Molecular Chaperones of the Endoplasmic Reticulum Promote Hepatitis C Virus E2 Protein Production in Plants

by

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ABSTRACT

Infections caused by the Hepatitis C Virus (HCV) are very common worldwide, affecting up to 3% of the population. Chronic infection of HCV may develop into liver cirrhosis and liver cancer which is among the top five of the most common cancers. Therefore, vaccines against HCV are under intense study in order to prevent HCV from harming people's health. The envelope protein 2 (E2) of HCV is thought to be a promising vaccine candidate because it can directly bind to a human cell receptor and plays a role in viral entry. However, the E2 protein production in cells is inefficient due to its complicated matured structure. Folding of E2 in the endoplasmic reticulum (ER) is often error-prone, resulting in production of aggregates and misfolded proteins. These incorrect forms of E2 are not functional because they are not able to bind to human cells and stimulate antibody response to inhibit this binding. This study is aimed to overcome the difficulties of HCV E2 production in plant system. Protein folding in the ER requires great assistance from molecular chaperones. Thus, in this study, two molecular chaperones in the ER, calreticulin and calnexin, were transiently overexpressed in plant leaves in order to facilitate E2 folding and production. Both of them showed benefits in increasing the yield of E2 and improving the quality of E2. In addition, poorly folded E2 accumulated in the ER may cause stress in the ER and trigger transcriptional activation of ER molecular chaperones. Therefore, a transcription factor involved in this pathway, named bZIP60, was also overexpressed in plant leaves,

aiming at up-regulating a major family of molecular chaperones called BiP to assist protein folding. However, our results showed that *BiP* mRNA levels were not up-regulated by bZIP60, but they increased in response to E2 expression. The Western blot analysis also showed that overexpression of bZIP60 had a small effect on promoting E2 folding. Overall, this study suggested that increasing the level of specific ER molecular chaperones was an effective way to promote HCV E2 protein production and maturation.

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LIST OF ABBREVIATIONS

ATF6	activating transcription factor 6
BiP	binding immunoglobulin protein
Blp	luminal binding protein
bZIP	basic leucine zipper protein
bZIP60ΔC	C terminal truncated basic leucine zipper protein 60
CD81	Cluster of Differentiation 81
CaMV	cauliflower mosaic virus
CNX	calnexin
CRT	calreticulin
dpi	days post-infiltration
DTT	dithiothreitol
E1	HCV envelope protein 1
E2	HCV envelope protein 2
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1α
eIF2	eukaryotic translation initiation factor 2
ER	endoplasmic reticulum
ERSE	endoplasmic reticulum stress response element
GP	glycoprotein
HCV	Hepatitis C virus
HRP	horseradish peroxidase

IgG	immunoglobulin G
IRE1	inositol-requiring enzyme 1
kDa	kilo Dalton
LB	left border
LIR	long intergenic region
mE2	membrane bound envelope protein 2
mRNA	messenger ribonucleic acid
NOS	nopaline synthase
ORF	open reading frame
PBST	phosphate-buffered saline containing 0.05% Tween 20
PERK	kinase R-like ER kinase
PKR	kinase R
p-UPRE	plant – unfolded protein response element
RB	right border
Rep	replication initiator protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sE2	soluble form of envelope protein 2
SIR	short intergenic region
T-DNA	transferred deoxyribonucleic acid
TEV	tobacco etch virus
TMV	tobacco mosaic virus
UPR	unfolded protein response

Chapter 1

INTRODUCTION

Overview

Vaccination is currently regarded as the most effective way of preventing infectious diseases by the public. Indeed, many good vaccines such as influenza vaccines and the Hepatitis B virus vaccine work very well on preventing their targeted viral infections, saving thousands of peoples' lives. Therefore, in order to better protect the public from infectious diseases, more efforts are being made to develop new effective and safe vaccines against more infectious diseases, especially those that are lethal but lack prevention methods. Recombinant viral protein vaccine is one type of vaccines that uses protein components of a virus which are immunogenic but not infectious to induce immune responses in the host. They are considered safer than live or killed virus vaccines because they lack the viral nucleic acid which is responsible for viral replication. Recombinant viral proteins are mostly produced in bacteria, yeast or mammalian cells. However, sometimes these systems have their own shortages. For example, bacteria cannot produce glycosylated proteins because they lack this post-translational modification process. But a large portion of viral proteins used as vaccines are glycosylated, such as viral envelope proteins found on the outmost surface of viruses. Plants are a relatively new system used for recombinant protein vaccine production. The advantages of using a plant expression system include rapid protein

expression, easy and safe manipulation, and low cost of vaccine production and manufacturing. The goal of this project is to efficiently produce the functional envelope protein E2 of HCV using plant expression system, in order to help developing a recombinant protein vaccine against HCV. Previous studies have showed that E2 protein often folded poorly in the Endoplasmic Reticulum (ER), reducing the yield of native form of E2. The strategy we use in this study is to increase the levels of several molecular chaperones in the ER which are responsible for helping glycoprotein to fold, thereby enhancing the ER's ability to fold newly synthesized or misfolded E2 polypeptides into native proteins. The ER molecular chaperones are thought to have functions in preventing intramolecular or intermolecular aggregation, suppressing pre-matured protein degradation, and facilitating ER folding factors to catalyze protein folding. We expect that increasing ER molecular chaperone levels can help to improve the quality and the quantity of HCV E2 produced in our plant system. For the experiments, we tried either overexpressing specific ER molecular chaperones involved in glycoprotein folding, or overexpressing a transcription factor that is thought to activate several genes encoding ER chaperones and ER folding factors. We tested their effects on HCV E2 folding and production, and the results indicated that they did promote HCV E2 production in the ER.

Statement of Problem

Though HCV E2 is likely to be a potent HCV vaccine candidate, inefficient folding of E2 in the ER has become a big problem in recombinant E2 vaccine development. Researches on recombinant E2 expression have shown that E2 expression often results in significant intermolecular aggregation which is stabilized by intermolecular disulfide bond (1). The aggregation of E2 usually generates stress in cells and cause cell death. Furthermore, those high-molecular-weight aggregates are not the active form of E2, but they occupy a large portion of the products. Studies on their structures and functions are very limited, and whether or not they can induce an antibody response in the host is unknown. But, at least we know that E2 aggregates bind poorly to CD81, the putative receptor of E2 broadly expressed on human cells (2). This means that those aggregates masked their binding sites to CD81, so they were not able to stimulate neutralizing antibody production in the host to effectively block the entry of HCV into human cells. They may still induce antibody response against HCV, but the effectiveness is not thought to be as good as neutralizing antibodies. In a word, aggregation is not desired in recombinant E2 protein production; new methods are needed to improve the E2 folding pathway in cells, so that we can acquire more correct form of E2 to make vaccines.

Significance

In this work, we aimed to increase the production of properly folded HCV E2 proteins in plant system. The method we use is to over-express

several ER molecular chaperones in plants to promote HCV E2 folding. This work allows us to find ER molecular chaperones that are important for facilitating HCV E2 folding, and to better understand their roles played in E2 processing. The expected result of the work is that elevated levels of ER molecular chaperones improve the productivity of correct form of E2. This may greatly benefit HCV E2 vaccines development because it saves time and labor to express enough amount of correct form of E2 proteins, and therefore lowers the total cost for HCV E2 vaccine production later on. Moreover, if our method of promoting E2 production by increasing ER chaperone levels is successful in our plant system, we can apply it to other viral glycoprotein productions in plants too. Since many viral envelope proteins are glycoproteins and also major antigens to the host during infection, our strategy may benefit vaccine development against other viruses as well.

Chapter 2

REVIEW OF LITERATURE

Hepatitis C Virus and Its Envelope Protein Vaccine Development

Hepatitis C Virus (HCV) is a single-stranded, positive-sense RNA virus that causes infection in the liver, leading to strong inflammation. Chronic HCV infection may develop into liver cirrhosis and hepatocellular carcinoma (liver cancer) (3, 4). It is globally infected, affecting about 200 million people worldwide with 3 to 4 million new cases per year, as reported by World Health Organization. HCV is mainly transmitted by exposure to contaminated blood, and more than 60% of the infected people are not able to fully recover from infection and become chronic carriers. Unfortunately, no vaccines against HCV are available for prevention and treatment so far. Therefore, HCV vaccines are currently in urgent need, and different strategies are being tried for HCV vaccine development.

To date, primary results from animal studies show that recombinant HCV envelope proteins are promising HCV vaccine candidates because some of them can induce relatively strong antibody responses which are able to protect the host from subsequent challenge with the homologous virus (4, 5). HCV genome encodes 2 envelope proteins named E1 (gp31) and E2 (gp70). E1 and E2 are both asparagine-linked glycoproteins (N-linked glycoproteins) and they form heterodimers on the surface of HCV, serving as major antigens which can be recognized by the immune system

of the host. E1 or E2 protein alone is also antigenic. Studies have shown that the E2 protein can bind to a cell receptor called CD81 on human cells, and this interaction can be blocked by anti-E2 antibodies produced from sera of animal model *in vitro* (6). This suggested that binding of E2 to CD81 may be relevant to HCV infection. Hence, E2 becomes a promising vaccine candidate to prevent HCV infection since it is likely to induce generation of naturalizing antibodies that can protect the host by inhibiting HCV's entry to host cells. However, a big challenge to develop HCV E2 vaccine is that it is difficult to produce sufficient amount of properly folded E2 proteins, mainly because of their complicated mature structure and heavy glycosylation modifications. We will discuss this in detail in the following sections. It is known that an antigen with incorrect structure may lose its ability to stimulate host immune cells to produce antibodies against it. Thereby, researchers are making effort to create several truncated forms of E2 in order to simplify the structure of E2 while maintaining its immunogenicity (2). Optimization of the expression systems that express E2 is another strategy to increase the yield of correctly folded E2.

Roles of ER Chaperons in Glycoprotein Folding

In order to become a functionally active protein, newly synthesized polypeptides must undergo folding and assembly in the endoplasmic reticulum (ER) to obtain a unique native structure. This process is usually coordinated with post-translational modifications such as N-linked glycosylation and disulfide bond formation. An incorrect structure of a

protein may disable the protein to interact with other molecules and play its function. Therefore, efficient protein folding is significantly essential to produce a functional protein. However, in the ER, nascent polypeptide chains are very likely to misfold and aggregate themselves, especially for those proteins whose mature structures are very complex. A big reason for that is protein folding is coupled with protein synthesis. Since synthesis of protein is a sequential process (from N-terminus to C-terminus), it is possible that polypeptides near the N-terminus already folded into an incorrect structure before the complete folding information encoded in a polypeptide chain is available. Besides the inherent complicated structure of protein, the efficiency of protein folding can also be strongly reduced by high concentration of macromolecules in the ER after protein translation, leading to a crowded environment which favors intermolecular associations among polypeptide chains. This hypothesis has already been confirmed experimentally; the result indicated that the folding rate of protein decreased and the danger of aggregation increased (7). Fortunately, the lumen of the ER contains many molecular chaperones which are designed to facilitate protein folding by increasing the efficiency of the folding process. They transiently bind to nascent and incompletely folded polypeptide chains, and release them in a regulated manner, preventing them from incorrect interactions. Therefore, the role of ER chaperones is thought to be preventing the tendency of aggregation

between non-native polypeptide chains, thereby ensuring efficient protein folding (8).

In the ER, there are different types of molecular chaperones that help protein folding, including general chaperones, lectin chaperones and non-classical chaperones. Among them, two lectin chaperons, calnexin and calreticulin, are major chaperones specifically facilitating glycoprotein folding. Calnexin is an ER resident membrane protein, and calreticulin is its soluble homolog in the ER lumen (9, 10). They preferentially and transiently associate with newly synthesized N-linked glycoproteins in a regulated manner, mainly due to their lectin-like affinity for monoglucosylated oligosaccharides (Glc1Man9GlcNAc2) found on pre-mature N-linked glycoproteins (11). Calnexin and calreticulin do not have a binding site on the correctly folded matured glycoprotein. After proteins are synthesized in the ER, glycans with three external glucose residues are linked to the asparagine residues of nascent proteins. The three glucose molecules are then trimmed by ER located enzyme glucosidase I and glucosidase II sequentially to make a mature glycoprotein that will export from the ER. Therefore, only the processing intermediate containing one glucose molecule can be recognized by calnexin and calreticulin. If a glycoprotein is misfolded, an ER-resident enzyme called UDP-glucose:glycoprotein glucosyltransferase (UGGT) can reglucosylate the N-linked glycan so that the glycoprotein can be re-associated with calnexin and calreticulin. How this binding cycle promotes glycoprotein

folding is yet to be studied. Some studies on folding of influenza hemagglutinin (HA), which is also an N-linked glycoprotein, demonstrated that calnexin and calreticulin bound to different but overlapping folding intermediates of influenza HA, slowing down the protein folding and assembly process, but increased the overall efficiency of HA maturation because of less aggregation and degradation of HA. They suggested that calnexin and calreticulin promoted protein folding by facilitating retention of misfolded proteins in the ER, and by preventing aggregation and degradation of incompletely folded proteins (12, 13).

Another important ER chaperon that helps glycoprotein folding is called Binding immunoglobulin protein (BiP), which is also called 78 kDa glucose-regulated protein (GRP-78) or heat shock 70 kDa protein 5. It is a general ER chaperone, so it does not specifically modulate glycoprotein folding. However, BiP is a central stress regulator of the ER. The expression of BiP protein can be remarkably induced by accumulation of unfolded or misfolded proteins in the ER, in order to promote protein folding and oligomerization. As other heat shock 70 kDa proteins, BiP is an ATPase which couples ATP hydrolysis to the binding and release of proteins. BiP has a peptide binding domain at C-terminus and an ATPase domain at N-terminus. When the ATPase domain of BiP interacts with ATP and triggers hydrolysis, a conformational change occurs at its C-terminal peptide binding domain and allows it to bind to unfolded or misfolded proteins. BiPs are thought to have high affinity binding to

hydrophobic regions that are exposed by non-native proteins. Under this condition, protein disulfide isomerase in the ER can come to catalyze incorrect disulfide bond reduction and correct disulfide bond formation of the trapped protein. After that, exchange from ADP to ATP at the N-terminus of BiP results in releasing the refolded protein to the ER environment (14). A corrected folded protein will no longer be targeted by BiP proteins. Hence, in regards to helping protein folding, the function of BiP is to stabilize the non-native structures of proteins until they can undergo subsequent folding, and to minimize incorrect interaction between molecules by shielding exposed hydrophobic regions of polypeptides.

Overall, calnexin, calreticulin and BiP have their own roles to promote glycoprotein folding in the ER. Particularly, the lectin binding site of calnexin was shown to have a significant advantage over BiP in suppressing aggregation of glycoproteins, indicating the importance of lectin-glycan binding in facilitating glycoprotein folding (15).

ER Stress Response in Plants

ER stress response, also known as unfolded protein response (UPR), is a conserved mechanism used by all eukaryotic cells to relieve the “ER stress” caused by accumulation of unfolded or misfolded proteins in the ER lumen (16). It triggers the protein quality control system to attenuate global protein translation and degrade proteins in the ER. It also induces a signaling pathway that result in up-regulation of ER molecular chaperones to promote protein refolding. If the ER stress cannot be relieved by these

actions, the programmed cell death will be activated. In mammalian cells, ER stress is generally sensed by three transmembrane proteins located in the ER: protein kinase R (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (17, 18). Under stress condition, the three sensors are activated to perform their functions in ER stress response. PERK acts on the eukaryotic translation initiation factor 2 (eIF2), leading to attenuation of protein translation (19). ATF6 moves to Golgi bodies and is cleaved by S_1P and S_2P proteases there, releasing its N-terminal domain to the nucleus to activate UPR genes such as genes encoding ER chaperone (20). IRE1 not only directs UPR genes activation but also specifically induces protein degradation (21).

In plants, although little information about ER stress response is available, two IRE1-like proteins and two ATF6-like stress transducers bZIP60 and bZIP28 have been identified (22). The function of plant IRE1 proteins as transcription inducer is yet to be determined, but the functions of basic leucine zipper (bZIP) transcription factor 60 and 28 have recently been characterized in *Arabidopsis* (23, 24). Both *Arabidopsis* bZIP60 (AtbZIP60) and bZIP28 (AtbZIP28) are type II transmembrane proteins localized in the ER membrane when they are inactive. When cells are stressed, they are activated by proteolytic cleavage of N-terminal domain at cytoplasmic side, and the free active forms of AtbZIP60 and AtbZIP28 are then translocated to the nucleus, at where they function as

transcription factors to induce expression of multiple UPR genes, including BiP, by binding to the ER stress response element (ERSE) or plant UPR element (p-UPRE) in their promoters (23, 24). Although both AtbZIP60 and AtbZIP28 are activated in response to ER stress, the activation of AtbZIP60 is much stronger than AtbZIP28. In addition, AtbZIP60 and AtbZIP28 proteins show little homology. Furthermore, AtbZIP28 contains S_1P and S_2P protease sites in its protein sequence, which suggests that it is cleaved by S_1P/S_2P system in the Golgi apparatus. Nevertheless, bZIP60 does not contain S_1P and S_2P sites, and its cleavage is not affected by mutations in the genes encoding Arabidopsis S_1P and S_2P proteases, indicating a different cleavage mechanism (25). Recently, Chika Tateda (2008) and his group reported a homolog of AtbZIP60 found in *Nicotiana tabacum*, named NtbZIP60 (26). The study showed that it had similar functions to AtbZIP60. Activated by ER stress, its N-terminal domain was cleaved and released from ER membrane, targeting to the nucleus. It could also transactivate the reporter gene containing p-UPRE cis-elements.

HCV Envelope Protein E2 Production in the ER

HCV envelope protein E2 is an N-linked glycoprotein with 11 glycosylation sites. It interacts non-covalently with the other HCV envelope protein E1 to form a heterodimer on the surface of HCV (27). Due to the complicated 3D structures of E2, its folding process and the subsequent E1-E2 complex assembly process in the ER is rather slow and error-prone.

Generally, significant aggregation and consequent degradation of unfolded and misfolded E2 proteins are shown during E2 maturation, which reduces the folding efficiency and increases the ER stress. Some studies suggested that this tendency of aggregation was intrinsic, not because of over-production of E2 proteins in the ER (28). Interaction of E2 with calnexin, calreticulin and BiP has already been reported in mammalian cells, suggesting important roles of the three ER chaperones in helping E2 folding (29). However, over-expression of each chaperone did not increase the level of native E1-E2 complexes in that study. Another research showed that high level of E2 could modulate the ER stress response by inhibiting the PERK pathway induced protein translational attenuation, so that it could promote its own synthesis (19). As a result, large amounts of non-native proteins together with inefficient protein folding make HCV envelope protein E2 very toxic to host cells.

Chapter 3

RESEARCH HYPOTHESIS

HCV E2 protein is a slow-folded glycoprotein and seems to have an intrinsic tendency of aggregation. Hence, we want to find ways to increase its folding efficiency so that we can acquire more non-aggregated and functional E2 proteins when we express them in plants. Since folding of proteins requires important assistance from the ER molecular chaperones, we hypothesize that overexpression of the ER molecular chaperones that are particularly essential to help glycoprotein folding will help to express more functional HCV E2 by increasing the efficiency of protein folding. Such molecular chaperones in the ER include calnexin and calreticulin. They take advantage over other ER molecular chaperones to facilitate glycoprotein folding mainly due to their lectin sites with a high affinity for the N-linked glycan side chains of glycoproteins. This interaction allows calnexin and calreticulin to suppress aggregation of some glycoproteins more effectively than other general molecular chaperones (15). Therefore, according to our hypothesis, we predict that overexpression of calnexin, calreticulin or both of them in our plant expression system will increase the yield of E2 and improve the quality of E2.

In addition to the overexpression of those two particular molecular chaperones, induction of several chaperons expressions involved in UPR by bZIP60 may also have a significant effect on folding nascent polypeptide chains and refolding the misfolded proteins. This is because

bZIP60 can activate many ER molecules responsible for protein folding at the same time, including BiP and calnexin, according to the studies done in *Arabidopsis* (30). These molecules can work together to promote protein folding to reduce the stress in cells. Hence, we have another hypothesis that is overexpression of bZIP60 in plant cells will make those cells more sensitive to the ER stress generated by E2 expression, and immediately induce more UPR genes expression such as BiP to participate the folding of newly synthesized and misfolded E2, therefore increasing the amount of properly folded E2 proteins.

Chapter 4

MATERIALS AND METHODOLOGY

Research Design

Although calnexin and calreticulin share functions in glycoprotein folding, a major distinction between them is that calnexin is a membrane protein but calreticulin is soluble in the ER lumen. This may has an effect on the type of protein they interact with, because some studies indicated that calnexin preferentially associated with membrane bound protein-folding intermediates rather than their truncated soluble forms (31). Therefore, we decide to test our first hypothesis on two versions of recombinant E2 proteins, one is a truncated soluble version lacking the transmembrane domain (sE2), and the other is a full-length insoluble version containing the membrane anchor (mE2). We planned to co-express *Arabidopsis* calreticulin and sE2 in leaves of *Nicotiana benthamiana* which is the plant model we use to express HCV E2 by transient transformation, and compare the resulting amount of total sE2 and properly folded sE2 to that from plant leaves expressing sE2 alone by Western blot. Same strategy also applied to co-expression of *Arabidopsis* calnexin and mE2, and the level of total and non-aggregated mE2 were measured and compared to that from leaves only express mE2.

To test our second hypothesis, bZIP60 cDNA was cloned from wild type *N. benthamiana* leaves to make the DNA construct overexpressing *N. benthamiana* bZIP60 (NbbZIP60). We also used the same method to

generate a DNA construct overexpressing the putative active form of NbbZIP60 which did not have the transmembrane domain and the C-terminal domain. The rationale is that the truncated NbbZIP60 (NbbZIP60ΔC) may activate the targeted UPR genes more efficiently because they are free to enter the nucleus, independent of ER stress. The NbbZIP60 or NbbZIP60ΔC construct was then transformed into plant leaves together with the construct expressing soluble form of E2. The leaves expressing these transgenes would transiently have an increased level of NbbZIP60 or NbbZIP60ΔC in the ER and also high level of the soluble HCV E2 proteins. Then we could determine the effect of overexpression of NbbZIP60 or NbbZIP60ΔC on E2 folding and production by comparing E2 protein level in NbbZIP60 overexpressed and normally expressed leaves. We could also compare the quantity and quality of E2 produced in NbbZIP60 overexpressed leaves to that produced in NbbZIP60ΔC overexpressed leaves, so that we could decide whether their effects on HCV E2 folding and accumulation are different. In addition to the usage of NbbZIP60 and NbbZIP60ΔC, we also tried AtbZIP60 and AtbZIP60ΔC in the same way to test their effects on HCV E2 folding and production.

Construction of Expression Vectors Used in This Study

In this study, a soluble form of HCV E2 and a membrane anchored HCV E2 were constructed in germiniviral replicon vectors. Calreticulin, calnexin, bZIP60 and bZIP60ΔC from *Arabidopsis*, and bZIP60 and

bZIP60ΔC from *N. benthamiana* were constructed in non-viral vectors. A schematic representation of the T-DNA region of the vectors was shown in figure 1.

Construction of Germiniviral vectors. pBYRsE2-711H (obtained from H. Mason, Arizona State University) contains the HCV E2 coding sequence truncated to use residues 384-711 of the HCV polyprotein, with a sequence encoding the peptide "HHHHHHDEL" added to its C-terminus. It contains the native HCV E2 signal peptide at its N-terminus. The plant-optimized coding sequence was based upon the native HCV sequence (Genbank accession M62321) and designed to use codons preferred by *N. tabacum* and to remove spurious mRNA processing signals. The coding sequence was amplified by high-fidelity PCR using primers sE2-Xba-F (5'-agcttctagaacaatggttggaaactggg) and sE2-711-Nhe-R (5'-cccgctagcaatacttgatcccacac) to create XbaI at 5' and NheI at 3', and ligated with annealed oligonucleotides Nhe-6HDEL-Sac-F (5'-ctagccaccatcaccatcaccatgacgagctttaagagct) and Nhe-6HDEL-Sac-R (5'-cttaaagctcgtcatggtgatggtgatggtgg). The resulting coding sequence was inserted into a geminiviral replicon pBYR1 (32) similar to pBYGFP.R (33).

pBYRsE2TR (obtained from H. Mason) contains the full-length HCV E2 coding sequence for residues 384-746 of the HCV polyprotein, including the C-terminal membrane anchor domain. It contains the native HCV E2 signal peptide at its N-terminus. The plant-optimized coding

sequence (H. Mason, unpublished) was inserted into a geminiviral replicon similar to pBYGFP.R (33).

Construction of ER chaperone vectors. pBYR-AtCRT was constructed by H. Mason as follows. The coding sequence of *Arabidopsis* calreticulin (AtCRT, Genbank accession number NM_104513) was amplified by high-fidelity PCR using primers AtCRT-Xba-F (5'-cctctagaacaatggcgaaactaaaccctaaa) and AtCRT-Kpn-R (5'-ggGGTACCttaaagctcgtcatgggcg) on the template pUNI-15759 (obtained from The Arabidopsis Information Resource Center, http://www.arabidopsis.org/index.jsp, stock U15759), digested with XbaI and KpnI and inserted into a geminiviral vector pBYR2 (32), to yield pBYR-AtCRT. The coding sequence was released from pBYR-AtCRT by XbaI and SacI restriction enzymes, digesting at 37°C for 2 h. The XbaI-SacI fragment of the binary vector psNV120 (obtained from H. Mason) was obtained by the same method, allowing *AtCRT* fragment to be inserted into psNV120 at XbaI and SacI sites to yield the vector psAtCRT-ext. The resulting AtCRT expression cassette contained the double enhancer cauliflower mosaic virus (CAMV) 35S promoter (2x35S) with tobacco mosaic virus (TMV) 5'UTR, AtCRT coding sequence and tobacco extensin 3' UTR.

To obtain *Arabidopsis* calnexin (AtCNX) expression vector, H. Mason first constructed pBYR-AtCNX as follows. The cDNA region of AtCNX

(Genbank accession number NM_120816) was amplified from pENTR- (TAIR stock U16625, Genbank accession AY059880) with primers AtCNX-Xba-F (5'- cctctagaacaatgagacaacggcaactattttc) and AtCNX-Kpn-R (5'- ggggtaccttgttctaattatcacgtctcg), digested with XbaI and KpnI, and inserted into the geminiviral replicon vector pBYR2, to yield pBYR-AtCNX. The coding sequence was released from pBYR-AtCNX vector (obtained from H. Mason) by digestion at XbaI and KpnI sites. The resulting fragment was inserted into the psAtCRT-ext vector at the corresponding digestion sites, replacing the *AtCRT* fragment in the vector to yield psAtCNX-ext.

For overexpression of NbbZIP60 and NbbZIP60ΔC, cDNA regions encoding full length of NbbZIP60 and truncated NbbZIP60 (amino acid positions 1-212) were amplified by Phusion® high-fidelity DNA polymerase (FINNZYMES) in PCR from total cDNA of wild type *N. benthamiana* leaves. The primers (see Appendix B, primer list) for *NbbZIP60* amplification were NbbZIP60-Nco-F which added an NcoI site at the 5' end, and NbbZIP60-SacI-R which added a SacI site at the 3' end. The primers for *NbbZIP60ΔC* amplification were NbbZIP60-Nco-F and NbbZIP60-S212 which added a SacI site at the 3' end. Since the coding sequence of *NbbZIP60* is not deposited in the Genbank, the primers were designed according to the *NtbZIP60* cDNA sequence (Genbank accession number AB281271), which was thought to share more than 96% sequence homology with *NbbZIP60* (26). The PCR products were digested by NcoI and SacI restriction enzymes and then inserted into

pIBT210.3 (34) at NcoI and SacI sites respectively. The resulting constructs were separately transformed into *E. coli* DH5α competent cells by electroporation to confirm the insertion and send for sequencing. Sequencing result showed that the *NbbZIP60* we obtained had a 95% homology to the *N. tabacum bZIP60* cDNA sequence. The constructs were then digested with NcoI and SacI restriction enzymes, releasing the *NbbZIP60* and *NbbZIP60ΔC* fragments. The binary vector pGPTV-Kan (35) was digested with BamHI (blunted by filling in with Klenow enzyme) and SacI restriction enzymes, and ligated with the *NbbZIP60* or *NbbZIP60ΔC* NcoI-SacI fragment and the PvuII-NcoI fragment from pGPTV-Kan containing the nopaline synthase (Nos) promoter, thus yielding plasmids with the coding sequences between Nos promoter and Nos 3' UTR, and they were called pNosNbZ60 and pNosNbZS212.

For AtbZIP60 and AtbZIP60ΔC expression vectors, cDNA regions encoding AtbZIP60 (full length) and AtbZIP60ΔC (truncated, 1-216) were amplified from the vector pUni51-AtbZIP60 (TAIR, The Arabidopsis Information Resource, http://www.arabidopsis.org/index.jsp stock number 4775801) by Phusion® high-fidelity DNA polymerase in PCR, using the primer pUni51-F and AtbZIP60-Kpn-R which added a KpnI site at the 3' end for AtbZIP60, and the primer pUni51-F and AtbZIP60-S216-K which added a KpnI site at the 3' end for AtbZIP60ΔC. The vector pIBT210.3 and the PCR products were respectively digested by NcoI and KpnI restriction enzymes, and the resulting *AtbZIP60* and *AtbZIP60ΔC* fragments were

separately ligated to pIBT210.3. The resulting constructs were transformed to *E. coli* DH5α competent cells and verified by PCR and digestion by NcoI and KpnI enzymes. After that, the *AtbZIP60* and *AtbZIP60ΔC* fragments were released from the constructs by NcoI and KpnI restriction digestion and inserted into a binary vector pPS1 respectively at the corresponding restriction sites, yielding psAtbZIP60 and psAtbZIPS216. The expression cassette contained the double enhancer cauliflower mosaic virus (CAMV) 35S promoter (2x35S), tobacco mosaic virus (TMV) 5'UTR, *AtbZIP60* or *AtbZIP60ΔC* cDNA sequence, and soybean vspB gene 3' element.

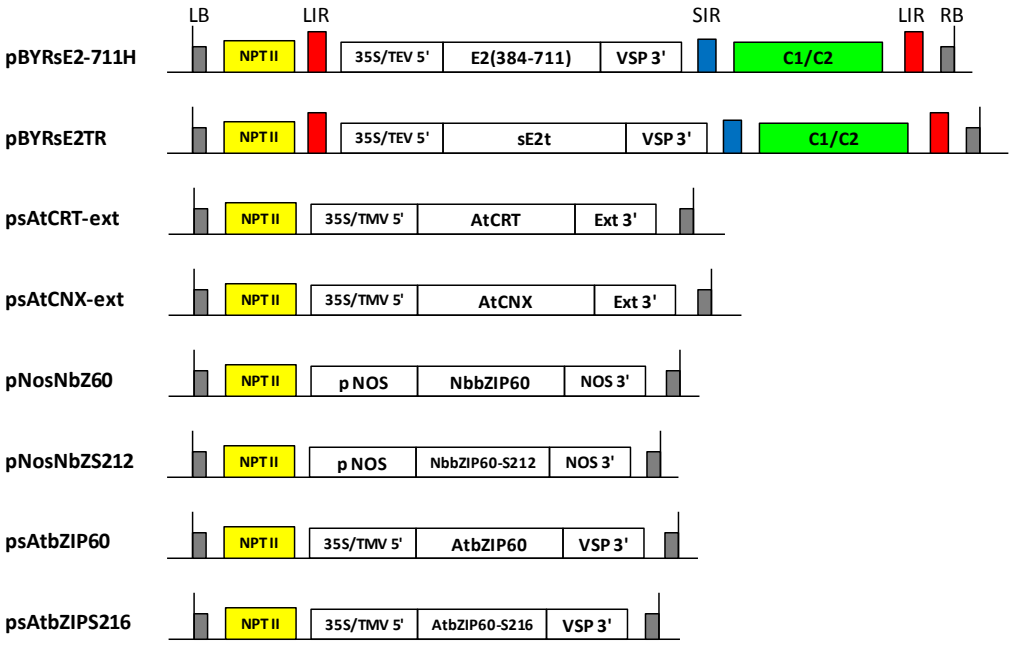

Figure 1. Schematic representation of the T-DNA region of the vectors used in this study. 35S/TEV5': CaMV 35S promoter with tobacco etch virus 5'UTR; VSP3': soybean vspB gene 3' element; 35S/TMV5': CaMV 35S promoter with tobacco mosaic virus 5'UTR; Ext 3': tobacco extensin 3' UTR; p NOS: nopaline synthase promoter; NOS 3': nopaline synthase 3' UTR; NPT II: expression cassette encoding nptII gene for kanamycin resistance; LIR: long intergenic region of bean yellow dwarf geminivirus (BeYDV) genome; SIR: short intergenic region of BeYDV genome; C1/C2: BeYDV ORFs C1 and C2, encoding Rep and RepA for viral replication; LB and RB: the left and right border of the T-DNA region.

Plant Materials and Agroinfiltration of Expression Vectors

Germiniviral vectors and non-replicating binary vectors were separately introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The resulting strains were verified by colony screening using PCR and restriction digestion of plasmids. Then they were grown in liquid culture medium for 1 to 2 days to be ready for agroinfiltration. 6 to 7 weeks old greenhouse-grown *N. benthamiana* plants were used as our expression host. For infiltration, the Agrobacteria were span down by centrifugation at 5000 rpm for 6 min and resuspended in infiltration buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.5 and 10 mM $MgSO_4$) to OD_{600} = 0.2. The plant leaves were then inoculated with one or mixed *Agrobacterium* strains by needle infiltration. The agroinfiltration procedure was performed as previously described (33). Infiltrated plants were maintained in a growth chamber for several days to allow transgenes expression.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from infiltrated plant leaves 48 h after infiltration, using a plant RNA purification reagent (Invitrogen) and chloroform:*iso*amyl-alcohol (24:1). Then the RNA was precipitated in isopropyl alcohol at room temperature for 10 min. The RNA pellet was washed with 75% ethanol and resuspended in 50 µl DEPC-treated water. The residual DNA in the RNA sample could be removed by DNase

included in the TURBO DNA-free™ system (Ambion) according to the manufacturer's instruction.

To perform RT-PCR, first-strand cDNA were synthesized from 1 μg purified total RNA using oligo$(dT)_{20}$ primer included in the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instruction. 2 μl of cDNA sample were directly used as templates in the PCR to amplify desired transcripts using gene-specific primer sets. RNA without reverse transcriptase was also amplified by PCR to confirm no genomic DNA contamination in samples.

Protein Extraction and Western Blot

Soluble proteins were extracted by grinding 100 to 200mg of leaf sample in 0.5 ml extraction buffer (20mM Tris pH8.0, 20mM KCl, 1mM EDTA, 0.1% Triton X-100, 50mM sodium ascorbate, 10μg/ml leupeptin) using the bullet blender® (Next Advance). The resulting leaf crude extracts were held on ice for 1 h to allow fully extraction. Then they were centrifuged at 12,000 rpm and 4℃ for 15 min and the supernatants were transferred to new tubes for subsequent analysis by Western blot. To extract proteins in the pellet of leaf crude extracts, same amount of the extraction buffer was added to the pellet to resuspend it. Total protein amount in a sample was determined by the Bradford assay (BIO-RAD). Usually, 15 μg of proteins per sample were added in the SDS-PAGE sample buffer either with 150mM DTT reducing reagent or without it, and

then loaded onto 4–15% gradient polyacrylamide gels for separation. Equivalent loading of total proteins in each sample to the gel was determined by Coomassie blue staining of the gel, and proteins separated on the gel could also be transferred to a polyvinlidene difluoride (PVDF) membrane (Amersham, NJ) for Western blot analysis. To detect denatured E2 proteins, the membrane was incubated with mouse monoclonal anti-E2 antibody against a linear epitope (Chiron/Novartis) diluted at 1:10000 in 1% skim milk in PBST at 37℃ for 1h, after washing the membrane with PBST for 4 times, the membrane was then incubated with goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate (Sigma) diluted at 1:5000 in 1% skim milk in PBST at 37℃ for another hour. To detect conformational E2 proteins, the membrane was probed with mouse monoclonal anti-E2 antibody against a conformational epitope (Chiron/Novartis) diluted at 1:5000 in 1% skim milk in PBST at 37℃ for 1h, and then they were washed with PBST for 4 times and detected with goat anti-mouse IgG-HRP conjugate diluted at 1:5000 in 1% skim milk in PBST at 37℃ for another hour. Finally, the membranes were washed again with PBST for 4 times and developed by chemiluminescence using ECL plus detection reagent (Amersham, NJ).

Chapter 5

RESULTS

HCV E2 transient expression in *Nicotiana benthamiana* leaves

Since no studies about HCV E2 expression in plants have been reported, we did a time course study to examine the expression of the soluble form of HCV E2 (sE2) in *N. benthamiana* leaves. The germiniviral vector pBYRsE2-711H containing the sE2 coding sequence was introduced into *Agrobacterium* GV3101, which was later infiltrated into 6 weeks old *N. benthamiana* leaves at the concentration of OD_{600} 0.2. The procedure of infiltration was previously described (33). An empty germiniviral vector without *E2* DNA called BYR1 was treated at the same way and was used as a negative control. The germiniviral vector contains the viral Rep protein (C1/C2 gene) cassette which is required for viral replicon amplification (36). The sE2 expression cassette, driven by the dual-enhancer CaMV 35S promoter, is inserted between the long intergenic region (LIR) and the short intergenic region (SIR) in the viral-sense orientation, replacing the viral movement and coat protein genes. When delivered into plant host, the viral vector can self-splice and become a viral replicon to highly express sE2 protein. We included three plants in the experiment to average the variability effects from plants. The expression of sE2 was monitored until 20 days post-infiltration (dpi). Leaf samples were harvested at 4, 8, 10, and 12 dpi and used for protein analysis. As shown in Figure 2, we observed that necrosis occurred in *N.*

benthamiana leaves after 3 dpi and become pretty strong from day 4 or 5, depending on the growth condition of plants. This indicated that expression of the soluble form of HCV E2 was very toxic to plant leaves and also suggested us to harvest leaf samples at an early time before global protein degradation occurred.

Figure 2. Phenotype observation of a leaf spot expressing soluble HCV E2 using pBYRsE2-711H at 4, 6, 8, and 10 days post infiltration. The upper leaf spot was infiltrated with an empty viral vector to be set as a negative control (pBYR1) for determination of the phytotoxic effect.

To determine the amount and confirmation of plant-derived sE2, we used the method of Western blot to detect denatured sE2 and conformational sE2 respectively. We extracted total soluble proteins from leaf samples at day 4, 8, 10 and 12 after infiltration and used 15μg of total soluble proteins from each sample for Western blot analysis. Correctly folded sE2 can be detected from total soluble protein samples by a conformation-sensitive mouse anti-E2 antibody (anti-conformational E2 antibody). The total amount of sE2 in the samples, including unfolded and misfolded molecules, can be determined by a mouse antibody targeting a linear epitope on E2 (anti-linear E2 antibody), but this required the protein samples to be denatured by DTT and boiling in order to expose the linear epitope. The results of Western blot for denatured sE2 and conformational sE2 are shown in Figure 3. On the blot for detecting denatured sE2, we observed a decrease of sE2 signal over time, indicating that protein degradation occurred at sometime between 4 and 8 dpi. The predicted weight of monomeric sE2 is about 37kDa for the unglycosylated molecule (http://www.expasy.org/cgi-bin/pi_tool), but with 11 potential N-linked glycosylation sites (N-X-S/T), glycosylated molecules could be as much as 20kDa larger (~57kDa). We observed a large amount of sE2 aggregates >57kDa at 4 dpi, which suggested low quality of sE2 production. Those high-molecular-weight aggregates seemed to be less stable as sE2 dimers and trimers, because they degraded faster than the dimers and trimers. In contrast, on the blot for detecting conformational

sE2, we saw an increase of sE2 signal at 8, 10, and 12 dpi compared to that at 4 dpi, although the total sE2 signal intensity was weaker than that of denatured sE2. This result firstly showed that only a small portion of sE2 produced in leaves were folded into their correct structures. In other words, the folding efficiency of sE2 is low. Secondly, the result indicated that sE2 folding was slow in the ER because the sE2 signal at 4 dpi was still rather weak. More correctly folded sE2 could be obtained from day 8 samples after infiltration, with a price of reducing the total yield of sE2 due to protein degradation. In all, plant expressed sE2 folded slowly and poorly in the ER; they tended to form aggregates, resulting in degradation of large portions of sE2 produced in leaf cells.

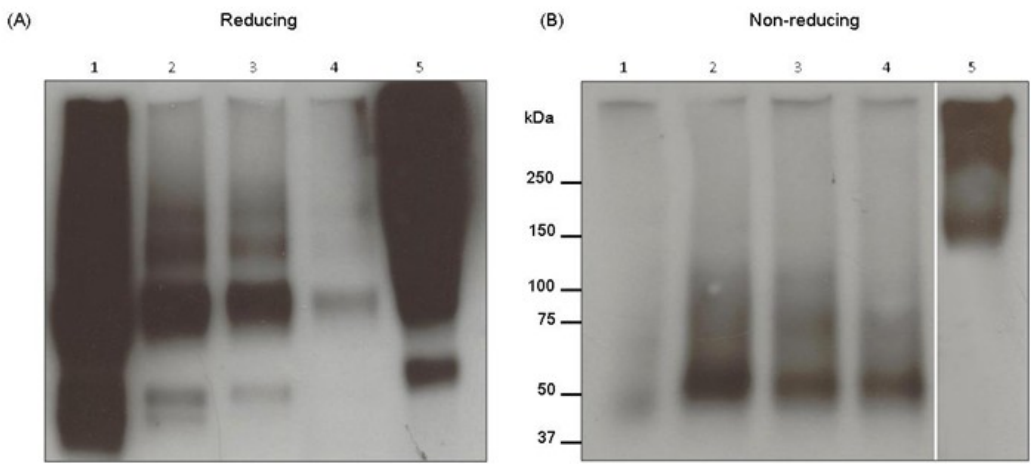

Figure 3. Western blot analysis of soluble forms of HCV E2 production in *N. benthamiana* leaves. (A) Analysis of denatured sE2 using a mouse linear E2 antibody. Lane 1 to 4: denatured total soluble proteins of crude leaf extracts from samples harvested at 4, 8, 10 and 12 dpi. Lane 5: purified E2-IgG heavy chain fusion protein, denatured (positive control). (B) Analysis of conformational sE2 using a mouse conformational E2 antibody. Lane 1 to 4: total soluble proteins of crude leaf extracts from samples harvested at 4, 8, 10 and 12 dpi. Lane 5: purified E2-IgG heavy chain fusion protein (positive control, obtained from H. Mason, expressed with vector pBYR-E2H2).

Increased Expression of HCV E2 with the Help of *Arabidopsis* calreticulin and calnexin

Expression of *Arabidopsis* calreticulin and calnexin in *N. benthamiana*. In order to express *Arabidopsis* calreticulin and calnexin in *N. benthamiana*, the coding sequences of the AtCRT and AtCNX were inserted into the non-replicating binary vector pPS1, driven by the dual-enhancer CaMV 35S promoter. The vspB 3' UTR element was replaced by extensin 3' UTR to improve the functions of 3' UTR. The expressions of *AtCRT* and *AtCNX* in leaves were measured by reverse-transcription PCR (RT-PCR) using primers AtCRT-Xba-F and AtCRT-Kpn-R for *AtCRT*, and primers AtCNX-Xba-F and AtCNX-Kpn-R for *AtCNX*. RNA was extracted from 100 mg leaves expressing calreticulin or calnexin 48 h after agroinfiltration. RNA extraction and purification from each sample were performed in the same way at the same time. The RT-PCR products were observed on agarose gels by electrophoresis (Figure 4). The electrophoresis result showed that *AtCRT* and *AtCNX* were successfully expressed in *N. benthamiana* leaves, and their expression did not cause necrosis of plant leaves (data not shown).

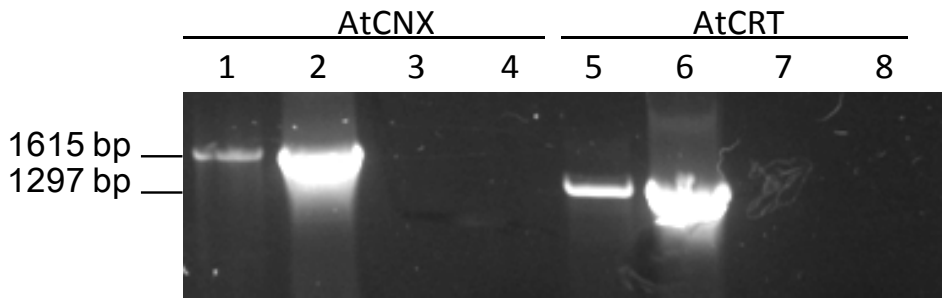

Figure 4. RT-PCR showing the abundance of *AtCNX* and *AtCRT* transcripts in samples infiltrated with psAtCRT-ext or psAtCNX-ext at 2 dpi. Lane 1: leaf infiltrated with psAtCNX-ext; Lane 2: pBYR-AtCNX plasmid, positive control; Lane 3: wild type leaf; Lane 4: negative control. Lane 5: leaf infiltrated with psAtCRT-ext; Lane 6: pBYR-AtCRT plasmid, positive control; Lane 7: wild type leaf; Lane 8: negative control. *AtCNX* was amplified using primers AtCNX-Xba-F and AtCNX-Kpn-R; *AtCRT* was amplified using primers AtCRT-Xba-F and AtCRT-Kpn-R.

Co-expression of the soluble form of HCV E2 with *Arabidopsis* calreticulin. The calreticulin construct and the sE2 construct were co-infiltrated into 6 to 7 weeks old leaves at 1:1 ratio to study the effect of calreticulin on sE2 production and structure. Two types of control were also infiltrated on the same leaves; one was an empty vector pPS1 and the other was sE2 construct plus pPS1 vector for expression of sE2 alone. The final OD_{600} value of *Agrobacterium* was 0.2 for all the three treatments, which means for those *Agrobacterium* with mixed constructs, the OD_{600} value for each construct was 0.1. We monitored the expression pattern of sE2 on leaves of three plants for 8 days after infiltration. We noticed that with calreticulin treatment, sE2 expression caused even stronger necrosis of leaf cells than sE2 expression alone (Figure 5). The necrosis began at 3 dpi and developed very quickly on the following day. At day 6 after infiltration, the whole infiltrated area turned yellow and was mostly dried, whereas the leaf spot expressing sE2 alone had much fewer yellow spots in the infiltrated area. The negative control spots infiltrated with pPS1 did not show any necrosis.

sE2 and sE2/calreticulin leaf samples were harvested at 4 dpi and 8 dpi for protein analysis. 15 µg of total soluble proteins extracted from each leaf sample were added in the SDS-PAGE sample buffer either with 150mM DTT reducing reagent or without it. For analysis of total sE2 level, the reduced protein samples were further boiled for 10 min so that they could be linearized and recognized by the anti-linear E2 antibodies in the

Western blot analysis. On the other hand, to analyze the conformation of sE2, the non-reduced protein samples were directly used in the Western blot to be recognized by the anti-conformational E2 antibodies. The result of the reducing Western blot (Figure 6A) showed that sE2/calreticulin co-expressing samples produced higher amount of sE2 than sE2/pPS1 samples at both day 4 and day 8 after infiltration. Also, compared of day 4 to day 8 samples, the degree of protein degradation was less in sE2/calreticulin samples than that in sE2/pPS1 samples. These indicated that calreticulin played a role in preventing protein degradation so that more sE2 could be accumulated in leaves. On the non-reducing Western blot (figure 6B), we observed higher amount of correctly folded sE2 in sE2/calreticulin samples compared to that in sE2/pPS1 samples at day 4 and day 8, with a higher amount in day 8 samples than in day 4 samples. We could also see some high molecular weight bands suggesting there were multimers of sE2 in day 4 samples, but they were gone in day 8 samples. This may be because some sE2 were not fully folded at 4 dpi so their hydrophobic regions could still interact with others, although they were in the correct folding track and already formed the conformational epitope, which could be detected by the anti-conformational E2 antibody. In summation, calreticulin greatly increased the yield of sE2 in plant leaves from early time point and efficiently suppressed protein degradation which was normally observed in sE2 expression. It also helped accumulation of

more of the correct form of sE2 at early time points, suggesting its role in facilitating protein folding.

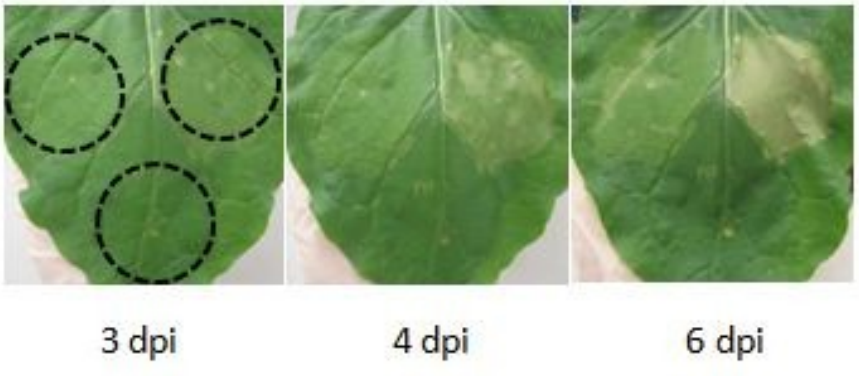

Figure 5. Phenotype observation of leaf spots at 3, 4 and 6 days post infiltration expressing soluble HCV sE2 alone (upper left), soluble HCV sE2 and calreticulin (upper right), and negative control (bottom). The upper left spot was co-infiltrated with pBYRsE2-711H and pPS1 at 1:1 ratio, the upper right spot was co-infiltrated with pBYRsE2-711H and psAtCRT-ext at 1:1 ratio, and the bottom spot was infiltrated with pPS1. The total OD_{600} for infiltration was 0.2.

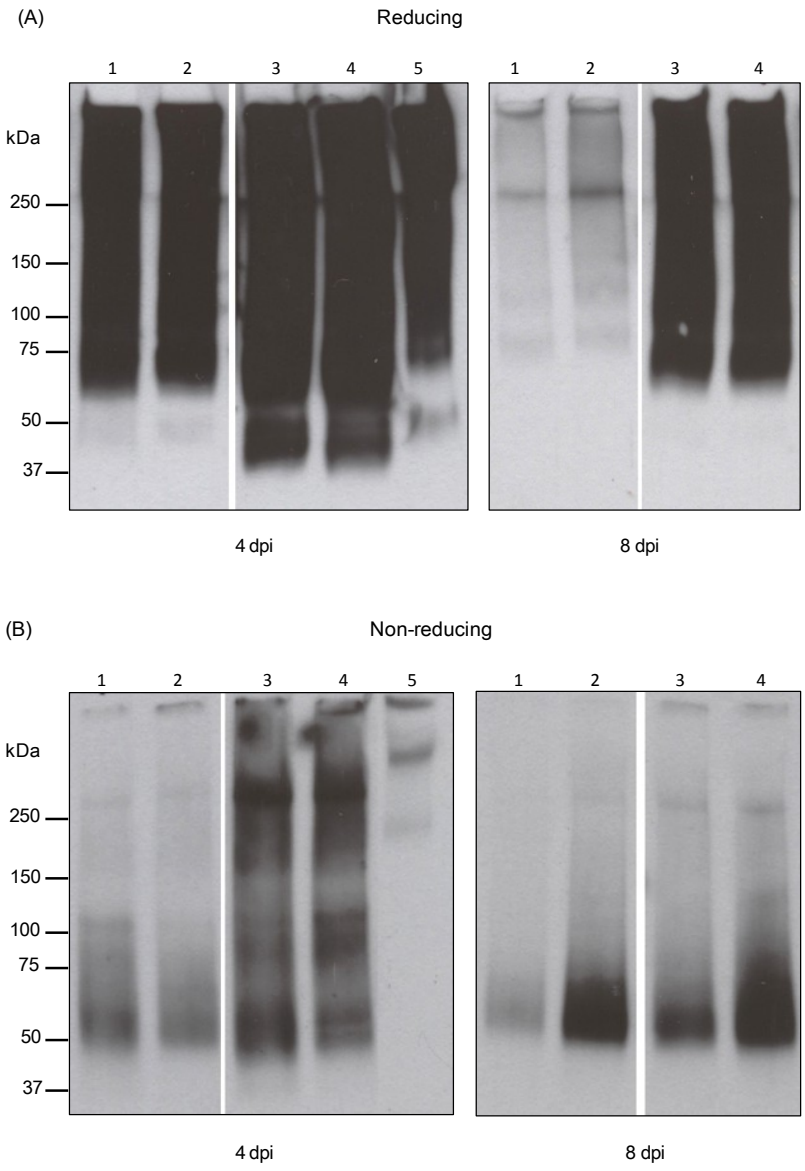

Figure 6. Western blot analysis of soluble form of HCV E2 production with or without co-expression of *Arabidopsis* calreticulin in *N. benthamiana* leaves. (A) Reducing Western blots comparing denatured sE2 levels in sE2/pPS1 samples and sE2/calreticulin samples from same leaves harvested at 4 dpi and 8 dpi, using mouse anti-linear E2 antibodies. Protein samples were denatured in SDS sample buffer containing 150 mM

DTT and boiling. (B) Non-reducing Western blots comparing correct folded sE2 levels in sE2/pPS1 samples and sE2/calreticulin samples from same leaves harvested at 4 dpi and 8 dpi, using mouse anti-conformational E2 antibodies. Protein samples were mixed with SDS sample buffer without DTT and were not boiled. Lane 1 and 2: soluble protein extracts from two different sE2/pPS1 samples. Lane 3 and 4: soluble protein extracts from two different sE2/calreticulin samples. Lane 5: purified E2-IgG heavy chain fusion protein (positive control).

Co-expression of the membrane bound HCV E2 with *Arabidopsis* calnexin. The construct pBYRE2TR expressing a membrane bound HCV E2 (mE2) protein was co-infiltrated with the calnexin construct or the empty binary vector pPS1 into 6 to 7 weeks old leaves at 1:1 ratio to study the effect of calnexin on mE2 production and structure. The empty vector pPS1 and the calnexin construct were also respectively infiltrated into the same leaves as negative controls. The final OD_{600} value of *Agrobacterium* to be infiltrated was 0.2 for all the treatments. Leaves were monitored for 5 days and harvested at day 5 after infiltration. Co-expression of mE2 and calnexin showed necrosis in leaves at 4 dpi and it became much stronger at 5 dpi (Figure 7). In addition, mE2/calnexin leaf spots have stronger necrosis than mE2/pPS1 leaf spots. Expression of calnexin alone in leaves showed very little necrotic effect. Proteins were freshly extracted from mE2/calnexin samples and mE2/pPS1 samples harvested at 5 dpi. Since mE2 is a membrane protein, we increased the amount of Triton X-100 to 1% in the extraction buffer to release more membrane proteins to the supernatant of the extracts. The level of denatured mE2 and conformational mE2 were compared between mE2/pPS1 samples and mE2/calnexin samples by Western blot. Both the supernatant and the pellet of protein extract for each sample were tested in the analysis in order to examine all the mE2 proteins produced in leaf samples. The reducing blot showed that a large portion of mE2 produced in mE2/calnexin samples were in the pellet, and the total mE2 signal in

mE2/calnexin samples was much stronger than that of mE2/pPS1 samples (Figure 8A). The mE2 produced in mE2/pPS1 was not well recognized by the anti-linear E2 antibody, because both the supernatant and the pellet had rather weak mE2 signals. However, the same mE2 protein co-expressed with calnexin had strong mE2 signals. It seemed that the linear epitopes on mE2 proteins were somehow masked. On the non-reducing blot shown in Figure 8B, we observed conformational mE2 signals in mE2/pPS1 samples, indicating that mE2 were expressed in those samples and the correct form of mE2 could be recognized by the anti-conformational E2 antibody. Also, we could see that the mE2 signals in mE2/calnexin samples were significantly stronger than those in mE2/pPS1 samples, and mE2 proteins were not aggregated. We interpret this to mean that calnexin could help accumulating more correctly folded mE2 in plants; it enhanced the ER's ability on mE2 folding and increased the efficiency of mE2 production.

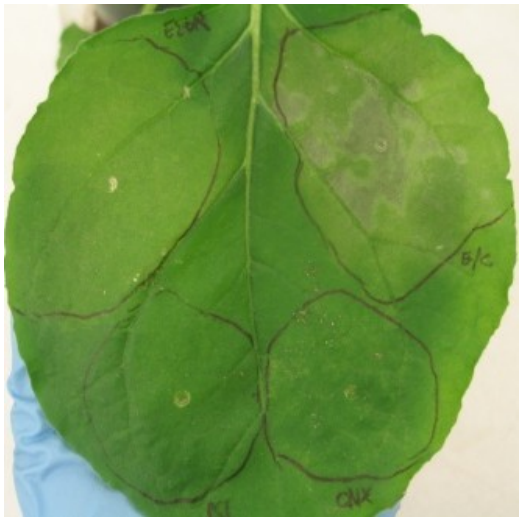

Figure 7. Phenotype observation of leaf spots expressing membrane bound HCV E2 alone (upper left), membrane bound HCV E2 and calnexin (upper right), empty vector pPS1 (bottom left), and calnexin (bottom right) at 5 dpi. The upper left spot was co-infiltrated with pBYRsE2TR and pPS1, the upper right spot was co-infiltrated with pBYRsE2TR and psAtCNX-ext, the bottom left spot was infiltrated with pPS1, the bottom right spot was infiltrated with psAtCNX-ext. The total OD_{600} for infiltration was 0.2, therefore the OD_{600} of each construct is 0.1.

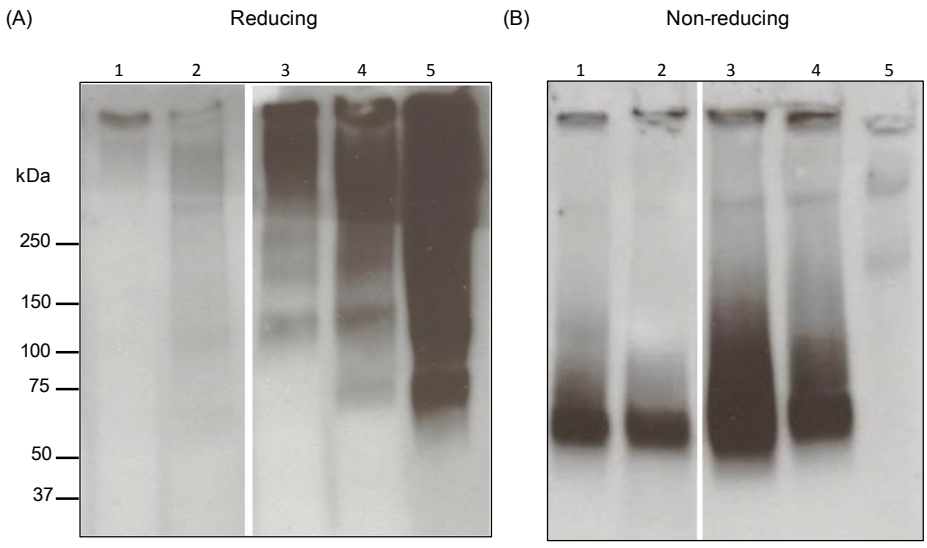

Figure 8. Western blot analysis of the membrane bound HCV E2 production with or without co-expression of *Arabidopsis* calnexin in *N. benthamiana* leaves. (A) A reducing Western blot comparing denatured mE2 levels in mE2/pPS1 samples and mE2/calnexin samples from same leaves harvested at 5 dpi, using mouse anti-linear E2 antibodies. Protein samples were denatured in SDS sample buffer containing 150 Mm DTT and boiled. (B) A non-reducing Western blot comparing correct folded sE2 levels in mE2/pPS1 samples and mE2/calnexin samples from same leaves harvested at 5 dpi, using mouse anti-conformational E2 antibodies. Protein samples were mixed with SDS sample buffer without DTT and were not boiled. Lane 1: supernatant of leaf crude extract from mE2/pPS1 sample. Lane 2: pellet of leaf crude extract from mE2/pPS1 sample. Lane 3: supernatant of leaf crude extract from sE2/calnexin sample. Lane 4: pellet of leaf crude extract from sE2/calnexin sample. Lane 5: purified E2-IgG heavy chain fusion protein (positive control).

Co-expression of the membrane bound HCV E2 with *Arabidopsis* calnexin and calreticulin. Since in previous experiments we have shown that calreticulin could increase the amount of total sE2 and correctly folded sE2, we were interested to know whether calreticulin could coordinate with calnexin to facilitate membrane bound E2 folding and production even better. Therefore, the constructs expressing mE2, calnexin and calreticulin were co-infiltrated into 6-7 weeks old leaves at 1:1:1 ratio to test the effect of combined calnexin and calreticulin treatment on mE2 expression. mE2 alone was expressed in a different spot on same leaves by co-infiltration of mE2 construct and pPS1 at 1:2 ratio. Besides, leaf spots infiltrated with pPS1 and co-infiltrated with calnexin and calreticulin were used as controls. The total OD_{600} of *Agrobacterium* for infiltration was 0.3 per treatment, and leaves of 3 different plants were infiltrated. Infiltrated leaves were monitored for 5 days and phenotype changes were recorded. Necrosis occurred at 3 dpi in all the leaf spots expressing mE2 transgenes, and at 5 dpi the leaf spots co-expressing mE2, calnexin, calreticulin were much more necrotic than those only expressing mE2 without chaperones (Figure 9). Expression of calnexin and calreticulin without mE2 also caused a little necrosis, compared to the pPS1 negative control.

mE2/calnexin/calreticulin samples and mE2/pPS1 samples were harvest at 5 dpi for mE2 protein analysis by Western blot. We added 1% Triton X-100 to the extraction buffer to destroy the membrane structures in cells and included both the supernatant and pellet of each leaf crude

extract in the analysis as we did in mE2/calnexin co-expression experiment. The reducing Western blot analysis again showed no or very low mE2 signal in mE2/pPS1 samples, indicating that the linear epitope of E2 was lost for some reason. Nevertheless, strong mE2 signals were observed in the supernatant and pellet of mE2/calnexin/calreticulin samples (Figure 10A). The pellet contained even more monomeric mE2 than the supernatant. The non-reducing Western blot (Figure 10B) showed that correctly folded mE2 were accumulated in both mE2/pPS1 samples and mE2/calnexin/calreticulin samples, but the level of correct form of mE2 was significantly higher in mE2/calnexin/calreticulin samples than in mE2/pPS1 samples. This indicated that a combination of calnexin and calreticulin treatment could also effectively increase the level of properly folded mE2. However, compared to Figure 7, it seemed that mE2 expression pattern was very similar between calnexin treatment and calnexin/calreticulin treatment. No better effect was observed when mE2 was co-expressed with calnexin and calreticulin together than mE2 expressed with calnexin alone.

Figure 9. Phenotype observation of leaf spots expressing membrane bound HCV E2, calreticulin and calnexin (upper left), membrane bound HCV E2 alone (upper right), calreticulin and calnexin (bottom left), and empty vector pPS1 (bottom right) at 5 dpi. The upper left spot was co-infiltrated with pBYRsE2TR, psAtCRT-ext and psAtCNX-ext, the upper right spot was co-infiltrated with pBYRsE2TR and pPS1, the bottom left spot was infiltrated with psAtCRT-ext and psAtCNX-ext, and the bottom right spot was infiltrated with pPS1. The total OD_{600} for infiltration was 0.3.

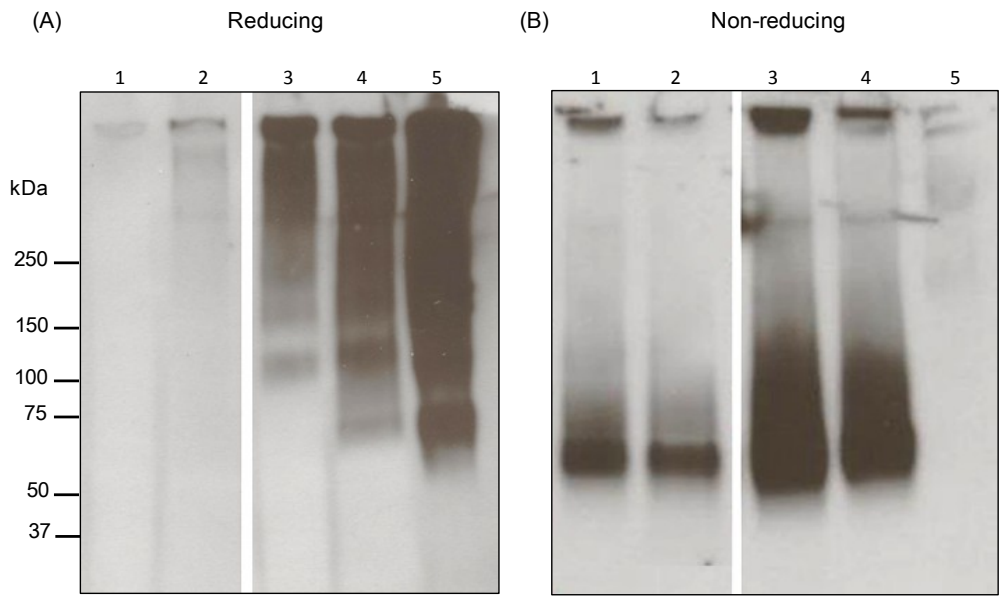

Figure 10. Western blot analysis of membrane bound HCV E2 production with or without co-expression of *Arabidopsis* calnexin and calreticulin in *N. benthamiana* leaves. (A) A reducing western blot comparing denatured mE2 levels in mE2/pPS1 samples and mE2/calnexin/calreticulin samples from same leaves, using mouse anti-linear E2 antibodies. Protein samples were denatured in SDS sample buffer containing 150 Mm DTT and boiled. (B) A non-reducing Western blot comparing correct folded sE2 levels in mE2/pPS1 samples and mE2/calnexin/calreticulin samples from same leaves, using mouse anti-conformational E2 antibodies. Protein samples were not denatured and boiled. Lane 1: supernatant of leaf crude extract of mE2/pPS1 sample. Lane 2: pellet of leaf crude extract of mE2/pPS1 sample. Lane 3: supernatant of leaf crude extract of sE2/calnexin sample. Lane 4: pellet of leaf crude extract of sE2/calnexin sample. Lane 5: purified E2-IgG heavy chain fusion protein (positive control).

Quality Improvement of Plant Produced HCV E2 with the Help of bZIP60 and bZIP60ΔC

Over-expression of bZIP60 and bZIP60ΔC in *N. benthamiana* leaves. The coding sequence of *NbbZIP60* and *NbbZIP60ΔC* were amplified by high-fetidity PCR from total cDNA of *N. benthamiana* wild type plant leaves. The PCR products were respectively inserted into a binary vector between Nos promoter and Nos 3' UTR, so that *NbbZIP60* and *NbbZIP60ΔC* could be constitutively expressed when they were transformed into *N. benthamiana* leaves. The coding sequences of *AtbZIP60* and *AtbZIP60ΔC* (amino acid 1-216) were acquired from Dr. Mason. The cDNA fragments of AtbZIP60 and AtbZIP60ΔC were respectively inserted into the non-replicating binary vector pPS1, between the dual-enhancer CaMV 35S promoter and vspB 3' UTR. Driven by the strong 35S promoter, the resulting constructs psAtbZIP60 and psAtbZIP60-S216 could highly express AtbZIP60 and AtbZIP60ΔC respectively in *N. benthamiana* leaves.

The four constructs were introduced into *N. benthamiana* leaves via *Agrobacterium* to examine whether they cause necrosis of plants. We checked the phenotypes of leaves for 1 week and the leaves stayed normal. Therefore, transient expression of bZIP60 or bZIP60ΔC did not cause toxic effects to plants (data not shown). At 2 dpi, leaf samples were harvested for RT-PCR in order to confirm gene overexpression. For each construct, 3 different leaf samples were used as replicates. Wild type

leaves were used as controls. RNA extraction and purification from each sample were performed in the same way at the same time. Same amount of total RNA from each sample was used in RT-PCR and the procedure was previously described in the Material and Methodology chapter. The RT-PCR results showed that *NbbZIP60* and *NbbZIP60ΔC* were overexpressed in leaves because their band signals were much higher than those from wild type samples representing the endogenous *NbbZIP60* and *NbbZIP60ΔC* mRNA levels (Figure 11A, B). The RT-PCR result shown in Figure 11C also indicated that *AtbZIP60* and *AtbZIP60ΔC* were highly expressed in *N. benthamiana* leaves.

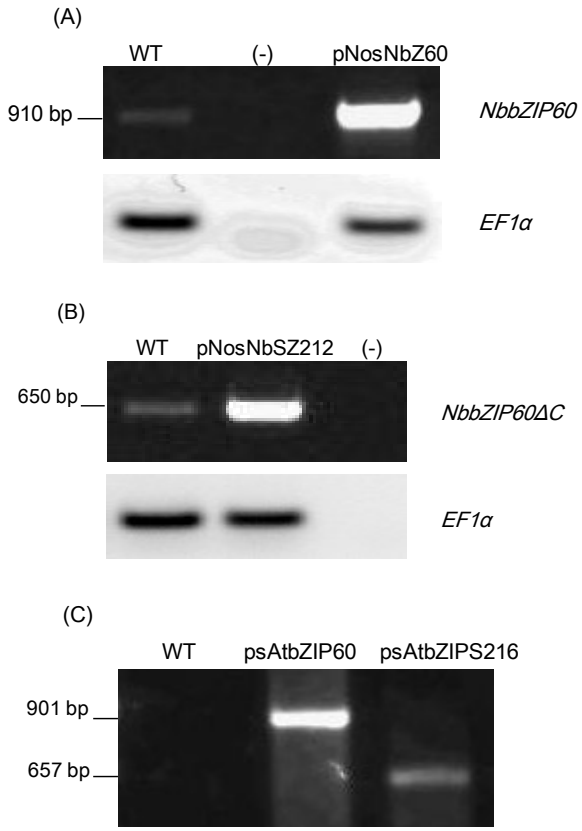

Figure 11. RT-PCR products amplified from samples expressing (A) *NbbZIP60*, (B) *NbbZIP60ΔC*, (C) *AtbZIP60* or *AtbZIP60ΔC*. Constitutively expressed *N. benthamiana EF1α* was used as the internal control and was amplified using primers EF1α-F and EF1α-R. WT stands for wild type sample. (-) stands for negative control. In (A), *NbbZIP60* was amplified using primers NbbZIP60-Nco-F and NbbZIP60-Sac-R. In (B), *NbbZIP60ΔC* was amplified using primers NbbZIP60-Nco-F and NbbZIP60-S212. In (C), *AtbZIP60* was amplified using primers pUni51-F and AtbZIP60-Kpn-R; *AtbZIP60ΔC* was amplified using primers pUni51-F and AtbZIP60-S216-K.

Co-expression of the soluble form of HCV E2 with bZIP60 or bZIP60ΔC. The construct expressing soluble form of HCV E2 (sE2) was co-infiltrated with NbbZIP60 construct, NbbZIP60ΔC construct, AtbZIP60 construct and AtbZIP60ΔC construct respectively into 6-7 weeks old *N. benthamiana* leaves in order to examine whether bZIP60 and bZIP60ΔC could promote HCV E2 folding. sE2 construct plus empty vector pPS1 were co-infiltrated into the leaves to express sE2 alone for comparison. *Agrobacterium* carrying different constructs were mixed at 1:1 ratio, and the total OD_{600} for infiltration was 0.2. Figure 12 showed phenotypes of leaf spots expressing sE2, sE2/NbbZIP60, sE2/NbbZIP60ΔC and sE2/AtbZIP60ΔC at 4, 6, and 8 dpi and leaf spot expressing sE2/AtbZIP60 at 8 dpi. sE2/NbbZIP60 treated leaf spot showed stronger necrosis than sE2 leaf spot and other treated leaf spots. However, sE2/NbbZIP60ΔC and sE2/AtbZIP60ΔC treated leaf spots had similar degrees of necrosis to that of sE2 leaf spot. sE2/AtbZIP60 was co-infiltrated into different leaves in the same growth condition, and co-expression of sE2/AtbZIP60 also showed similar necrotic effect to the sE2 leaf spot.

Day 4 and day 8 samples were harvested for analysis of sE2 production with the help of each bZIP60 protein and bZIP60ΔC protein. Reducing and non-reducing Western blots were performed to compare the total sE2 levels and correctly folded sE2 levels between sE2 samples and sE2/bZIP60 or sE2/bZIP60ΔC samples. The results of reducing and non-reducing Western blots were shown in Figure 13. The reducing blots

showed that sE2 alone and sE2 with treatments had similar expression levels of sE2, although sE2/NbbZIP60ΔC, sE2/AtbZIP60, and sE2/AtbZIP60ΔC samples seemed to express more monomeric sE2. A portion of sE2 was degraded in all samples at 8 dpi, and the degradation degrees are similar for all samples. Therefore, we did not see any benefit of bZIP60 and bZIP60ΔC in regarding to the yield of sE2. On the other hand, the non-reducing blots showed that at day 4, expression of sE2 with treatments did not increase the level of correct form of sE2. However, in day 8 samples, sE2/NbbZIP60 and sE2/AtbZIP60ΔC samples seemed to have more correctly folded sE2 than sE2 samples, although the benefits were not significant. To ensure NbbZIP60 and AtbZIP60ΔC could help accumulating more properly folded sE2, more sE2/NbbZIP60 and sE2/AtbZIP60ΔC samples were tested by reducing and non-reducing Western blots (Figure 14). The repeated experiments confirmed that sE2/NbbZIP60 and sE2/AtbZIP60ΔC samples contains more correct form of sE2 proteins than sE2 alone samples at 8 dpi but not 4 dpi, indicating that the helping effects of NbbZIP60 and AtbZIP60ΔC on sE2 production took time. However, compared to the total sE2 level suggested by the reducing Western blots, correct form of sE2 were still very little with NbbZIP60 or AtbZIP60ΔC treatments. Overall, NbbZIP60 and AtbZIP60ΔC did not seem to increase the amount of plant produced sE2, but they helped to improve the quality of sE2 to some extent.

Figure 12. Phenotype observation of a leaf expressing soluble HCV E2 and NbbZIP60 (spot 1), soluble HCV E2 alone (spot 2), HCV E2 and NbbZIP60ΔC (spot 3), and HCV E2 and AtbZIP60ΔC (spot 4) at 4, 6 and 8 days post infiltration. Another leaf expressing HCV E2 alone (spot 5) and HCV E2 and AtbZIP60 (spot 6) at 8 dpi was also shown on the right. Spot 1 was co-infiltrated with pBYRsE2-711H and NosNbZ60, spot 2 and 5 were co-infiltrated with pBYRsE2-711H and pPS1, spot 3 was co-infiltrated with pBYRsE2-711H and NosNbZS212, spot 4 was co-infiltrated with pBYRsE2-711H and psAtbZIP60 and spot 6 was co-infiltrated with pBYRsE2-711H and psAtbZIP60-S216. The total OD_{600} for infiltration was 0.2, so that the OD_{600} of each construct is 0.1.

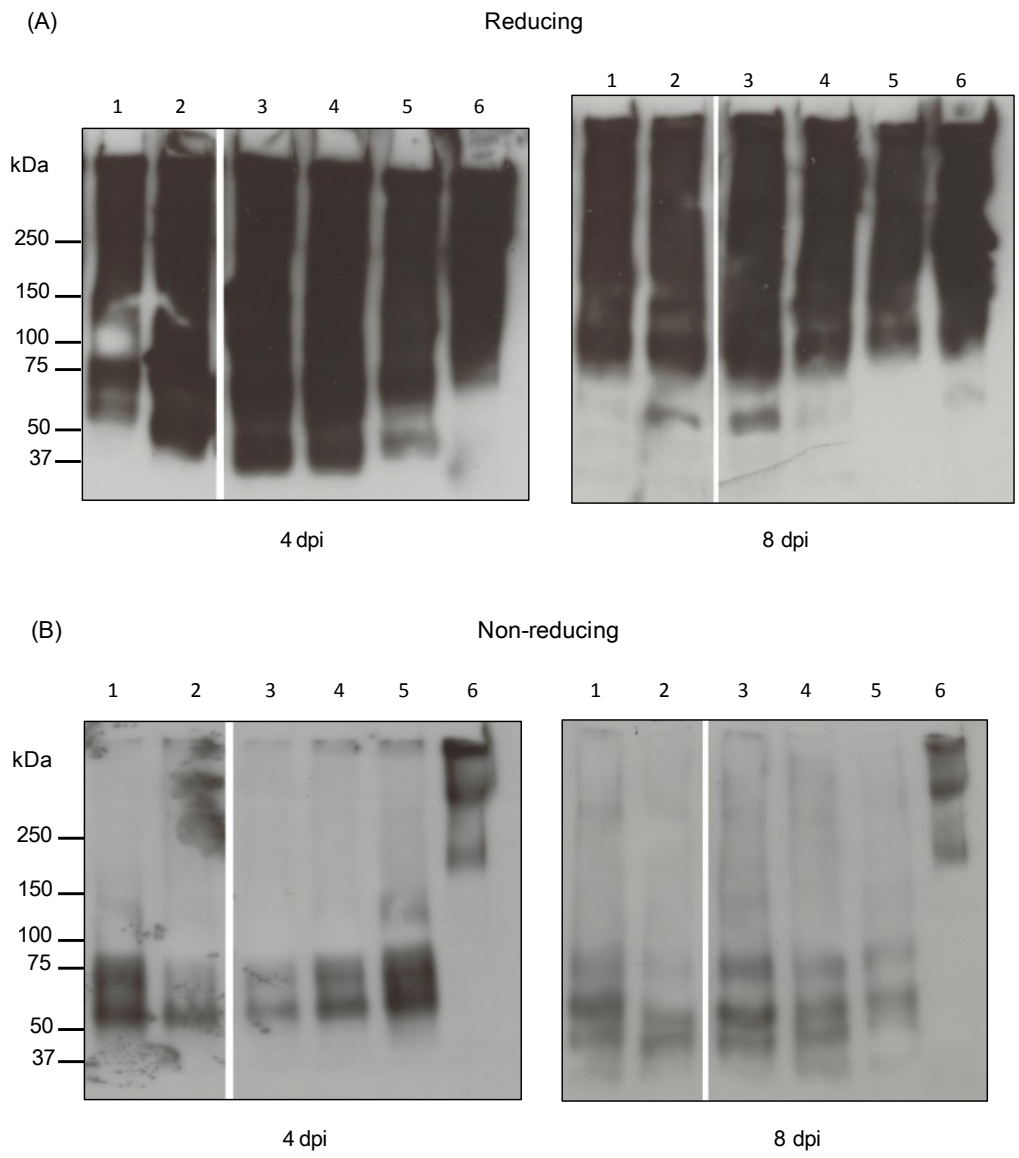

Figure 13. Western blot analysis of expression of soluble form of HCV E2 with bZIP60 or bZIP60ΔC treatments at 4 and 8 dpi. (A) Reducing Western blots comparing denatured sE2 levels in sE2/pPS1 samples and sE2/treatment samples from same leaves harvested at 4 dpi and 8 dpi, using mouse anti-linear E2 antibodies. Protein samples were denatured in SDS sample buffer containing 150 mM DTT and boiled. (B) Non-reducing Western blot comparing correct folded sE2 levels in sE2/pPS1 samples

and sE2/treatment samples from same leaves harvested at 4 dpi and 8 dpi, using mouse anti-conformational E2 antibodies. Protein samples were mixed with SDS sample buffer without DTT and were not boiled. Lane 1: sE2/NbbZIP60 sample. Lane 2: sE2/AtbZIP60 sample. Lane 3: sE2/AtbZIP60ΔC sample. Lane 4: sE2/NbbZIP60ΔC sample. Lane 5: sE2/pPS1 sample. Lane 6: purified E2-IgG heavy chain fusion protein (positive control).

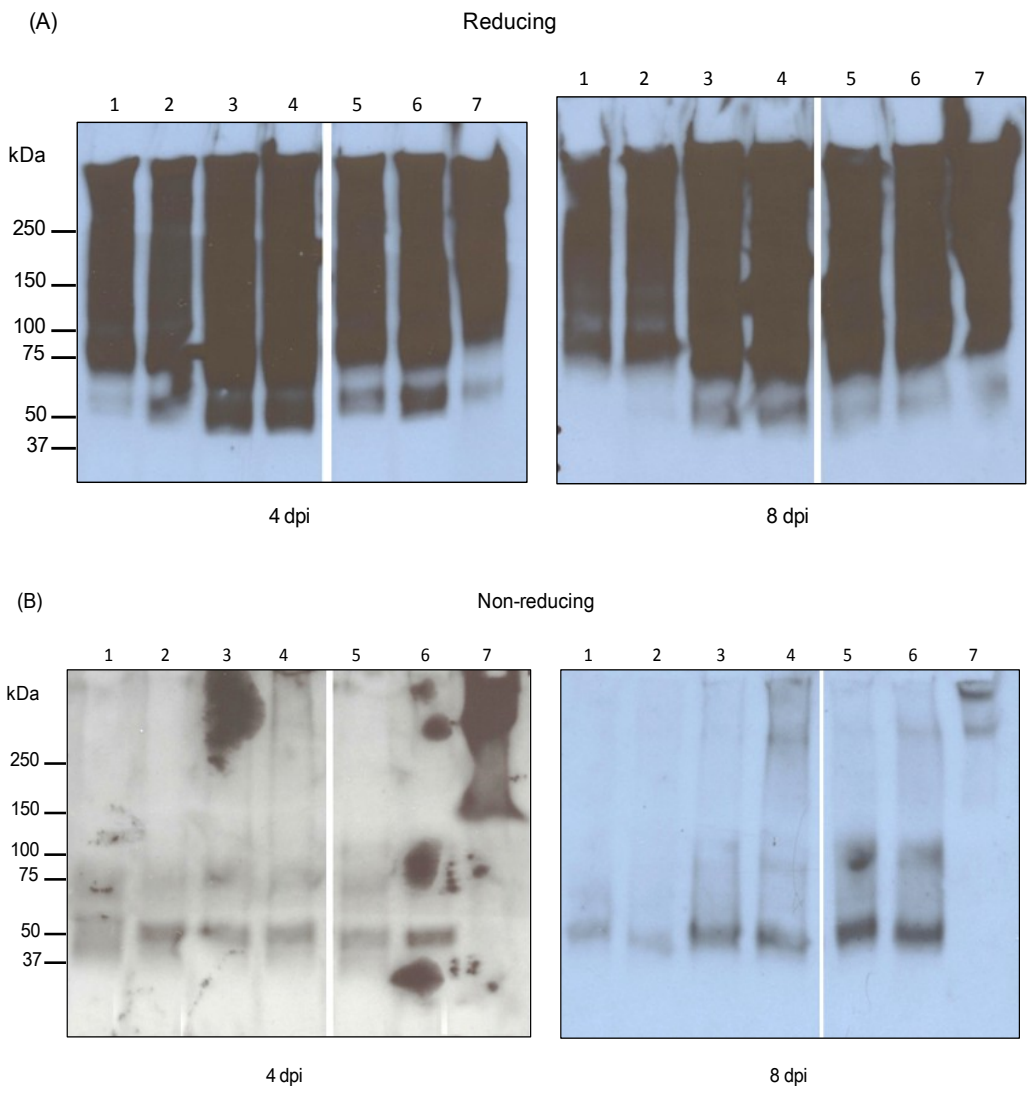

Figure 14. Western blot analysis of expression of soluble form of HCV E2 with NbbZIP60 or AtbZIP60ΔC treatments at 4 and 8 dpi. (A) Reducing Western blots comparing denatured sE2 levels in sE2/pPS1 samples and sE2/treatment samples from same leaves harvested at 4 dpi and 8 dpi, using mouse anti-linear E2 antibodies. Protein samples were denatured in SDS sample buffer containing 150 Mm DTT and boiled. (B) Non-reducing Western blot comparing correctly folded sE2 levels in sE2/pPS1 samples

and sE2/treatment samples from same leaves harvested at 4 dpi and 8 dpi, using mouse anti-conformational E2 antibodies. Protein samples were mixed with SDS sample buffer without DTT and were not boiled. Lane 1 and 2: two different sE2/pPS1 samples. Lane 3 and 4: two different sE2/NbbZIP60 samples. Lane 5 and 6: two different sE2/AtbZIP60ΔC samples. Lane 7: purified E2-IgG heavy chain fusion protein (positive control).

Relationship between bZIP60 and *BiP* expression in *N. benthamiana*. From our observations in the previous experiment, bZIP60 and its putative active form bZIP60ΔC did not significantly promote the folding and production of sE2 in plants. To find out possible reasons, we examined *BiP* expression levels in leaves infiltrated with sE2/pPS1, sE2/NbbZIP60 or sE2/AtbZIP60ΔC constructs to see if NbbZIP60 or AtbZIP60ΔC treatment under ER stress condition increases the *BiP* genes expression, since these two treatments showed a little help on sE2 folding. We also examined *BiP* expression levels in leaves infiltrated with only NbbZIP60 or AtbZIP60ΔC construct to see whether overexpression of *NbbZIP60* or *NbbZIP60ΔC* without ER stress can induce the expression of *BiPs*. In *N. benthamiana*, only one *BiP* cDNA sequence was found in the Genbank, which was called luminal binding protein 4 (Blp4) (Genbank accession number FJ463755). But, we found 5 more cDNA sequences of *Blp* genes in *N. tabacum*. Since *N. benthamiana* and *N. tabacum* are closely relative species, we designed primers based on tobacco *Blp* sequences and used them to amplify the orthologs of tobacco *Blp1*(Genbank accession # X60060.1), *Blp2* (X60059.1), *Blp4* (X60057.1) and *Blp8* (X60062.1) in *N. benthamiana*. Leaf samples were harvested 48 h after infiltration and total RNA were extracted from them for RT-PCR analysis. Three different leaf samples were tested for each construct to ensure the reliability of the result. Wild type leaves infiltrated with pPS1 were used as controls. RNA extraction and purification from samples were

performed at the same time, following the procedures described in the Material and Methodology chapter. One μg total RNA extracted from each sample was used to perform RT-PCR, and cDNA of *Blp* genes were amplified with their corresponding pairs of primers. A fragment of constitutively expressed *EF1α* was also amplified from each sample to serve as internal control. The result of *Blps* expression was observed by electrophoresis of RT-PCR products (Figure 15). The result showed that *N. benthamiana Blp* expression levels increased slightly only in response to HCV E2 treatment, but not to NbbZIP60 or AtbZIP60ΔC treatment. In addition, *Blp* levels were about the same between wild type samples and NbbZIP60 or AtbZIP60ΔC samples, indicating that overexpression of NbbZIP60 or AtbZIP60ΔC in leaves without ER stress did not activate *Blp* genes expression. Furthermore, among those samples expressing sE2, the expression levels of *Blps* were also very similar, which suggested that overexpression of NbbZIP60 or AtbZIP60ΔC under ER stress condition could not induce *Blps* expression, either. Expression of *Blps* was increased in response to the ER stress generated by HCV E2 expression, but not induced by NbbZIP60 or AtbZIP60ΔC. This may be the reason why overexpression of bZIP60 and bZIP60ΔC in leaves expressing sE2 did not significantly promote HCV E2 folding and accumulation.

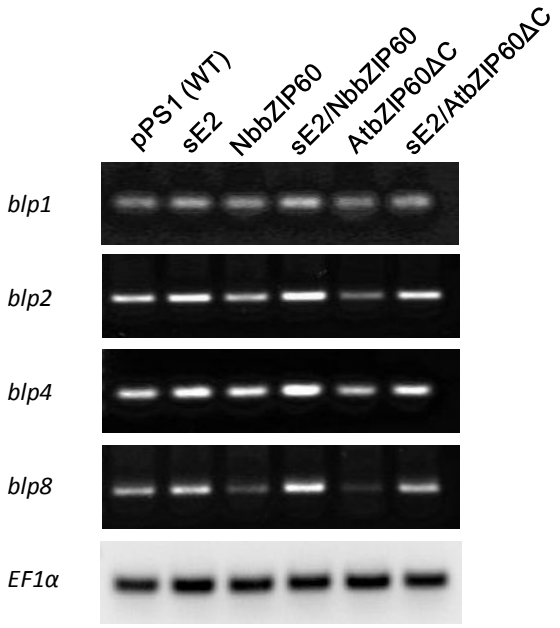

Figure 15. RT-PCR showing the abundance of *Blp1, Blp2, Blp4* and *Blp8* transcripts from samples with indicated treatments harvested at 2 dpi. Constitutively expressed *N. benthamiana EF1α* was used as the internal control and was amplified using primers EF1α-F and EF1α-R. Blp1 was amplified using primers NtBlp1-F and NtBlp1-R; Blp2 was amplified using primers NtBlp2-F and NtBlp2-R; Blp4 was amplified using primers NtBlp4-F and NtBlp4-R; Blp8 was amplified using primers NtBlp8-F and NtBlp8-R.

Chapter 6

DISCUSSION

Arabidopsis calreticulin and calnexin promote HCV E2 protein production in *N. benthamiana*

The most important result in this study was that *Arabidopsis* calreticulin and calnexin did help to increase the yield of HCV E2 and also improve the folding quality of HCV E2. We found that with calreticulin or calnexin treatment, protein degradation appeared to be suppressed, which was consistent with other published studies (12. 31). The suppression of protein degradation may be because the unfolded or misfolded proteins were stabilized by calnexin and calreticulin when associated with them, escaping from digestion by proteases. It is also possible that with the help of calreticulin and calnexin, protein folding efficiency was increased so that the ER stress was effectively reduced, weakening the signal that triggered protein degradation in the ER. Also, we were excited to see that more HCV E2 proteins were expressing in their correct conformation in calreticulin or calnexin treated samples, especially at early time point. This may be simply because fewer proteins were degraded, or because calreticulin and calnexin directly assisted E2 folding and maturation by recruiting folding factors such as ERp57 to catalyze protein folding (37). This result is encouraging because our goal is to rapidly produce large amounts of functional HCV E2 proteins so as to save time and money when manufacturing this vaccine candidate in the future.

Previous studies also showed that calreticulin and calnexin could efficiently prevent protein aggregation in the ER. However, in our experiment, it is hard to tell whether calreticulin and calnexin prevented protein aggregation. In the Western blot analysis using the linear epitope antibody, the protein aggregates in E2/calreticulin or E2/calnexin samples were even more than those in E2 alone samples. But we should notice that the total E2 level in calnexin or calreticulin treated samples was also much higher than that in E2 alone samples, so it is difficult to determine the percentage of aggregates in total E2 proteins from Western blot. In our future work, we need to further test the quality of our plant produced HCV E2 by CD81 binding assay. If the recombinant E2 proteins produced in our plant system are correctly folded, they should be able to bind CD81, the putative receptor of HCV E2 on human cells. Otherwise, our plant-derived HCV E2 proteins cannot interact with CD81, and the antibodies generated against our recombinant E2 proteins cannot effectively block the entry of HCV into cells.

Overexpression of bZIP60 or bZIP60ΔC has small effect on facilitating HCV E2 folding in the ER

In our second hypothesis, we hypothesized that overexpression of the ER stress transducer bZIP60 or its active form bZIP60ΔC in *N. benthamiana* leaves expressing HCV E2 could up-regulate the expression of a group of UPR genes especially *Bip*, which could then efficiently assist

HCV E2 folding and maturation. However, our experiment showed that *Bips* (or *Blps*) expression could not be up-regulated by either NbbZIP60 or AtbZIP60ΔC, no matter whether HCV E2 caused ER stress or not, but their expression was slightly increased in response to HCV E2 expression. As a result, we did not receive a significant help from overexpression of bZIP60 or bZIP60ΔC for HCV E2 folding and accumulation. Only the truncated forms of AtbZIP60 and NbbZIP60 helped a little in expressing more correctly folded E2, but the total yield of correct form of E2 was still relatively low.

From these results, we can confirm that HCV E2-induced ER stress creates a response in leaf cells due to the increased expression level of *BiP* genes, but it seems that *BiPs* are not activated by the NbbZIP60 pathway. This is possible because there are other bZIP proteins in *Arabidopsis* that also have the capability of *Bip* activation, such as bZIP28. It is possible that in *N. benthamiana* bZIP60 loses the function to activate *BiP*, and that function is maintained in other bZIP proteins. It is also possible that NbbZIP60 can activate some other *BiP* genes or UPR genes that are not tested in our experiment. But even if that is the case, those UPR genes do not seem to be effective in facilitating glycoprotein folding, because the protein level of correctly folded E2 in E2/AtbZIP60ΔC and E2/NbbZIP60 co-expressed samples were not increased a lot. Therefore, in future work, we want to design experiments to test whether NbbZIP60 can activate UPR genes in *N. benthamiana*. *BiP* and other UPR genes

which are activated by AtbZIP60 in *Arabidopsis* contain the ER stress response element (ERSE) or the plant unfolded protein response element (p-UPRE) before their promoters (23). Thus, we can make reporter constructs that have the ERSE or the p-UPRE element inserted before the promoter, then test if NbbZIP60ΔC can transactivate the expression of reporter genes. If it can, that means NbbZIP60 has the ability to activate UPR genes containing ERSE or p-UPRE element. Then maybe there are other unidentified *Bip* genes in *N. benthamiana* that can be induced by NbbZIP60. If it is unable to, that means NbbZIP60 may not play roles in UPR gene activation in *N. benthamiana*. Therefore, we have to try other methods to up-regulate *Bip* expression in order to promote HCV E2 production.

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APPENDIX A

CODING SEQUENCE OF *NbbZIP60* GENE

ATGGTGGGTGACATCGATGATATCGTTGGACACATCAATTGGGACGA

TGTAGATGACCTCTTCCACAATATTCTAGAAGATCACGCCGACAATCT

CTTCTCTGCTCATGATCCGTCCGCGCCGTCTATCCAGGAGATAGAGC

AGCTTCTCATGAAAGACGATGAAATCGTCGGTCACGTGGCTGTCAGG

GAGCCTGATTTTCAACTTGCTGATGACTTTCTCTCCGACGTGCTGGC

CGATTCTCCTGTTCAGTCCGATCATTCTCACTCTGATAAAGTCAATGG

ATTCCCCGATTCCAAGGTTTCAAGTGGCTCCGAGGTTGATGATGACG

ACAAAGACAAGGAGAAGGGTTCCCAGTCGCCGACTGAGTCTAAGGA

CGGCTCCGACGAACTAAACAGTAACGATCCCGTCGATAAAAAGCGCA

AGAGGCAATTGAGAAACAGGGATGCAGCTGTCAGGTCACGAGAGCG

GAAGAAGTTGTATGTTAGGGATCTTGAGTTGAAGAGTAGATACTTTGA

ATCAGAGTGCAAGAGGTTGGGGTTAGTTCTCCAGTGTTGTCTTGCAG

AAAATCAAGCTTTGCGTTTTTCTTTGCAGAATGGAAGTGCTAATGGTG

CTTGTATGACCAAGCAGGAGTCTGCTGTGCTCTTGTTGGAATCCCTG

CTGTTGGGTTCCCTGCTTTGGTTCTTGGGCATCATATGCCTGCTCATT

CTTCCCAGCCAACCCTGGTTAATTCCAGAAGAAAATCAACGAAGCAG

AAACCACCGTCTTCTGGTTCCAATAAAGGGAGGAAATAAGAATGGTC

GGATTTTTGAGTTCGTGTCCTTCATGATGGGCAAGAGATGCAAAGCT

TCAAGATCGAGGATGAAGTTCAATCCCCATTATTTGGGAATTGTGATG

TGA

APPENDIX B

PRIMERS USED IN THIS STUDY

Table 1.

Primers Used In This Study

Name	Sequence
NbbZIP60-Nco-F	5’ TGGCATGGTGGGTGACATCGATGATATC 3’
NbbZIP60-Sac-R	5’ CCGAGCTCTCACATCACAATTCCCAAATA 3’
NbbZIP60-S212	5’ CCGAGCTCTCAAGACTCCTGCTTGGTCAT 3’
pUni51-F	5’ CGGGTACCTCAAGACTCCTGCTTCGACATC 3’
AtbZIP60-Kpn-R	5’ GCGGTACCCGTTGTCACGCCG 3’
AtbZIP60-S216-K	5’ CGGGTACCTCAAGACTCCTGC 3’
NtBlp1-F	5’ GCTGCTGTTCAAGGTGGTA 3’
NtBlp1-R	5’ TGGTTGGGATGACGGTGTT 3’
NtBlp2-F	5’ GCAACCCAATTATCACAGC 3’
NtBlp2-R	5’ GTAACCCTCACCTCAACCT 3’
NtBlp4-F	5’ ACGGAAAGGACATCAGCAAG 3’
NtBlp4-R	5’ GTGCCCGAGTAAGTGGTTCA 3’
NtBlp8-F	5’ GCAACCCAATTATCACAGC 3’
NtBlp8-R	5’ GTAACCCTCACCTCAACCT 3’
AtCRT-Xba-F	5’ CCTCTAGAACAATGGCGAAAC 3’
AtCRT-Kpn-R	5’ GGGGTACCTTAAAGCTCGTCA 3’
AtCNX-Xba-F	5’ CCTCTAGAACAATGAGACAAC 3’
AtCNX-Kpn-R	5’ GGGGTACCTTGTTCTAATTAT 3’
EF1α-F	5’ CTGGTGGTTTTGAAGCTGGTA 3’
EF1α-R	5’ GGTGGTAGCATCCATCTTGTT 3’