Full Length Research Paper

Expression of chimeric HCV peptide in transgenic tobacco plants infected with recombinant alfalfa mosaic virus for development of a plant-derived vaccine against HCV

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Hepatitis C virus (HCV) is the major etiologic agent of blood transfusion–associated and sporadic non-A non-B hepatitis affecting more than 180 million worldwide. Vaccine development for HCV has been difficult and there is no vaccine or effective therapy against this virus. In this paper, we describe the development of an experimental plant-derived subunit vaccine against HCV. Our subunit vaccine originates from a consensus HCV-HVR1 epitope (R9) that antigenically mimics many natural HVR1 variants. This HVR1 sequence was cloned into the open reading frame of a plant virus, Alfalfa Mosaic Virus (ALMV) coat protein (CP) . The chimeric ALMV RNA4 containing sequence-encoding R9 epitope was introduced into full-length infectious ALMV-RNA3 that was utilized as an expression vector. The recombinant chimeric protein is expressed in transgenic tobacco plants (P12) expressing ALMV RNA1 and 2. Plant–derived HVR1/ALMV-CP reacted with HVR1 and/or ALMV-CP specific monoclonal antibodies and immune sera from individuals infected with HCV. Using plant-virus based transient expression to produce this unique chimeric antigen will facilitate the development and production of an experimental HCV vaccine. A plant derived recombinant HCV vaccine can potentially reduce expenses normally associated with production and delivery of conventional vaccine.

Key words: Hepatitis C virus (HCV), transgenic tobacco plants (P12), consensus HCV HVR1 epitope (R9), and chimeric ALMV-RNA4.

INTRODUCTION

The development of plants and plant viruses for expression and delivery of vaccine antigens is a particularly promising approach. Plant-derived antigenic proteins have delayed or prevented the onset of disease in animals and have proven to be safe and functional in human clinical trials (Amanda et al., 2000). The list of plant-derived vaccinogens continues to grow and includes viral, bacterial, enteric and non-enteric pathogen antigens. In an effort to increase expression levels, stability and ease of harvest, synthetic genes have been constructed (Mason et al., 1998) and expression has been targeted to specific tissues (Brennan et al., 1999). A recombinant ALMV was engineered to express two rabies virus epitopes (Fleysh et al., 2001). Modelska et al. (1998) were the first to detect a mucosal immune

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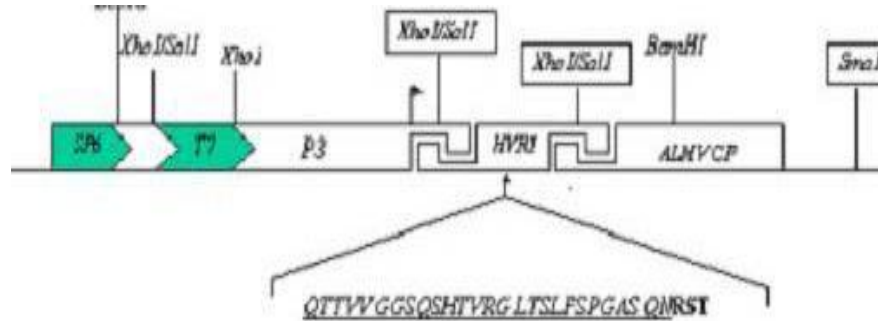
Figure 1. Schematic representation of ALMV-HVR1, an Alfalfa mosaic virus RNA3 (recRNA3) encoding a coat protein (CP) gene fused with the sequences encoding chimeric HCV-HVR1 peptide. SP6 and T7, promoters. P3, cell-to-cell movement protein. Arrow indicates the subgenomic promoter for RNA4. Amino acid sequences of the chimeric HVR1 peptide is underlined.

response after oral induction with a plant- virus-derived vaccinogen. This report describes our efforts to develop a subunit vaccine candidate for hepatitis C virus (HCV) using a modified ALMV to produce recombinant vaccine antigens in transgenic tobacco plants. Hepatitis C virus (HCV) is the major etiologic agent of blood transfusion–associated and sporadic non-A non-B hepatitis affecting more than 180 million worldwide including nearly 4 million in the United States (Cohen, 1999; Di Bisceglie et al., 1999; Purcell, 1997). Despite a wide array of humoral and cell- mediated host immune responses, HCV infection leads to chronic disease in about 70% of cases, among which a significant proportion eventually develop cirrhosis and hepatocellular carcinoma (Alter et al., 1995). Vaccine development for HCV has been difficult. Presently, the virus cannot be grown in tissue culture and there is no vaccine or effective therapy against this virus. Interferon treatment (also in combination with ribavirin) is the only antiviral therapy available at the moment, but it is effective only in 20% to 40% of patients (Fried et al., 1995; Fried et al., 1998), thus making the development of an HCV vaccine a high priority target. It seems possible that many of the "evolving" escape epitopes are scattered throughout the HCV genome preventing the development of an effective anti-viral response (Eckels et al., 1999). In fact, HCV displays a high rate of mutation during replication and exists in the bloodstream of infected patients as quasispecies, (Weiner et al., 1991; Bukh et al., 1995) which fluctuates during the course of the disease mainly as a result of immune pressure (Bukh et al., 1995; Farci et al., 2000). The major target of this immune response is the 27 amino acid N-terminal segment of the E2 glycoprotein. This protein fragment is the most variable region of the whole HCV polyprotein (Weiner et al., 1991) and contains a principal neutralization determinant (Farci et al., 1996).Recent studies described a novel approach to this problem by selecting highly cross-reacting "synthetic variants" of HCV-HVR1 from a vast repertoire of HVR1 surrogates as fusion to the major coat protein of bacteriophage M13 (Puntoriero et al., 19980). When used in the form of multiple antigenic peptides (MAP) (Pessi et al., 1990), the

selected HVR1 peptide mimics (mimotopes) induced antibodies in mice and rabbits that recognizeda large number of HVR1 variants from natural isolates (Puntoriero et al., 1998). HCV HVR1 (R9)/cholera toxin B subuinte (CTB) protein expressed by recombinant tobacco mosaic virus (TMV) reacted with HVR1-spesific monoclonal antibodies as well as with sera from HCV infected patients (Nemchinov et al., 2000). HVR1 epitope (R9) expressed protein in plant using Cucumber Mosaic Virus (CMV) display the epitope demonstrated significant immunoreactivity to HCV serum from infected patients (Natilla et al., 2004). In our research, we chose a vaccine development approach that is predicted to be effective against extremely high antigen variability. This HVR1 sequence was cloned into the open reading frame of ALMV CP (Yusibov et al., 1995) in an intermediate of vector that contained the cDNA of ALMV RNA4. This chimeric sequence then was introduced into a full-length ALMV-RNA3 vector that was used to produce *in vitro* transcripts to inoculate P12 tobacco plants. This recombinant chimeric protein is expressed in transgenic tobacco plants (P12) expressing ALMV- RNA1 and 2 (Thole et al., 2001).

MATERIALS AND METHODS

Plasmid DNA construction

HVR1 consensus sequences coding 27 amino acids (R9 epitope) were synthesized using polymerase chain reaction. Two oligonucleotides were designed with 12 overlapping nucleotides and served as a template for each other. Nucleotide sequence for the upstream (5`-cgg ctc gag atg tcc caa acc acc gtg gtc ggc gga tct caa tct cat acc gtg cgt ggc ctc-3`) with XhoI engineered restriction site (underlined) and the downstream (5` gcg gtc gac cta ttt tga gaa gcg cca gga gag aag aga gat gtg agg cca cgc ac-3`) with SalI engineered restriction site (underlined). The resulting PCR product was cloned into pGEM-T-Easy vector (Promega, Madison, WI). The HCV-HVR1 PCR-amplified product was digested XhoI and SalI, and then cloned into XhoI site pSPΔAUG (Yusibov et al, 1995). After sequence confirmation, the chimeric ALMV RNA4 containing sequence encoding R9 epitope was introduced into full-length RNA3 cDNA construct that was utilized as the expression vector (Figure 1).

A

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1    ATGTCCCAAACCACCGTGGTCGGCGGATCTCAATCTCATACCGTGCGTGGCCTCACATCT
61   CTCTTCTCTCCTGGCGCTTCTCAAAATAGGTCGAGTTCTTCACAAAAGAAAGCTGGTGGG
121  AAAGCTGGTAAACCTACTAAACGTTCTCAGAACTATGCTGCCTTACGCAAAGCTCAACTG
181  CCGAAGCCTCCGGCGTTGAAAGTCCCGGTTGTAAAACCGACGAATACTATACTGCCACAG
241  ACGGGCTGCGTGTGGCAAAGCCTCGGGACCCCTCTGAGTCTGAGCTCTTTTAATGGGCTC
301  GGCGTGAGATTCCTCTACAGTTTTCTGAAGGATTTCGCGGGACCTCGGATCCTCGAAGAG
361  GATCTGATTTACAGGATGGTGTTTTCCATAACACCGTCCTATGCCGGCACCTTTTGTCTC
421  ACTGATGACGTGACGACTGAGGATGGTAGGGCCGTTGCGCATGGTAATCCCATGCAAGAA
481  TTTCCTCATGGCGCGTTTCACGCTAATGAGAAGTTCGGGTTTGAGTTGGTCTTCACAGCT
541  CCTACCCATGCGGGAATGCAAAACCAAAATTTCAAGCATTCCTATGCCGTAGCCCTCTGT
601  CTGGACTTCGACGCGCAGCCTGAGGGATCTAAAAATCCCTCATACCGATTCAACGAAGTT
661  TGGGTCGAGAGAAAGGCGTTCCCGCGAGCAGGGCCCCTCCGCAGTTTGATTACTGTGGGG
721  CTGCTCGACGAAGCTGACGATCTTGATCGTCATTGATG
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B

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1    MSQTTVVGGSQSHTVRGLTS
21   LFSPGASQNRSSSSQKKAGG
41   KAGKPTKRSQNYAALRKAQL
61   PKPPALKVPVVKPTNTILPQ
81   TGCVWQSLGTPLSLSSFNGL
101  G  V  R  F  L  Y  S  F  L  K  D  F  A  G  P  R  I  L  E  E
121  D  L  I  Y  R  M  V  F  S  I  T  P  S  Y  A  G  T  F  C  L
141  T  D  D  V  T  T  E  D  G  R  A  V  A  H  G  N  P  M  Q  E
161  F  P  H  G  A  F  H  A  N  E  K  F  G  F  E  L  V  F  T  A
181  P  T  H  A  G  M  Q  N  Q  N  F  K  H  S  Y  A  V  A  L  C
201  L  D  F  D  A  Q  P  E  G  S  K  N  P  S  Y  R  F  N  E  V
221  W  V  E  R  K  A  F  P  R  A  G  P  L  R  S  L  I  T  V  G
241     L  L  D  E  A  D  D  L  D  R  H  *
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Figure 2. A Nucleotide sequence of HVR1/ALMV CP chimeric gene. B Translated amino acid sequence of the recombinant protein. The HVR1 fragment is underlined.

In vitro transcription and plant inoculation

Plasmid DNA corresponding to recombinant RNA3 of ALMV-HVR1 was linearized with SmaI at the 3` end of the cDNA sequence of the viral construct and purified using buffered - saturated phenol/chloroform/isoamyl alcohol (25:24:1). One µg of linearized DNA was used to produce *in vitro* transcripts using T7 Cap Scribe according to manufacture's guidelines (Roche, Indianapolis, IN).

Transgenic P12 tobacco plants were inoculated with a mixture of *in vitro* synthesized transcripts of recombinant ALMV-HVR1 RNA3 and subgenomic RNA4.

Reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from systemically ALMV-HVR1-infected (20 days post inoculation) and uninfected leaves by RNeasy plant kit according to the manufacturer recommendation (Qiagen, Valencia, CA). The purified RNA was eluted in water and kept at -70ºC for conventional RT-PCR. Five µg were reverse transcribed in 20 µl reaction mixture containing 100 µM of complementary primer specific for ALMV CP (5`-cgcggatccgaggtcccgcgaaatcct-3`), 5 µl of first stranded buffer and 200 units of M-MLV reverse transcriptase enzyme (Life Technologies, Gaithersburg, MD). Five µl of the cDNA, 5 µM of forward (5`-atgtcccaaaccaccgtggtcggcg-3`) and complimentary primers were mixed with PCR reaction mixture containing 2.5 units of Ampli Taq enzyme. The PCR parameters: 94˚C for 2 min one cycle, 94˚C for 30 sec (denaturing) 55˚C for 30 sec, (annealing) and 72˚C for 45 sec (extension) for 35 cycles, with final extension 72˚C for 10 min.

Western blot analysis for the recombinant protein

One hundred milligram of ALMV-HVR1 infected leaves were placed in Eppendorf tubes and homogenized in 1X PBS containing 1% plant protease inhibitor cocktail (sigma), and boiled for 10 min in 2X loading buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, and 50 mM Tris-HCl, pH 6.8). The mixtures containing ALMV-HVR1were separated on 12% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% dry non-fat milk in 1X PBS buffer, and then incubated in diluted monoclonal antibodies (1:10,000) against ALMV CP conjugated with horseradish peroxidase (Agdia, Elkhart, IN) for 1 h. The membrane was washed 6X with PBST 5 min each and ALMV CP signal was detected by super signal west pico chemiluminescent substrate (Pierce, Rockford, IL). Recombinant ALMV CP containing chimeric R9 HVR1 mimotope, in addition, was detected with ALP213 monoclonal antibodies for HVR1 that recognizes epitope GAARSTLQLAGLFQPGAKQN, a 390-409 ALP213 was generously provided by Dr. Jane McKeating of University of Reading and Dr. Arvin Patel of MRC, Glasgow, UK. Mabs were used at 1:5,000 dilution and sera at 1:10,000 dilutions. Infected human serum was also used for the detection of HVR1 mimotope.

ELISA assay for detection of HVR1 epitope

ELISA plates were coated with 100 µl per well of anti-ALMV CP monoclonal antibodies (1:5,000) dilution in carbonate buffer (50 mM Na2CO3, pH 9.0) for 2 h at 37˚C, then blocked with 1X I block (1X PBS, 0.5% I-block, 0.1 % Tween) at room temperature for 1 h. Plates were incubated overnight with the plant samples at 4˚C, followed by incubation with anti-ALMV CP Mabs, or anti-HVR1 Mab (ALP213), or infected human serum, or normal human serum at 37˚C for 2 h. All washing steps between incubations were performed with 1X PBS-Tween buffer. The plates were incubated at room temperature for 1 h with anti-mouse or anti-human IgG-HRP conjugate, and then freshly prepared horseradish peroxidase substrate was added to the wells. The plate was covered with foil and incubated at room temperature for 30 min. The reaction was stopped by the addition of 50 µl of l.5 M sulphuric acid to each well and the absorbance was measured at 492 nm.
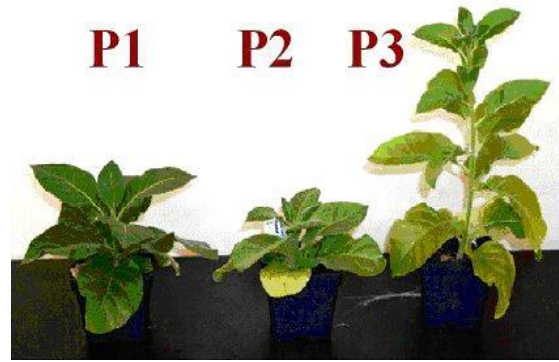
Figure 3. Shows the stunting of P12 plants infected with recombinant ALMV. P1; P12 plant infected with recombinant ALMV. P2; P12 plant infected with ALMV. P3 is a healthy plant.

RESULTS

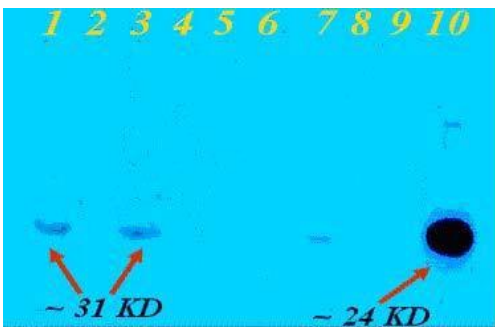### Construct engineering and nucleotide sequencing

We chose cross-reactive HVR1 mimotope R9, which antigenically mimics a large number of natural HCV-HVR1 variants and induces a broadly cross-reactianti-HVR1 response (Puntoriero et al., 1998). The sequence encoding the 27 amino acid-long HVR1 mimotope (engineered to contain XhoI at 5` and SalI at 3` ends) was fused to the amino-terminus of the ALMV CP gene in pSPΔAUG using an XhoI site. DNA sequencing confirmed the ligation of HVR1 mimotope, in-frame, to the ALMV CP gene in pSPΔAUG vector. Translation of recombinant ALMV CP in recRNA3 starts from the AUG codon introduced at the 5` end of the DNA sequence encoding the chimeric HVR1 mimotope after introduction of the chimeric cDNA sequence for ALMV RNA4 into the full-length infectious RNA3. The recombinant RNA3 carrying the HVR1 sequence was placed under control of the SP6 promoter. The assumption was that HVR1/ALMV CP subgenomic message transcribed from recombinant viral RNA will be translated into ALMV coat protein particles displaying the HVR1 epitope on their surface.
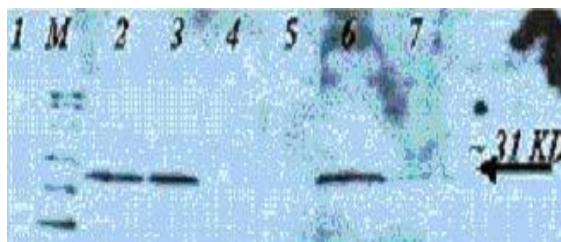
Infectivity and stability of recombinant ALMV

In-vitro synthesized capped RNA transcripts of recombinant ALMV RNA3 and subgenomic RNA4 were used to inoculate the three upper leaves of transgenic tobacco plants (P12) that were at the four-leaf stage. To assess the stability of recombinant virus, we prepared extracts from systemically infected leaves of plants at 20 days post inoculation (dpi) and used it as inoculum for new P12 plants. Infection with the recombinant virus resulted in mild mosaic symptoms in the upper uninoculated leaves and stunting of plant growth is similar, but not as severe, to that observed in the wild-type ALMV (Figure 3).
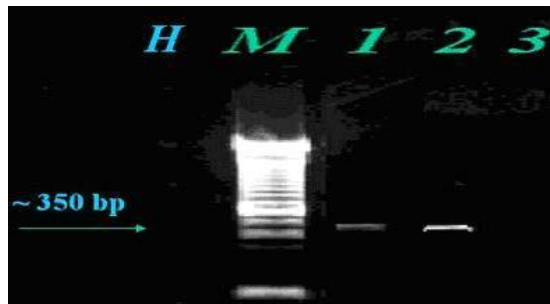
Figure 4. RT-PCR with total RNA from P12 transgenic plants inoculated with hybrid ALMV vector. M; 100 bp DNA marker. L1& L2; P12 plants inoculated with rec. RNA3. H; healthy plant control. L3 P12 plant inoculated with wield type ALMV.

Figure 7. Shows the autoradiographic from the Western Blot analysis for the recombinant protein probed with HCV infected human serum. 1: Uninfected plant; 2, 3, 6, 7: different plants infected with recombinant ALMV. 4, 5: different plants infected with wild type ALMV**.**
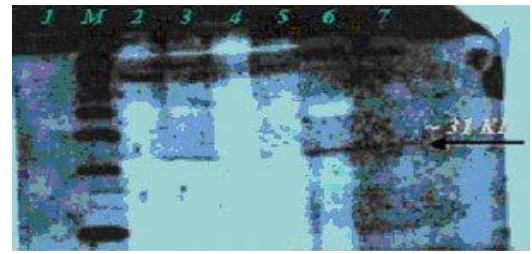

Figure 5. Shows the autorediographic from the Western Blot analysis for the recombinant ALMV coat protein Probed with anti ALMV antibodies. 1 to 8 Different Plants infected with recombinant ALMV. 8 and 9: Uninfected plants. 10: ALMV purified virus.

Figure 8. Shows the autoradiographic from the Western Blot analysis of total protein examined infected normal human serum: 1 Uninfected plant; 2, 3, 6, 7 different plants infected with recombinant ALMV; 4,5: plants infected with wild type ALMV.

obtained from several P12 plants infected with the recombinant virus (Figure 4 lanes 1 and 2). No PCR amplification obtained from P12 plants infected with wiled type ALMV or healthy P12 plants (Figure 4 lanes 3 and H, respectively)

Accumulation of recombinant ALMV in infected plants and western Blotting

Western blot analysis was performed for tissue samples from locally and systemically infected leaves collected at 7, 15 and 20 dpi. Only samples collected at 15 and 20 dpi revealed a band of the size expected for the ALMV CP (24.0 kDa) with different band intensities but not in samples collected at 7 dpi or samples from healthy plants, as detected by reactivity with ALMV CP-specific antibodies. Similar analysis of tissue samples from systemically infected leaves collected at 20 dpi demonstrated expression of recombinant protein, with antibodies specific for ALMV CP (Figure 5) and monoclonal antibodies specific for HCV (Figure 6) and human serum infected with HCV (Figure 7) revealing the expected size protein (~31 KD) but not with normal human serum (Figure 8). Wild-type ALMV CP reacted
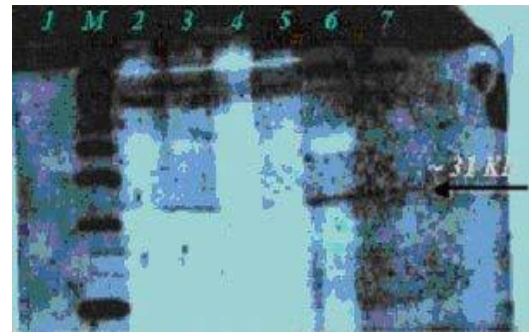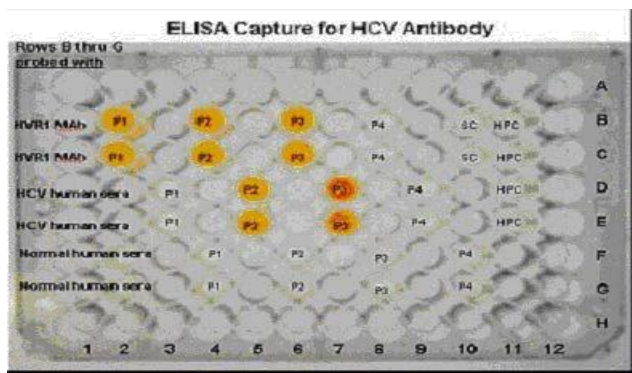

Figure 6. Shows the autoradiographic from the Western Blot analysis for the recombinant ALMV coat protein probed with anti HVR1 monoclonal antibodies. 1: Uninfected plant; 2, 3, 6, 7: different plants infected with recombinant ALMV; 4,5: different plants infected with ALMV.

Amplification of HVR1 epitope by RTPCR

To confirm that the ALMV RNA3 from infected plants indeed carried the engineered epitope, we performed an RT-PCR assay in total RNA from infected P12 plant. The amplified fragment of the expected size (350 bp) was

Figure 9. Shows the ELISA plate coated with anti ALMV CP antibodies and propped with monoclonal anti-HVR1 antibodies (rows B and C) or HCV infected human sera [rows D and E] or normal sera (rows F and G). P1, P2 and P3 are plant samples infected with recombinant ALMV. P4, plant sample infected with wild type ALMV. SC, substrate controls. HPC, healthy plant controls.

only with ALMV CP-specific antibodies and not with monoclonal antibodies specific for HCV or Infected human serum.

Elisa tests

ELISA tests using anti-ALMV CP monoclonal antibodies clearly demonstrated that transgenic tobacco plants (P12) infected with recombinant ALMV RNA3 and subgenomic RNA4 transcripts produce the chimeric ALMV coat protein (Figure 9). To prove that the ALMV coat protein carried the HVR1 peptide fused to its surface; we performed an ELISA assay using anti-ALMV CP monoclonal antibodies as primary antibodies to capture the ALMV-CP/HVR1 fusion protein on the ELISA plates and ALP 213 anti-HVR1 monoclonal antibodies or infected human serum to specifically detect the HVR1 epitope on the captured chimeric protein. As shown in Figure 9, only plants infected with the recombinant ALMV were positive indicating that the HVR1 epitope expressed was associated with the ALMV CP.HCV infected human sera [rows D and E] or normal sera (rows F and G). P1, P2 and P3 are plant samples infected with recombinant ALMV. P4, plant sample infected with wild type ALMV. SC, substrate controls. HPC, healthy plant controls.

DISCUSSION

Plants have been actively targeted for the production of medically important proteins, including vaccine antigens and monoclonal antibodies (Hiatt et al., 1989). A variety of antigens have been expressed in transgenic plants; including hepatitis B surface antigen (Mason et al., 1992), *Escherichia coli* heat-labile enterotoxin (Haq et al., 1995),

rabies virus glycoprotein (McGarvey et al., 1995), and Norwalk virus capsid protein (Mason et al., 1996). In addition to transgenic plants, alternative approach to express high levels of foreign proteins uses viruses that infect plants (Dalsgaard et al., 1997; Hamamoto et al., 1993; Modelska et al., 1998; Porta et al., 1994; Turpen et al., 1995; Usha et al., 1993; Yusibov et al., 1997; Nemchinov et al., 2000; Natilla et al., 2004). An important aspect of using plant viruses as production tools is screening and establishing appropriate host species for manufacturing large quantities of polypeptides. The present study suggests the usefulness of plants as a vehicle for producing anti-HCV vaccine utilizing an ALMV-based expression vector.

Plant-synthesized recombinant ALMV CP/HVR1 protein appears to be in the biologically active configuration and immunoreactive for its ALMV CP and HVR1 epitopes. The ALMV CP-displayed HVR1 antigenic determinant cross-reacted with different serum samples derived from HCV-infected patients. This is a direct confirmation of immunological similarity between the R9 HVR1 "mimotope" expressed in plants and the natural HVR1 variants. Thus, if HVR1 indeed contains a principal neutralization epitope for HCV, a plant-produced, purified HVR1 surrogate antigen will be able to induce cross-neutralizing antibodies against most HCV quasispecies. Monoclonal antibodies specific for aa 384-391 within HVR1 interfere with the interaction of HCV E2 glycoprotein with CD81 (Flint et al., 1999), which is the putative HCV cellular receptor (Pileri et al., 1998) suggesting a functional role of HVR1 in viral attachment and entry into the host cells. Hence, HVR1 may be a prominent target for host neutralizing activity. The latter observation has been postulated and experimentally shown to be true (Shimizu et al., 1996; Zibert et al., 1995; Zibert et al., 1997). An experimental vaccine has also been examined in chimpanzees (Esumi et al., 1999). If the capacity of HCV to consistently escape immune recognition is indeed related to HVR1 heterogeneity, an HVR1-consensus approach developed by Puntoriero et al. (1998) is an attractive strategy for obtaining cross-neutralizing HCV antibodies.

The cross-reactivity of plant produced ALMV CP-displayed HVR1 epitope with different HCV infected sera could potentially make this chimeric protein a simple and inexpensive diagnostic tool for the virus.

The large scale production of biopharmaceuticals in plants is clearly a matter of the near future (Cunningham et al., 1998; Hammond et al., 1999). The recombinant ALMV used in this study does not represent a biohazard and the "foreign sequence" will not remain in the environment because of subsequent reversion of the recombinant virus to the wild type (Hammond et al., 1999) . The use of transgenic P12 plants expressing RNA1 and RNA2 components of ALMV minimize the risk of recombinant ALMV virus infecting or being transmitted to other susceptible plants (other than P12 plants) in the

environment because the recombinant ALMV developed in this study contains only the recombinant RNA3 and sub-genomic RNA4 but not RNA1 or RNA2. This is an added attractive feature of this system.

We have demonstrated that sufficient quantities of recombinant virus containing chimeric HCV peptide can be recovered from systemically infected leaves at 20 dpi. In addition, HVR1 consensus sequence fused to ALMV CP can be recovered in a very cost-effective manner utilizing standard virus isolation procedure. Introduction of foreign sequences, however, has resulted in some attenuation of virus accumulation and severity of symptoms. Reduction of symptom severity, however, results in the accumulation of larger amounts of source material (e.g. leaves and roots). Thus, considering the quantities of virus we are able to recover, utilizing a very economical procedure (virus isolation), and this approach has great potential for producing foreign sequences in plants in a safe, time- and cost-efficient manner.

ACKNOWLEDGEMENTS

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