J. Plant Biol. (2018) 61:241-252
DOI 10.1007/s12374-018-0133-4

ORIGINAL ARTICLE

Development of Plant-produced E2 Protein for Use as a Green Vaccine Against Classical Swine Fever Virus

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Received: April 23, 2018 / Accepted: May 17, 2018

Abstract Plants are promising host systems for recombinant protein production. However, progress in the commercialization of plant-made proteins (PMPs) has been slow. Only one PMP drug is commercially available. In this study, we explored the possibility of using plants to produce E2 of classical swine fever virus (CSFV) and the use of this plant-produced E2 as a vaccine. We designed high-level expression vectors for transgenic plants by considering the transcription, translation, and storage of E2 in the cell. We incorporated a cellulose-binding domain sequence into the expression vector as an affinity tag for cost-effective, one-step purification. Using this vector, we generated multiple lines of transgenic *Arabidopsis thaliana* plants expressing a fusion protein of E2 from CSFV at high levels (0.7% of total soluble proteins). ER-targeted E2 fusion protein was successfully purified via a one-step purification process using amorphous cellulose resin. Arabidopsis-produced E2 was recognized by an antibody that detects CSFV antigen. Finally, antisera from mice immunized with E2 fusion protein reacted strongly to the antigens in a CSFV antibody detection kit. Therefore, we propose that plant-produced E2 fusion proteins could be further developed for use as a green vaccine against CSFV in animals.

Keywords: *Arabidopsis thaliana*, Classical swine fever virus (CSFV), Green vaccine, Molecular farming, Plant-made proteins (PMPs)

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Introduction

Plants have been attracting a great deal of attention for their potential use as bioreactors for the production of recombinant proteins. This interest is based on several promising attributes of plants; they are easy to grow and do not require large, highly expensive facilities, and it is easy to scale up production, depending on demand (Sabalza et al. 2014; Lomonossoff and D'Aoust 2016; Rybicki 2017). Thus, much effort has focused on developing tools to produce recombinant proteins in plants. One of the most important tools is the expression vector. Numerous vectors have been developed for high-level protein production in plants (Hefferon 2012; Peyret and Lomonossoff 2013; Mortimer et al. 2015; Salazar-González et al. 2015). For the expression of heterologous genes, many different approaches have been explored involving the mode (transient expression vs stable transgenic expression) and location (nucleus or chloroplast) of the gene expression, and the use of plant tissues (whole plants, cell cultures, leaf tissues, or seeds) (Daniell et al. 2016; Sheshukova et al. 2016; Kopertekh and Schiemann 2017). As a result of such efforts, several products are currently available for various applications, and many more are at the development stage (Grabowski et al. 2014; Lomonossoff and D'Aoust 2016; Komarova et al. 2017). However, despite the great promise of using plants as recombinant protein production systems, there is still room for improvement, and there are obstacles that must be overcome before plants are widely utilized for recombinant protein production.

Plant-made proteins (PMPs), like recombinant proteins produced in other organisms such as animal cells and bacteria, have many applications. Of these, pharmaceutical drugs and vaccines represent the most valuable products. For example,

an enzyme taliglucerase-a is currently on the market as a protein drug to treat Gaucher disease, and plant-produced ZMapp is used to treat Ebola virus infection (Sheshukova et al. 2016; Kopertekh and Schiemann 2017). Efforts have been made to develop PMPs as vaccines against infectious viruses or cancers (Daniell et al. 2016; Rybicki et al. 2017; Wong-Arce et al. 2017). Hemagglutinin-neuraminidase (HN) protein from chicken Newcastle disease virus has been successfully developed as a PMP-based vaccine (Gómez et al. 2009). Plant-produced hemagglutinin (HA) from H5N1 is effective in eliciting an immune response in mice and provides protection against viral challenge (Bertran et al. 2015; Lee et al. 2015). Many PMP-based vaccines are currently undergoing clinical trials (Lomonossoff and D'Aoust 2016). Thus, plants are excellent systems for producing viral proteins that could be developed as vaccines. Moreover, PMP-based vaccines, otherwise known as green vaccines, are thought to be safer than standard vaccines because there is no possibility of spreading the virus of interest to the field (Rybicki 2017). Another important application of PMPs is their use as functional additives to cosmetics or foods (Yemets et al. 2014; Stephan et al. 2017). In fact, EGF produced in barley seeds has been developed as a cosmetics additive (Schouest et al. 2012). Canine interferon-alpha was successfully expressed in strawberry, and transgenic strawberry has recently been developed as an animal drug to treat canine tooth disease and was commercialized in Japan (Tabayashi and Matsumura 2014).

Classical swine fever virus (CSFV) is a small, enveloped RNA virus of the genus Pestivirus within the Flaviviridae family that causes severe fever, leading to death (Blome et al. 2017). This virus is highly contagious. Depending on the policies of individual countries, various measures are taken to control this virus. Live or attenuated virus is used to control classical swine fever in endemically infected regions in Asia, Eastern Europe, the Americas, and some African countries. In Korea, this viral disease is treated as a first-level animal viral disease, and all animals must be vaccinated at an early stage of growth. Currently, live attenuated LOM vaccine is widely used in the field in Korea (Lim et al. 2016). However, although live attenuated vaccines are effective for animal vaccination, their use in the field leads to trade restrictions for pigs between countries. This can be addressed using differentiation of infected and vaccinated animals (DIVA) vaccines. The use of live attenuated vaccines might also increase the danger of viral spread. Due to these issues, E2 subunit marker vaccines have been developed using recombinant E2 proteins expressed in insect cells (Ahrens et al. 2000; Bouma et al. 2000), which are currently on the market.

In this study, we investigated whether E2 from CSFV could be expressed at high levels in plants and used to develop plant-produced E2 as a vaccine against CSFV. We provide evidence that E2 can be expressed as a fusion protein at high levels in transgenic *Arabidopsis thaliana* plants, is easily purified from plant extracts via CBD-based one-step purification, and can be detected using an ELISA kit designed to detect CSFV antigens. Finally, we found that E2 in the fusion protein was highly immunogenic in mice and that antisera from immunized mice were recognized by an ELISA kit designed to detect CSFV antibodies.

Results

Design of a High-level Expression Construct in Arabidopsis

One of the most crucial steps in developing plants as protein production systems is to achieve high levels of expression of the target protein. To obtain high expression levels of a target gene, we considered many issues, such as transcription, translation, and storage of translated proteins in the cell (Buyel et al. 2013). Fig. 1A shows the various components used to design the expression vectors used in this study. To induce high levels of expression, we used the strong, double-enhanced CaMV S35 promoter (Comai et al. 1990). To obtain high translational efficiency, we used a highly efficient 5' UTR (untranslated region) sequence immediately upstream of the AUG in the chimeric gene; we previously showed that the

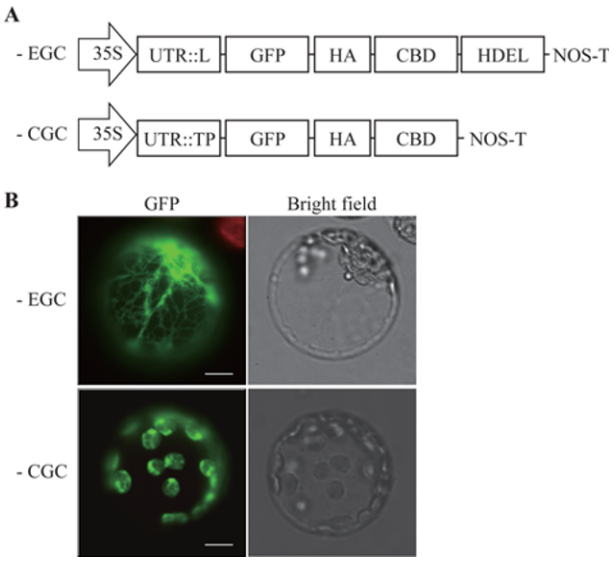

Fig. 1. Design of plant expression vectors and localization of proteins to the ER and chloroplasts in protoplasts. (A) Schematic diagram of expression vectors. 35S, double CaMV 35S promoter; UTR, 5' untranslated region, L, BiP1 leader sequence; GFP, green fluorescent protein; HA, HA epitope; CBD, cellulose-binding domain of xynA; HDEL, ER retention signal; UTR, 5' untranslated region; TP, Cab transit peptide; NOS-T, nos terminator. (B) Localization of GFP fusion proteins. Protoplasts were transformed with the ER-targeted *EGC* or chloroplast-targeted *CGC* constructs, and the localization of GFP fusion proteins was examined by fluorescent microscopy. Scale bar = 20 μm.

nucleotide sequence of the 5' UTR at positions -1 to -20 greatly influences the translational efficiently of a gene (Kim et al. 2014). Next, we decided to target the protein to the ER or chloroplasts because proteins accumulate to high levels in these compartments (Staub et al. 2000; Jha et al. 2012). For ER targeting, we used the BiP leader sequence (L) (Song et al. 2006). To induce protein accumulation in the ER, we added the ER retention signal HDEL (Gomord et al. 1997). For chloroplast targeting, we used the transit peptide (TP) of chlorophyll a/b binding protein (Cab) (Lee et al. 2015). Finally, we included an affinity tag for easy purification of recombinant protein. As an affinity tag for purification, we used the cellulose-binding domain (CBD) from *xynA* of *Clostridium stercorarium* (Sakka et al. 1993). CBD shows a high affinity for cellulose beads and can be used as an affinity tag for purification (Wan et al. 2011; Sugimoto et al. 2012). To investigate the expression levels and localization of proteins expressed from these constructs, we used GFP as a model protein. To detect the target protein by western blot analysis, we included the small epitope HA in front of the C-terminal CBD. We transiently expressed the final fusion constructs, *L:GFP:HA:CBD:HDEL* (*EGC*) and *TP:GFP:HA:CBD* (*CGC*), in protoplasts from Arabidopsis leaf tissues and examined their localization under a fluorescent microscope. EGC and CGC were detected in the ER and chloroplasts, respectively (Fig. 1B), confirming that they were properly targeted to the chosen organelles. Next, we investigated the expression of these constructs in transgenic plants. We generated transgenic Arabidopsis plants by the floral dip method and screened T0 plants on kanamycin

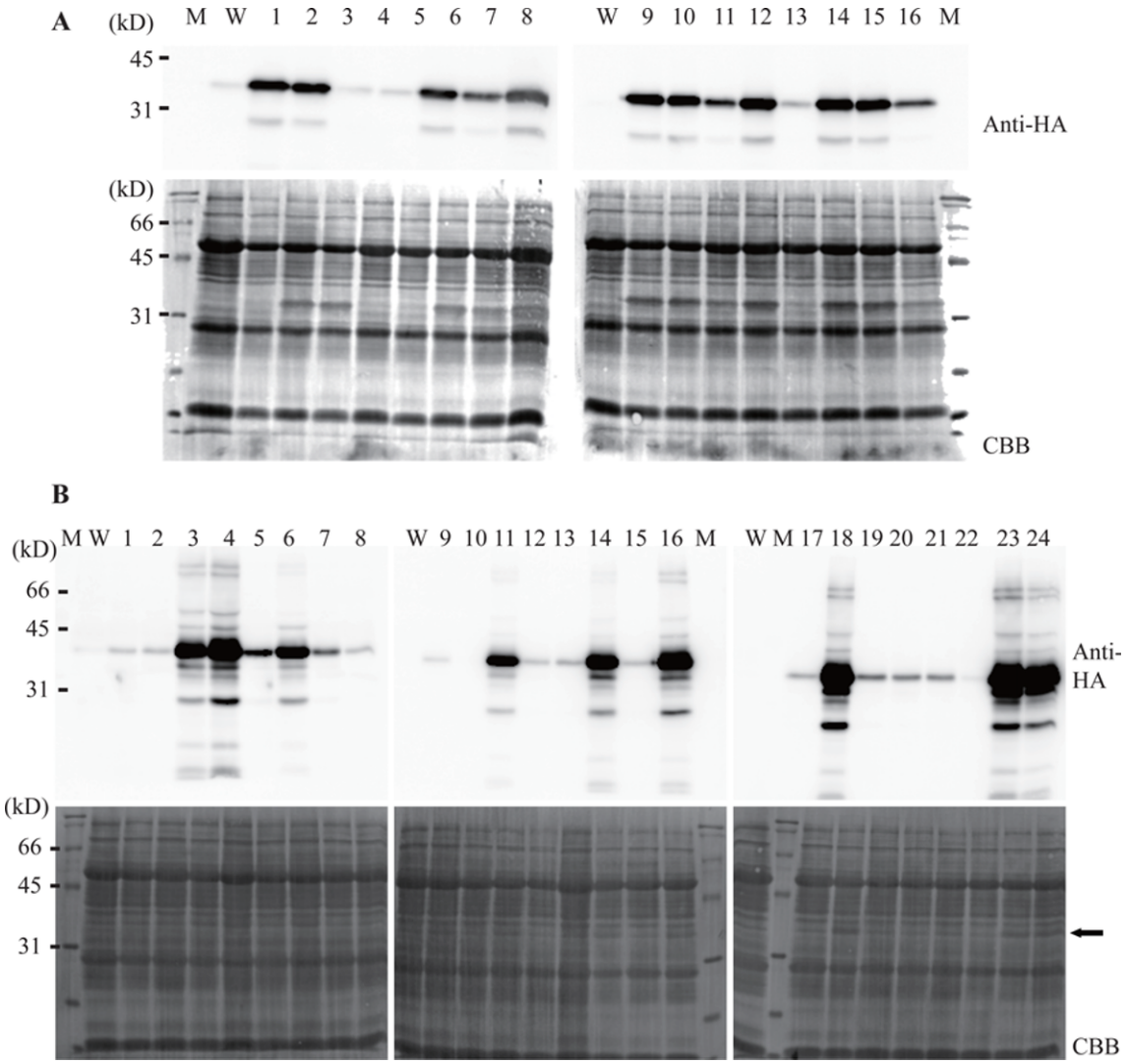

Fig. 2. Generation of transgenic plants expressing GFP fusion proteins targeted to the ER and chloroplast. (A, B) Western blot analysis of T1 lines. Protein extracts (50 μg) from T1 plants expressing ER-targeted EGC (A) or chloroplast-targeted (B) CGC fusion proteins were analyzed by western blotting using anti-HA antibody. Blots were stained with Coomassie brilliant blue (CBB) after immunoblotting. Arrow, GFP fusion proteins.

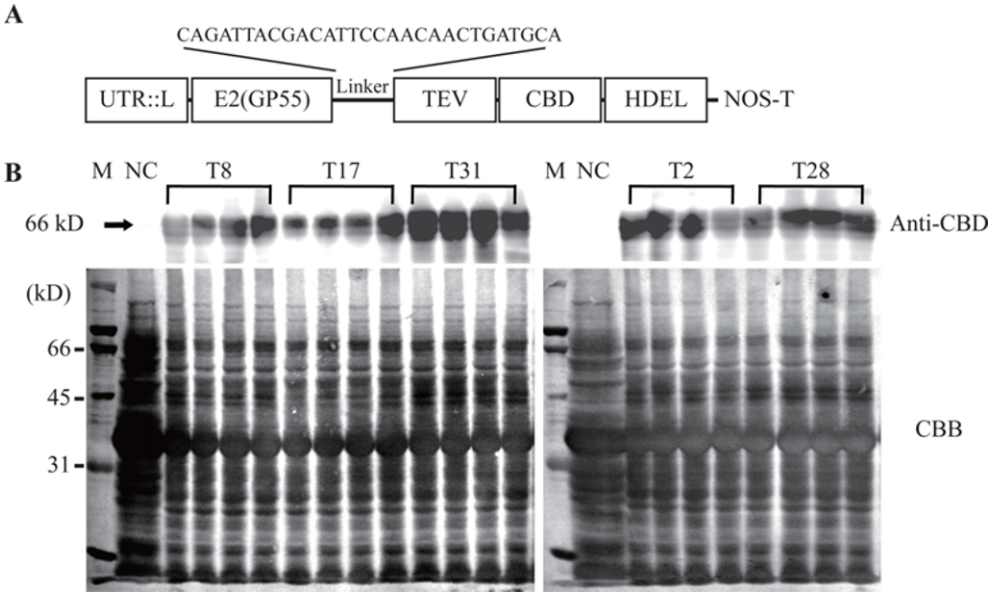

Fig. 3. Identification of transgenic plants expressing E2 fusion proteins at high levels. (A) Schematic diagram of *E2* fusion construct. UTR, 5' untranslated region; L, BiP1 leader sequence; E2, classical swine fever virus E2; TEV, tobacco etch virus protease site; CBD, cellulose-binding domain of xynA; HDEL, ER retention signal; NOS-T, nos terminator. (B) Western blot analysis of E2 fusion proteins expressed in transgenic plants. Proteins were prepared from five independent homozygous lines of the T3 generation, and 50 μg protein was subjected to western blot analysis using anti-CBD antibody. The immunoblots were stained with Coomassie brilliant blue (CBB).

plates. To examine the expression of chimeric proteins in Arabidopsis, we analyzed T1 transgenic lines by western blotting using anti-HA antibody. For both constructs, multiple T1 plants showed high levels of expression (Fig. 2).

E2-containg Chimeric Gene is Expressed at High Levels in Transgenic Arabidopsis Plants

Next, we investigated whether the newly designed expression vectors could be used to produce a target protein at high levels and if the PMP could be successfully developed as a green vaccine. We used classical swine fever virus (CSFV) E2 as the target antigen. E2 antigen was previously used as an antigen to elicit protective immune responses in animals (Ahrens et al. 2000; Bouma et al. 2000). We decided to use the ER-targeting vector for two reasons: first, E2 is a glycoprotein, and second, the ER-targeting vector showed more uniform GFP expression among independent T1 lines than the other vector. Uniform expression among transgenic lines suggests that there was less of a positional effect when the transgene was inserted into the nuclear genome compared to the other vector.

To stably express *L:E2:CBD:HDEL* (*EEC*) in plants, we introduced *E2:TEV* into *L:GFP:HA:CBD:HDEL* (*EGC*) by replacing the *GFP:HA* fragment (Fig. 3A) and used the resulting construct to generate transgenic plants via the floral dip method (Clough and Bent 1998). The tobacco etch virus protease site (TEV) was included for the possibility of removal of CBD. Multiple independent T0 lines were selected on kanamycin plates and transferred to soil to generate T1 seeds. We screened homozygous T2 plants on MS plates containing kanamycin. We obtained multiple T3 lines, which we grew on soil, and analyzed protein extracts from the T3 plans by western blotting using anti-CBD antibody (Hyunjong et al. 2006). Of these lines, transgenic lines 2, 8, 17, 28, and 31 showed high levels of CBD expression and were selected for further study. The transgenic lines showed a

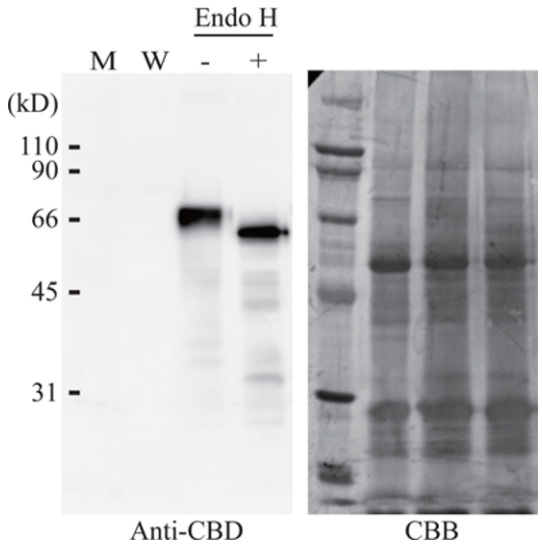

Fig. 4. E2 fusion proteins have endo H-sensitive N-glycans. Protein extracts were treated with or without endo H and analyzed by western blotting using anti-CBD antibody. M, molecular weight standard; W, wild-type plants.

protein band at the 66 kD position (Fig. 3B), which is slightly larger than the calculated molecular mass of EEC (58 kD).

E2 is an N-glycosylated protein (Kumar et al. 2015). Thus, EEC is likely N-glycosylated in the ER of Arabidopsis. Moreover, the N-glycan should be of the high mannose type, as the EEC in these plants contained an ER retention signal at its C-terminus. To test this, we treated the EEC with endo H (endoglycosidase H) and analyzed it by western blotting using anti-CBD antibody. Endo H can remove ER-type N-glycans, but not Golgi or post-Golgi-localized complex type N-glycans (Park et al. 2004). Endo H treatment led to the more rapid migration of EEC on SDS/PAGE (Fig. 4), confirming that EEC localized to the ER.

Plant-produced E2 Proteins are Recognized by an Antibody That Detects CSFV Antigen

We assessed whether E2 produced in plants as a fusion protein with many additional domains had the same antigenicity as the native antigen of CSFV. We estimated the expression levels of EEC in five independent transgenic lines. Specifically, we performed indirect measurements using western blot analysis based on the signal intensity compared to a control protein containing the same CBD, i.e., GFP:CBD expressed and purified from *E. coli*. Indirect measurement by western blot analysis using anti-CBD antibody (Hyunjong et al. 2006) revealed that EEC comprised approximately 0.35-0.7% of total soluble proteins, depending on the individual

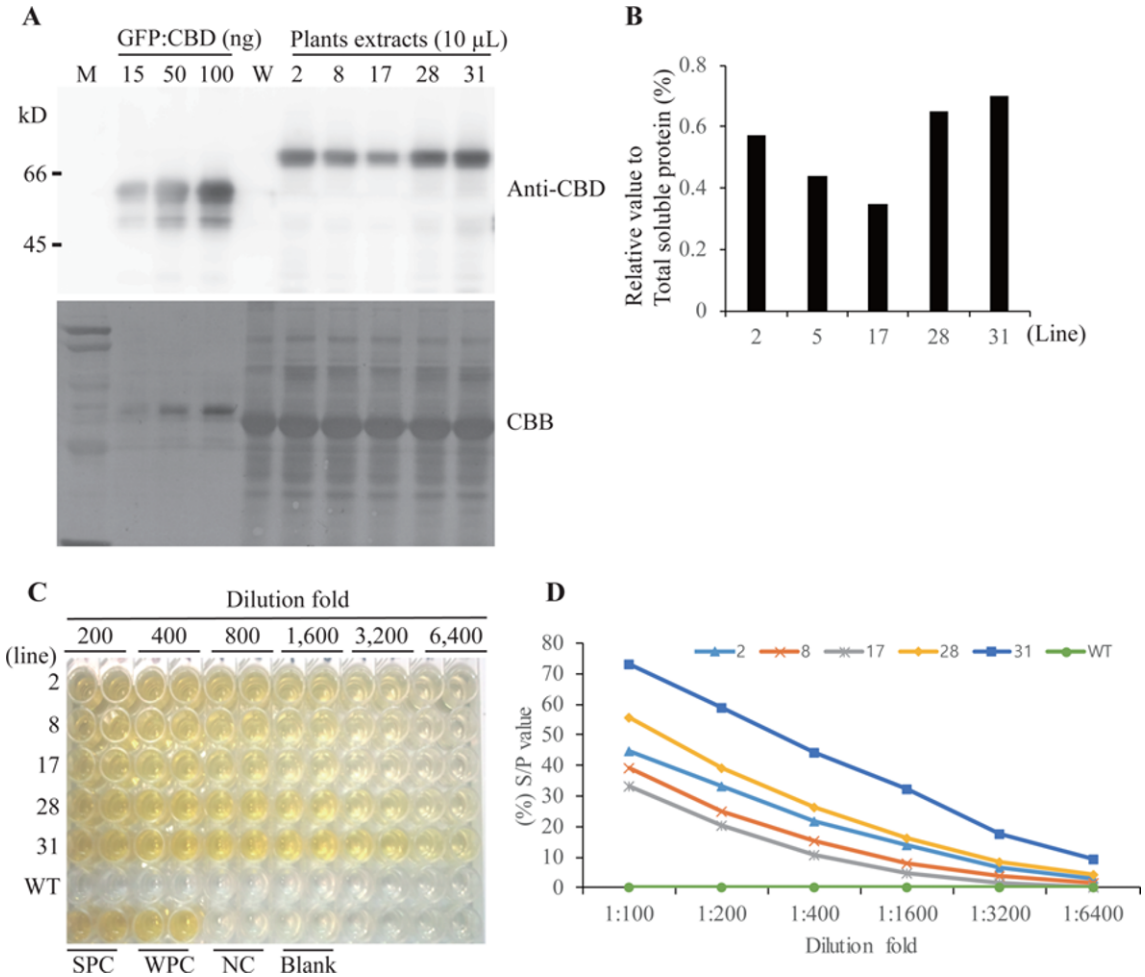

Fig. 5. E2 fusion proteins expressed in Arabidopsis are recognized by the antibody used to detect the antigen of classical swine fever virus. (A) Western blot analysis of E2 fusion proteins. Total soluble protein extracts (20 μg in 10 μL) together with varying amounts of GFP:CBD protein (10, 50, and 100 ng) were separated by SDS/PAGE and analyzed by western blot analysis using anti-CBD antibody. M, molecular weight standard; W, wild-type plants; the numbers indicate independent transgenic lines. (B) Quantification of E2 fusion proteins in transgenic plants. Signal intensity in (A) was quantified using the software provided with the LAS3000 and compared to the standard (GFP:CBD) to estimate the amount of E2 fusion protein in transgenic plants. E2 fusion protein levels are represented as values relative to total soluble proteins (TSP). (C) Antigenicity of E2. Protein extracts were prepared from transgenic plants, and serially diluted protein extracts were analyzed by ELISA with a kit used to detect CSFV antigens. (D) Quantification by ELISA. Signal intensity was measured at A_{405}. CPC = A_{SP}-A_{NC}. S/P (%) = A_{SP}-A_{NC}/CPC x100. A_{SP}, sample absorbance; A_{NC}, negative control. S/P, sample compared to the positive control value.

transgenic line (Fig. 5A, B).

Next, we measured the antigenicity of E2 in recombinant EEC protein by ELISA using a kit designed to detect the E2 antigen of CSFV. We prepared plant extracts diluted 200- to 6,400-fold, and subjected them to ELISA. Wild-type plant extracts did not show any signals, confirming that Arabidopsis does not contain any non-specific proteins that react with the antibody in the antigen detection kit. However, plant extracts from all five transgenic lines showed strong signals to levels as high as those of the positive control included in the kit (Fig. 5C, D). These results indicate that E2 in EEC recombinant protein expressed in plants had the same antigenicity as that of native E2. Moreover, the signal intensity was proportional to the expression levels of EEC in the various transgenic plant lines.

Purification of EEC from Arabidopsis Plant Extracts

We purified recombinant EEC protein from Arabidopsis plants using protein extracts prepared from plants of the T3 generation. Since CBD was incorporated into the EEC recombinant proteins, we used CBD as an affinity tag for purification (Wan et al. 2011; Sugimoto et al. 2012). CBD binds strongly to amorphous cellulose beads and can be released by di-saccharide sugar molecules. Based on these properties, CBD was previously used in protein purification from *E. coli* extracts (Sugimoto et al. 2012). We loaded total protein extracts onto a column containing cellulose beads and collected the unbound flow-through to estimate binding efficiency. After washing the cellulose resin with washing buffer, the proteins that bound to the cellulose beads were eluted with 1% cellobiose. To examine the behavior of E2 in the cellulose bead column, we analyzed total protein extracts, the unbound fraction, washing-off fractions, elution fractions, and proteins from boiled cellulose beads by western blotting using anti-CBD antibody. Most protein was detected in the elution fractions (Fig. 6A), indicating that E2 binds well to cellulose resin and can readily be eluted from cellulose beads using cellobiose. However, a small amount of protein was detected in the unbound fraction as well as the washing-off fractions. However, over 70% of the protein was recovered in the two elution fractions. This purification process yielded 40 μg purified protein from 2 g fresh plant tissue. Next, using these conditions, we performed a large-scale purification of EEC (80 g fresh weight) for use in immunization of mice. The purified protein appeared as a major band at the 66 kD position and only a minor band at the 54 kD position, i.e., the size of the large subunit of the Rubisco complex (Fig. 6B).

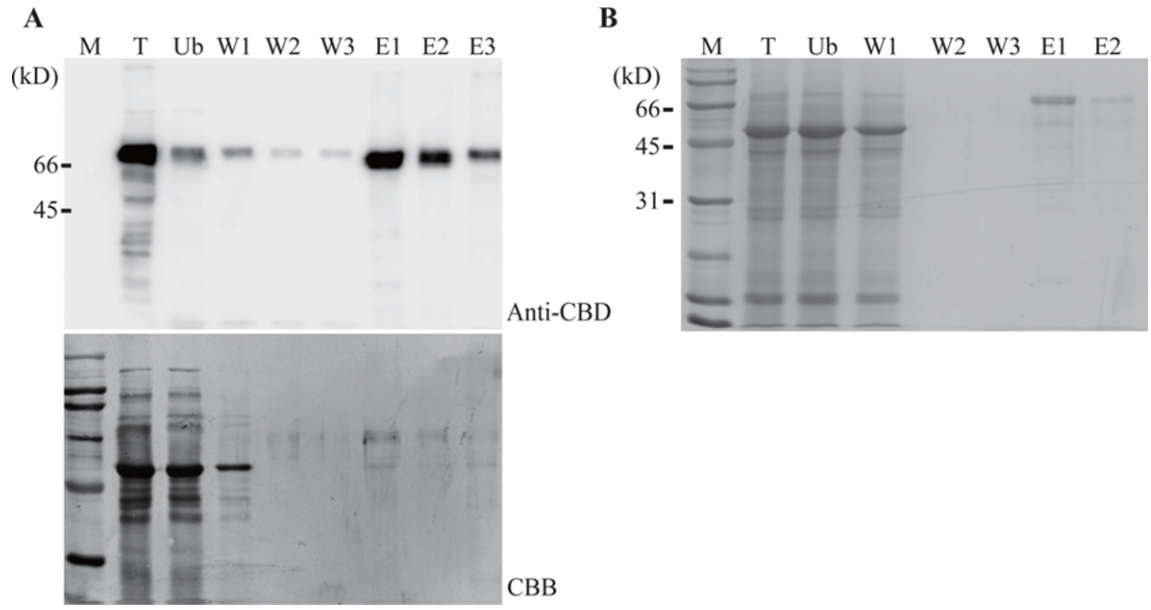

Fig. 6. Purification of E2 fusion protein using cellulose resin column chromatography. (A) E2 fusion protein purification. Protein extracts (25 mL) prepared from transgenic plants (5 g fresh weight) were loaded onto a column containing 1.4 g cellulose beads (AMC) at a flow rate of 100 mL/min. The cellulose column was then washed three times with 15 mL washing buffer (50 mM NaOAc, pH 5.2, 50 mM NaCl, 1 mM $CaCl_2$, and 0.1% Triton X-100) (flow rate, 500 mL/min) each time. After washing, proteins were eluted twice with 10 mL elution buffer (50 mM Tris-HCl, pH 8.8, 50 mM NaCl, 1 mM $CaCl_2$, and 1% cellobiose) (flow rate, 200 mL/min) each time. The unbound fraction (Ub), washing-off solutions (W1 to W3), and elution fractions (E1 and E2) were separately collected at each step and analyzed by western blotting using anti-CBD1 antibody. The immunoblot was stained with Coomassie brilliant blue (CBB). T, total protein extracts; B, proteins bound to bead after elution proteins were prepared by boiling cellulose beads in loading buffer. (B) Large-scale protein purification. Protein extracts were prepared from 80 g plant tissue in five volumes of extraction buffer. Total (T), unbound (Ub), washing off (W1 to W3), and eluted fractions (E1 and E2) were separated by SDS/PAGE and stained with Coomassie brilliant blue.

E2 Induces a Strong Immune Response in Mice

We then investigated whether E2 in the EEC fusion protein produced in Arabidopsis could be developed as a vaccine for CSFV in animals. We immunize mice (C57BL/6, 6-week-old females) with purified EEC protein at two concentrations, 0.2 μg and 1 μg. We subcutaneously immunized the mice three times at 2-3 week intervals (Fig. 7A). For immunization, the protein was mixed with Freund's complete adjuvant (Yoshikai et al. 1990). Sera were collected four times, including immediately before each injection and at 2 weeks after the final injection. To examine the immune responses to the antigen, we detected antibody against EEC protein by ELISA. Sera were diluted at 10^2 to 10^4, depending on injection conditions. Pre-immune sera, which were used as a control, did not show any response to the antigen. By contrast, both

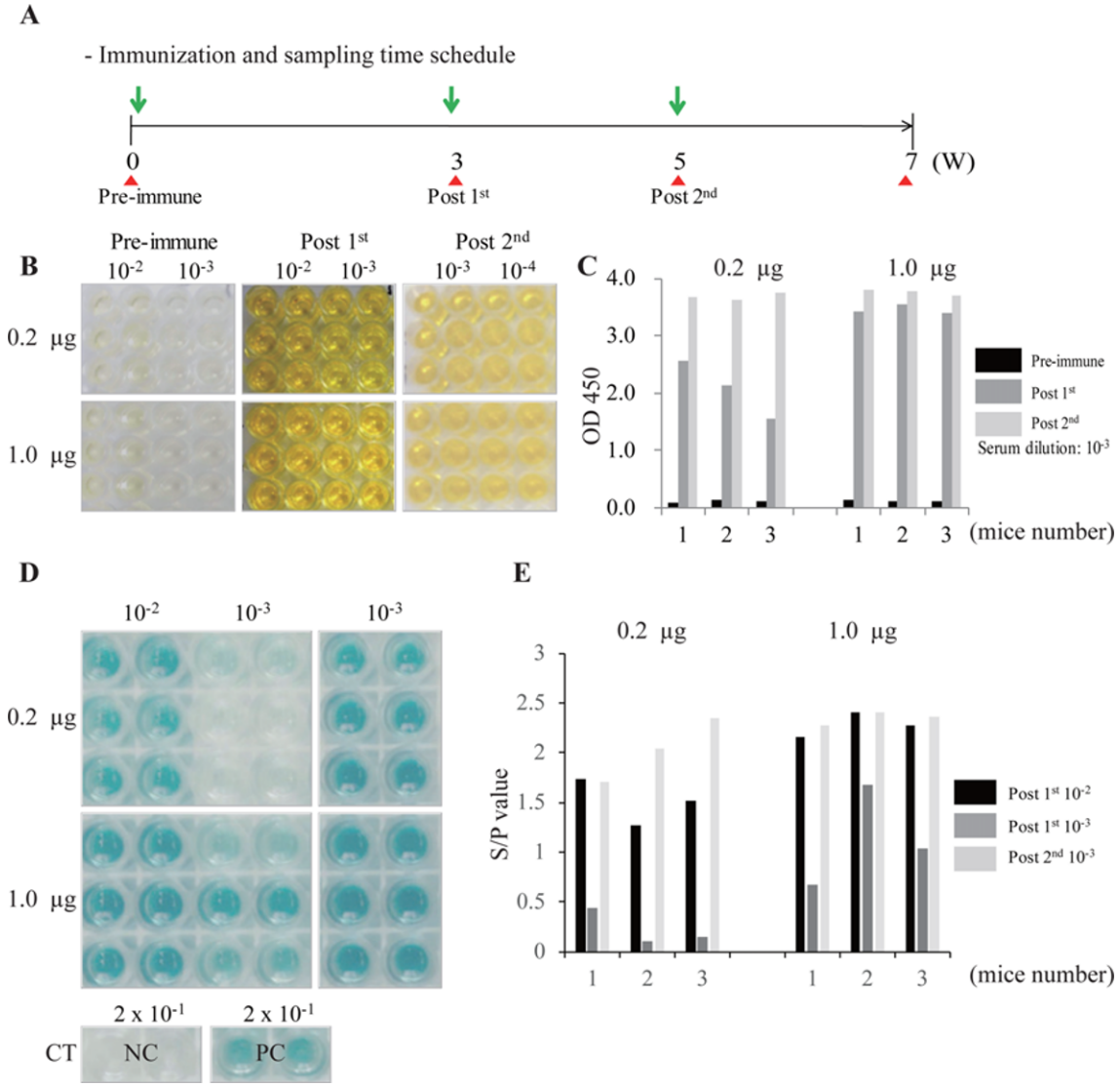

Fig. 7. E2 fusion proteins are highly immunogenic in mice. (A) Immunization protocol. Mice (C57BL/6, 6-week-old females) were injected three times with 0.2 or 1.0 μg E2 fusion proteins mixed with Freund's complete adjuvant according to the schedule. For each dose, three mice were immunized. Immediately before each injection, sera were collected. Green arrows, injection time points; red triangles, sera collection time points; W, week. (B) Self-antigenicity test. Sera collected from mice immunized with E2 fusion proteins were diluted 100- to 10,000-fold and used for ELISA. Pre-immune serum was included as a negative control. Using post-1st and post-2nd sera, antibody titers were measured by ELISA using E2 fusion proteins produced from Arabidopsis. Optical density was measured at a wavelength of 450 nm. (C) Quantification of antigenicity. Signal intensity in (B) was quantified. The graph represents 1,000-fold diluted values. (D) Antigenicity to commercial CSFV antigen. Sera collected from immunized mice were diluted 100- to 10,000-fold and used for ELISA. Pre-immune serum was included as a negative control. Using post-1st and post-2nd sera, antibody titer was measured by ELISA using the antigen included in the commercial CSFV test kit. Optical density was measured at a wavelength of 405 nm. The control test (CT) was performed using the negative and positive controls in the ELISA kit. NC, negative control; PC, positive control. (E) Quantification of antigenicity. Signal intensity in (D) was quantified. The graph represents 1,000-fold diluted values.

the post-first and post-second injection sera showed strong signals with the antigen (Fig. 7B, C). We quantified the titer of the sera, finding that the signal strength increased with increasing injection time as well as with increasing antigen concentration, indicating that EEC fusion protein is highly immunogenic in mice. Next, we examined whether the mice sera obtained from immunized mice contained antibodies that react with CSFV antigens using a commercial ELISA kit designed to detect antibodies against CSFV in animals. Again, the post-first and post-second injection sera showed strong signals at dilutions of 10^2 to 10^3 (Fig. 7D, E). The response was stronger than the signal from the positive control at a dilution of 10^2, indicating that the mouse sera contained antibodies that strongly reacted with the native E2 antigen of CSFV. The sample-to-positive (S/P) values were much greater than 0.14, which is considered to be a sufficient level to provide protective immunity.

Discussion

In this study, we investigated whether PMPs could be developed for use as vaccines against infectious viruses. To date, the potential use of PMPs for development of vaccines against infectious viruses has not been fully explored. Here, the most crucial aspect is whether the recombinant proteins have the same antigenicity as the native viral proteins. Only a few of them have been tested and shown that they have the same antigenicity with native proteins. These include plant-produced HN of chicken Newcastle disease virus (Gómez et al. 2009) and plant-produced HA of H5N1 (Bertran et al. 2015; Lee et al. 2015). Moreover, plant-produced HA of H5N1 is currently undergoing clinical trials for use as a human vaccine (Lomonossoff and D'Aoust 2016). We explored whether E2 produced in plants had the same antigenicity to native protein. The E2 fusion protein that was expressed in transgenic Arabidopsis plants contained multiple domains, such as a BiP leader sequence, E2, CBD, and the ER retention motif HDEL. Originally, we intended to use the target protein without any extra domains as antigen. However, finally we decided to use the entire fusion protein as antigen because the target region in the entire fusion protein showed strong antigenicity (Fig. 5). More importantly, the CBD which was highly immunogenic in mice (Fig. 7) can function as a DIVA marker for the vaccination. In addition, removal of the CBD from the fusion proteins requires enterokinase treatment, leading to an increase in the cost and a loss in the yield of target proteins. For these reasons, we decided to use the entire protein instead of E2 protein as antigen. E2 proteins produced in insect cells were previously developed for use as DIVA vaccines (Ahrens et al. 2000; Bouma et al. 2000). These findings strongly suggest that the E2 protein produced in Arabidopsis had the same antigenicity as the native protein and that it could be developed as a vaccine against CSFV in animals, specifically pigs.

The successful commercialization of PMPs as vaccines strongly depends on the expression level of the protein of interest. In the case of HN from chicken Newcastle disease virus, HN produced in plants was successfully developed as a vaccine, but it is not available on the market, perhaps due to its low expression levels (Gómez et al. 2009). Compared to animal cells and bacteria, many different strategies can be used to express proteins in plants. Gene expression can occur transiently or stably in plants (Maliga 2004; Makhzoum et al. 2014; Canto 2016). In addition, genes can be housed in the nuclear or chloroplast genomes (Hefferon 2012; Peyret and Lomonossoff 2013; Mortimer et al. 2015; Salazar-González et al. 2015). Of the many approaches available for foreign gene expression in plants, we chose to express *E2* in transgenic plants. Moreover, we decided to use plant leaf tissues as a source of biomass for protein production. Indeed, the use of transgenic plants harboring a transgene integrated into the nuclear genome is the most cost-effective approach due to the ease of biomass production and the low cost of plant growth facilities compared to cell culture and transient expression after *Agrobacterium*-mediated infiltration. Also, it is easier to scale up production capacity using stably transformed plants compared to other plant systems. However, the gene expression in chloroplasts generally yields the highest levels of protein among systems (Staub et al. 2000; Maliga 2004; Abdoli et al. 2016; Daniell et al. 2016). Transient expression using RNA virus-based expression vectors also leads to high-level protein production (Gleba et al. 2007). Thus, when expressing genes in transgenic plants harboring an integrated, single copy gene in their nucleus, it is quite challenging to achieve high levels of protein production. The expression level of a protein is controlled at many levels, such as transcription, translation, and storage of the protein in the cell. In the current study, we included a 5'-UTR translational enhancer immediately upstream of the AUG codon. We previously showed that the 20 bp region immediately upstream of the initiation codon greatly affects translational efficiency (Kim et al. 2014). Another consideration is the storage of proteins after translation. The ER and chloroplasts accumulate proteins at high levels (Staub et al. 2000; Jha et al. 2012). Proteins in the ER are likely less to be subject to proteolysis than proteins in other compartments because protease activity is not very high in the ER. By incorporating these multiple components, we were able to produce E2 fusion protein at a level of up to 0.7% of total soluble proteins.

Another critical step in protein production is purification; this is the most cost step in recombinant protein production. One of the most efficient methods for protein purification is affinity column chromatography (Arora et al. 2017). Many

affinity tags have been developed for use in protein purification (Łojewska et al. 2016; Yadav et al. 2016; Singh and Herzer 2017; Oliveira and Domingues 2018), some of which are based on a specific property of the target protein. For example, antibodies have a high affinity for protein A or protein G (Hernández-Velázquez et al. 2015' Madeira et al. 2016). Thus, these proteins can be used as affinity tags to purify antibodies or fusion proteins containing an antibody or a part of an antibody. Since a specific binding protein that can be used for affinity purification is not available for E2, we decided to add an extra domain as an affinity tag. Certain artificial epitopes or protein domains (such as His_6, GST, flag, HA, and T7) that can be fused to target proteins as affinity tags have been developed, along with the corresponding resins (Valdez-Ortiz et al. 2005; Kittur et al. 2015; Abdoli et al. 2016). However, not many of these systems have been tested for their suitability for protein purification from plant extracts. We chose CBD as an affinity tag for the purification of E2 fusion protein. CBD has also been used as an affinity tag in *E. coli* and other expression systems (Boraston et al. 2001; Wan et al. 2011; Sugimoto et al. 2012). CBDs show high affinity for either the amorphous or crystalline region of cellulose, depending on their types (Armenta et al. 2017). The CBD derived from xynA of *Clostridium stercorarium* has high affinity for amorphous cellulose. CBD has been shown to be useful for purifying proteins from bacterial extracts (Hong et al. 2008; Fig. S1). In addition, we also found that it is an excellent system to purify protein from plant extracts. CBD is one of cheapest biomaterials. Thus, compared to other commonly used affinity resins, cellulose resin is one of the cheapest resins, thereby leading to the lower purification cost. Vaccines for pigs and other animals are cheaper than human vaccines. Thus, the purification cost is one of important concerns in the vaccine development. In addition, the binding capacity is comparable to other resins. We observed that rubisco subunits are not tightly bound to cellulose resin in our experiments. Thus, the use of CBD as an affinity tag for purification is a cost-effective method for protein purification. In the current study, we successfully used CBD-based protein purification to purify E2 fusion protein from Arabidopsis extracts. Using our one-step CBD-based purification, we obtained protein with >90% purity and a yield of >70%, suggesting that CBD-based affinity purification using cellulose as a resin is an efficient method for protein purification from plant extracts. When developing PMPs for use as animal vaccines for commercialization, one important consideration is the cost of protein production. Thus, using cellulose as a resin can greatly reduce protein production costs.

In summary, we investigated whether E2 from CSFV expressed in plants could be developed for use as a green vaccine against CSFV in pigs. We tested this possibility at several levels: protein expression in plants, protein purification, and the immunogenicity of E2 in the fusion protein. We demonstrated that 1) E2 fusion protein can be expressed in Arabidopsis at a level as high as 0.7% of total soluble proteins; 2) E2 fusion proteins can be purified in a single step with over 70% yield and 90% purity; 3) E2 in the fusion protein is highly immunogenic in mice and is recognized by an ELISA kit used to detect CSFV antibody; and 4) antisera from immunized mice can be recognized by the ELISA kit used to detect CSFV antigen. Thus, we propose that E2 fusion protein produced in Arabidopsis could be further developed into a green vaccine for CSFV. Further studies are needed using the target animal, pig, in the future.

Materials and Methods

Plant Growth Conditions

Arabidopsis thaliana (ecotype Columbia) plants were grown on ½ MS plates at 23°C in a culture room under a 16 h/8 h light/dark cycle (Ahn et al. 2017).

Plasmid Construction

To generate the ER-targeting expression vector, the *5'-UTR::L* was generated by PCR. The forward primer consisted of three regions: the 6 bp Xba I restriction site, a 20 bp 5' UTR translational enhancing sequence, and 20 bp of *BiP1* (nucleotide positions 1 to 20; Table S1). The reverse primer consisted of two parts: the 6 bp BamH I restriction site and 20 bp of *BiP1* (nucleotide positions 252 to 272). The fusion construct, *HA:CBD:HDEL*, was generated by PCR. The forward primer consisted of four regions: the 6 bp Xma I restriction site, 27 bp of the HA epitope, and 20 bp of *Clostridium stercorarium xynA* (nucleotide positions 1554 to 1574). The reverse primer consisted of three regions: 20 bp of *xynA* (nucleotide positions 1954 to 1974), 15 bp of HDEL and termination codon, and the 6 bp Xho I restriction site. The two PCR fragments were inserted into the 326-GFP vector containing the double CaMV 35S promoter and nos terminator using restriction endonuclease pairs Xba I and BamH I, and Xma I and Xho I, respectively.

To generate the chloroplast-targeted expression vector, the *5'-UTR::TP* was generated by PCR. The forward primer consisted of three regions: the 6 bp Xba I restriction site, 20 bp of the 5' UTR, and 20 bp of *Cab* (nucleotide positions 1 to 20). The reverse primer consisted of 20 bp of *Cab* (nucleotide positions 241 to 261) and the 6 bp BamH I restriction site. HA:CBD was generated by PCR. The forward primer consisted of the 6 bp Xma I restriction site, 27 bp of the HA epitope, and 20 bp of *xynA* (nucleotide positions 1554 to 1574). The reverse primers consisted of the 6 bp Xho I restriction site and 23 bp of *xynA* (nucleotide positions 1954 to 1974) with a termination codon. The two fragments were inserted into the 326-GFP vector using restriction endonuclease pairs Xba I and BamH I, and Xma I and Xho I, respectively.

To generate *UTR::L:GFP:HA:CBD:HDEL* and *UTR::TP:GFP:HA:CBD*, the *GFP* fragment was inserted downstream of the BiP leader sequence and Cab transit peptide sequence, respectively, using restriction endonucleases Bam H1 and Sma I. To generate *UTR::L:E2:TEV:CBD:HDEL*, *E2* was PCR amplified using primers E2-F and E2-R and *TEV:CBD:HDEL* was PCR amplified using primers TEV/CBD-F and HDEL/CBD-R, and the resulting PCR product was inserted into *UTR::L:GFP:HA:CBD:HDEL* by replacing *GFP:HA: CBD:HDEL*.

To express GFP:CBD in *E. coli*, the *GFP* and *CBD* sequences were fused by sequential PCRs. First, *GFP* was amplified by PCR using

primers GFP-F and GFP-R, and *CBD* was amplified by PCR using primers CBD-F and CBD-R. The second PCR was performed with primers GFP-F and CBD-R using the two PCR products as template. Finally, *GFP:CBD* was subcloned into the pRSET-A vector to generate pRSET-GFP:CBD.

Generation of Transgenic Plants

Expression vectors were introduced into *Agrobacterium* and subsequently transformed into Arabidopsis using the floral dip method (Clough and Bent 1998). T0 plants were screened on MS plates containing kanamycin (50 mg/L). Homozygous plants were screened at the T2 generation, and E2 fusion protein was purified from homozygous T3 plants.

Transient Expression in Protoplasts and Imaging of GFP

Plasmids were purified using a Qiagen column and used for polyethylene glycol-mediated protoplast transformation (Kang et al. 2012)

Images of GFP fluorescence and red chlorophyll autofluorescence were acquired by fluorescence microscopy (Lee et al. 2011; Park et al. 2016. Images were obtained using a cooled CCD camera and a Zeiss Axioplan-fluorescence microscope at 40x magnifications. The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23) and XF137 (exciter, 540AF30; dichroic, 570DRLP; emitter, 585ALP) (Omega, Inc., Brattleboro, VT, USA) for GFP and chlorophyll autofluorescence, respectively. Adobe Photoshop software was used to adjust the brightness and contrast of the images and to process the images.

Protein Purification and Western Blot Analysis

Leaf tissues were frozen in liquid nitrogen and ground to a powder. The sample was mixed with 5 volumes of protein extraction buffer (50 mM NaOAc, pH 5.2, 50 mM NaCl, 1 mM $CaCl_2$, and 0.1% Triton X-100) and homogenized with a mortar and pestle and by brief sonication. The homogenates were centrifuged at 20,000 × g for 30 min and the supernatant was collected. Total protein extract (25 mL) was loaded onto a column containing 1.5 g amorphous cellulose beads (AMC) at a flow rate of 100 mL/min. The flow-through was collected as the unbound fraction. The column was washed three times with 15 mL extraction buffer at a flow rate of 500 mL/min, and each washing-off solution was collected separated. Finally, 10 mL elution buffer (50 mM Tris-HCl, pH 8.8, 50 mM NaCl, 1 mM $CaCl_2$, and 1% cellobiose) was added to the column at a flow rate of 200 mL/min to release bound proteins from the cellulose resin. Elution was performed twice, and each elution fraction was collected separately. For large-scale protein purification, 80 g (fresh weight) plant tissue was harvested, ground in liquid nitrogen, and combined with 5 volumes of extraction buffer.

Total proteins and the fractions from the protein purification steps were analyzed by 10% SDS/PAGE and western blotting using anti-HA or anti-CBD antibodies (Hyunjong et al. 2006).

Immunization of Mice

Mice (C57BL/6, 6-week-old females) were used for immunization. E2 fusion protein (0.2 μg or 1.0 μg) was combined with Freund's complete adjuvant and injected subcutaneously into mice (Yoshikai et al. 1990; Lee et al. 2015). Sera were collected from mice just prior to each injection and at 2 weeks after the final injection. The mice were given three injections at 2- or 3-week intervals.

ELISA of Serum from Immunized mice and E2 Expressed in Arabidopsis

A VDPro® CSFV AB ELISA kit (Median, Cat. No. ES-CSF-01) was used to detect antibody production against CSFV. The serum was obtained from blood after centrifugation at 3,000 × g for 15 min and diluted to 1:2000 with serum dilution buffer. The CSFV E2 antibody-coated 96-well microplate and reagents were placed on a bench to reach room temperature before use. Positive and negative controls were diluted 20-fold. Each 100 μL aliquot of sample was added to a microplate well and incubated at 37°C for 1 h. The liquid was discarded from the microplate. The wells were washed three times with 300 μL washing buffer, and 100 μL HRPO Anti-mouse IgG Conjugate (1:5000 dilution) was added to each well. After 1 h incubation at 37°C, the wells were washed three times with 300 μL washing buffer, and 100 μL ABTS Substrate was added to each well, followed by incubation for 10 min at room temperature. The reaction was inhibited by adding 100 μL Stop Solution. The absorbance of each well was read at 405 nm on an ELISA Reader.

To test whether E2 in the fusion proteins expressed in Arabidopsis had the same antigenicity as native E2, ELISA was performed as described for the analysis of sera from mice immunized with E2 fusion proteins, except that a VDpro® CSFV AG ELISA kit (Median, Cat. No. ES-CSF-03) and protein extracts from Arabidopsis plants were used. Protein extracts were diluted 200- to 6,400-fold in CB buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3), combined with 100 μL HRPO Anti-CSFV conjugate, and incubated at 37°C for 1 h. After washing three times with PBST buffer (PBS buffer + 0.1% Tween-20), 100 μL TMB Substrate was added to each well and incubated for 10 min at room temperature. After the addition of 50 μL Stop solution, the absorbance of each well was measured at 450 nm on an ELISA Reader.

Expression and Purification of GFP:CBD in *E. coli*

The pRSET-GFP:CBD construct was introduced into *E. coli* strain BL21 (DE3). A single colony from the plate was cultured in 5 mL LB liquid medium overnight in a shaker. The following day, the 5 mL culture was transferred to 200 mL LB liquid medium containing ampicillin. When the culture reached an optical density at 600 nm (OD600) of 0.6, the culture temperature was reduced to 30°C. IPTG was added to the culture to a final concentration of 1 mM, and the culture was incubated overnight at 30°C. The *E. coli* cells were collected by centrifugation at 5,000 × g for 10 min at 4°C. The pellet was re-suspended in 5 mL ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) containing 1 mM PMSF, 1 mM DDT, and proteinase inhibitors (1 μg/mL leupeptin and 1 μg/mL pepstatin). The *E. coli* cells were disrupted by sonication using 3–5 sec pulses at 40% amplitude for 5-10 min on ice (Ultrasonic Generator VC 505/VC 750 Sonics, Newtown, CT, USA). The cell extracts were separated by centrifugation at 14,000 × g for 20 min, and the supernatant was collected and stored at 4°C for purification.

For protein purification, a 1 mL Ni^+-NTA slurry (0.5 mL bed volume) was added to a 15 mL tube and briefly centrifuged. The supernatant was removed, and 2 mL lysis buffer was added to the tube. After mixing by gently inversion, the Ni^+-NTA resin was centrifuged again and the supernatant was discarded. The cleared bacterial lysates were added to the Ni^+-NTA resin and mixed gently by shaking (200 × g on a rotary shaker) at 4°C for 60 min. The lysate–Ni^+-NTA mixture was loaded onto a column with the bottom outlet capped. After removing the bottom cap, the column flow-through was collected. The flow-through fractions were saved for SDS/PAGE analysis. The column was washed twice with five bed volumes (2.5 mL) of washing buffer alone and then at least four times with 30 mM imidazole-containing washing buffer. The washing fractions were also collected for SDS/PAGE analysis. Proteins were eluted with 250 mM imidazole-containing elution buffer.

Acknowledgements

This work was supported by Technology Commercialization Support

Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (grant no. 2015300022) and was supported by a grant (2016R1E1A1A02922014) from the National Research Foundation, Ministry of Science and ICT, Korea.

Author's Contributions

HI, SE and LY designed and organized the overall study; PM carried out cloning and generated constructs; PN and KN performed immunization-related work and antibody analysis; PS, MK and GS carried out western blot analysis; PY analyzed subcellular localization of constructs; SJ and AD provided information for immunization experiments; HI wrote the paper. All the authors agreed on the contents of the paper and post no conflicting interest.

Supporting Information

Fig. S1. Expression of GFP:CBD in *E. coli*.
Table S1. Nucleotide sequences of primers used in this study.

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