxon rank-sum test, with R version 3.2.3. Statistical significance in the analysis of HCV-E2 binding to CD81 by capture ELISA was calculated using Student's t-test and GraphPad Prism version 6 software. Comparisons of HCVpp infection in the neutralization assay between HCV-E2- and control-immunized groups was evaluated using Mann-Whitney test, in GraphPad Prims version 6. A *P* value < 0.05 was considered statistically relevant.

### Acknowledgement

The research leading to these results has received funding from the EEA Grants 2014-2021, the GreenVac project no. 5/2014 and SmartVac project no. 1/2019.

### Conflict of interest

The authors have no conflicts of interest to declare.

### Author contributions

N.B.N., J.L.C., and C.S. conceived the study and analysed the data. M.O.D performed HCV antigen extraction, purification and functional characterization, N-glycan sequencing, and HCVpp neutralization studies. J.L.C, R.B., L.P., A.E., H.S., and S.H made the HCV gene constructs for plant expression and carried out the production of HCV antigen in *N. benthamiana*. N.Z., D.A., and M.O.D. designed the N-glycan sequencing experiments and interpreted the data. M.O.D and C.I.P. designed the HCVpp neutralization experiments. J.D. contributed reagents and materials. C.S. and A.O. conceived animal experiments. I.C., C.T., E.V and C.S. performed the animal experiments. C.T., S.C. and A.O. analysed immunology data. N.B.N. and M.O.D wrote the manuscript. J.L.C., C.S., C.I.P, C.T., R.B., J.D., N.Z., D.A., and A.E. reviewed the manuscript and provided detailed comments and suggestions for improvement.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** CD81 binding of the HCV-E2 antigen produced in *N. benthamiana*.

**Method S1** Vacuum concentration of antigen samples.

**Method S2** CD81 pull-down assay.