Plant Biotechnology Journal

Plant Biotechnology Journal (2015) **13**, pp. 62–72 doi: 10.1111/pbi.12235

Oral immunization of haemaggulutinin H5 expressed in plant endoplasmic reticulum with adjuvant saponin protects mice against highly pathogenic avian influenza A virus infection

Goeun Lee[1,2], Yun Jeong Na[3], Bo-Gie Yang[2], Jun-Pyo Choi[3], Yong Bok Seo[3], Chun-Pyo Hong[2,4], Chang Ho Yun[2], Dae Heon Kim[3], Eun Ju Sohn[4], Jeong Hee Kim[5], Young Chul Sung[3,4], Yoon-Keun Kim[3,†], Myoung Ho Jang[2,4,*] and Inhwan Hwang[1,3,4,*]

[1]*School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, Pohang, Korea*

[2]*Academy of Immunology and Microbiology (AIM), Institute for Basic Science (IBS), Pohang, Korea*

[3]*Department of Life Science, Pohang University of Science and Technology, Pohang, Korea*

[4]*Division of Integrative Biosciences and Biotechnology, Pohang University of Science and Technology, Pohang, Korea*

[5]*Department of Oral Biochemistry and Molecular Biology and Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul, Korea*

Received 24 March 2014;
accepted 26 June 2014.
**Correspondence* (IH, Tel +82 054 279 2128; fax +82 054 279 8159; email ihhwang@postech.ac.kr and MHJ, Tel +82 054 279 0614; fax +82 054 279 8744; email jangmh@postech.ac.kr)
[†]Present address: School of Medicine, Ehwa Woman's university, Seoul, Korea.

Keywords: highly pathogenic avian influenza virus, plant edible vaccine, ER-targeted haemagglutinin, adjuvant Saponin, mucosal immunity, influenza infection.

Summary

Pandemics in poultry caused by the highly pathogenic avian influenza (HPAI) A virus occur too frequently globally, and there is growing concern about the HPAI A virus due to the possibility of a pandemic among humans. Thus, it is important to develop a vaccine against HPAI suitable for both humans and animals. Various approaches are underway to develop such vaccines. In particular, an edible vaccine would be a convenient way to vaccinate poultry because of the behaviour of the animals. However, an edible vaccine is still not available. In this study, we developed a strategy of effective vaccination of mice by the oral administration of transgenic *Arabidopsis* plants (HA-TG) expressing haemagglutinin (HA) in the endoplasmic reticulum (ER). Expression of HA in the ER resulted in its high-level accumulation, *N*-glycosylation, protection from proteolytic degradation and long-term stability. Oral administration of HA-TG with saponin elicited high levels of HA-specific systemic IgG and mucosal IgA responses in mice, which resulted in protection against a lethal influenza virus infection with attenuated inflammatory symptoms. Based on these results, we propose that oral administration of freeze-dried leaf powders from transgenic plants expressing HA in the ER together with saponin is an attractive strategy for vaccination against influenza A virus.

Introduction

Influenza A virus is one of the most serious respiratory pathogens that cause severe illness and are potentially fatal (Cox *et al.*, 2004). In recent outbreaks of new swine H1N1 influenza virus, the virus infected a large number of humans and caused significant concern as a global pandemic (Garten *et al.*, 2009). Highly pathogenic avian influenza (HPAI) A virus subtype H5N1, HPAI A (H5N1), is regarded as being the next global pandemic threat for humans because these viruses spread easily at the global level by avian hosts across the species barrier and can directly infect humans (Munster and Fouchier, 2009; Webster *et al.*, 2006). Once the virus infects humans, it causes extraordinarily high mortality and shows a high mutation rate (Abdel-Ghafar *et al.*, 2008). As the first case of human infection with HPAI A (H5N1) in 1997, 386 deaths of a total of 650 cases of human infection have been reported (WHO, 2014). Human infection of HPAI A (H5N1) has been sporadic, but the risk of human infection is increasing because of wide circulation and rapid evolution of the virus (Webster and Govorkova, 2006; Webster *et al.*, 1992). Therefore, there is increasing demand for HPAI A (H5N1) vaccines to prevent the onset of the pandemic situation.

The most common method of vaccination against influenza viruses is parenteral injection. Injectable vaccines induce mainly a systemic IgG response, but only a little mucosal IgA response which plays an important role for the protection against respiratory virus infection (Holmgren and Czerkinsky, 2005). By contrast, mucosal vaccination provokes both mucosal (Secretory IgA) and systemic (Serum IgG) immune responses, thus being obviously a better strategy against respiratory pathogens (Lycke, 2012; Ogra *et al.*, 2001). Indeed, intranasal vaccination, a type of the main types of mucosal vaccination, induces strong mucosal immune responses. However, the intranasal vaccination has potential risk of patients with asthma or Bell's palsy which may exacerbate the disease (Hodge *et al.*, 2001; Mutsch *et al.*, 2004). The oral route is another choice for the mucosal vaccination. Moreover, oral vaccination is easier, cheaper and safer than parenteral vaccination and has a better patient compliance (Mitragotri, 2005).

Many different approaches have been used to produce vaccine antigens for oral vaccination. These include bacterial fermentation, insect cell cultures, mammalian cell cultures and plant tissue

cultures (Chu and Robinson, 2001; Swartz, 2001; Wang *et al.*, 2006). However, these cell culture–based systems are not feasible propositions for antigen production in terms of cost, product quality and safety (Ma *et al.*, 2003). Recently, transgenic plant systems have been recognized to have many advantages over the traditional systems (Guan *et al.*, 2013; Streatfield *et al.*, 2001; Yusibov and Rabindran, 2008): inexpensive and easy handling, complete exclusion of microbial and animal products by appropriately controlling the microbial contamination, potential for post-translational modifications, long-term storage in transgenic plants as seeds and most importantly a potential for the oral administration of edible plant parts without additional processing. Therefore, the development of a plant-based edible vaccine for HPAI A (H5N1) has been the subject of great interest as a preventive measure against influenza pandemics (Streatfield, 2005; Walmsley and Arntzen, 2003).

In this study, we investigated whether haemagglutinin (HA) of HPAI A (H5N1), expressed in the ER of transgenic *Arabidopsis* plants can be used as an edible vaccine. At the same time, we identified an adjuvant suitable for oral immunization using plant tissues. From this study, we provide evidence that oral administration of freeze-dried leaf powders from plants expressing HA in the ER together with saponin as an adjuvant induced strong immune responses that conferred on mice the protective immunity against a lethal avian influenza virus challenge.

Results

Transgenic *Arabidopsis* plants expressing influenza A virus haemaggulutinin in the ER

To develop an edible vaccine against avian influenza A virus, we tested whether haemagglutinin (HA) of the A/Hong Kong/213/03 (H5N1) influenza virus strain expressed in the ER of *Arabidopsis* plants is suitable for edible vaccination. We reasoned that the ER is a suitable location for storing the antigen because proteins can be *N*-glycosylated and the ER membrane may provide a protection against proteolytic degradation. For targeting to the ER, the HA lacking the transmembrane domain (amino acid positions 14 to 530 of HA) was fused to the ER-targeting signal of BiP at the 5′-end. Additionally, the cellulose-binding domain (CBD) and an ER retention motif HDEL were sequentially fused to the C-terminus of HA as an affinity tag for purification and ER retention, respectively (Figure 1a). The resulting fusion construct, *BiP:HA:CBD:HDEL,* was used to generate transgenic plants (HA-TG) and its expression levels were examined in T4 or T5 generations. Total protein extracts were analysed by Western blotting using anti-CBD and anti-H5 antibodies. The BiP:HA:CBD:HDEL protein level calculated from the band intensity was 700 μg/g in the dried leaf powders (140 μg/g in fresh leaf tissues) of HA-TG plants in both generations (Figure 1b). By contrast, no visible band was detected in protein extracts of wild-type plants with these antibodies, confirming the specificity of the antibody we used.

Next, we examined its localization by immunostaining. Protoplasts from the transgenic plants at T4 or T5 generation were immunostained using the CBD antibody. Both samples produced a network pattern (Figure 1c), indicating that BiP:HA:CBD:HDEL localizes to the ER. To confirm the ER localization of BiP:HA:CBD:HDEL at the biochemical level, we examined its glycosylation pattern. Sequence analysis revealed that the HA contains multiple *N*-glycosylation sites (Das *et al.*, 2010). Protein extracts from the transgenic plants were treated with endoglycosidase H (Endo H) and analysed by Western blot analysis using anti-CBD and anti-H5 antibodies. Endo H is an endoglycosidase which cleaves asparagine (*N*)-linked mannose-rich oligosaccharides. The CBD-positive band migrated faster in Endo H-treated extracts than in control extracts (Figure 1d), indicating that BiP:HA:CBD:HDEL

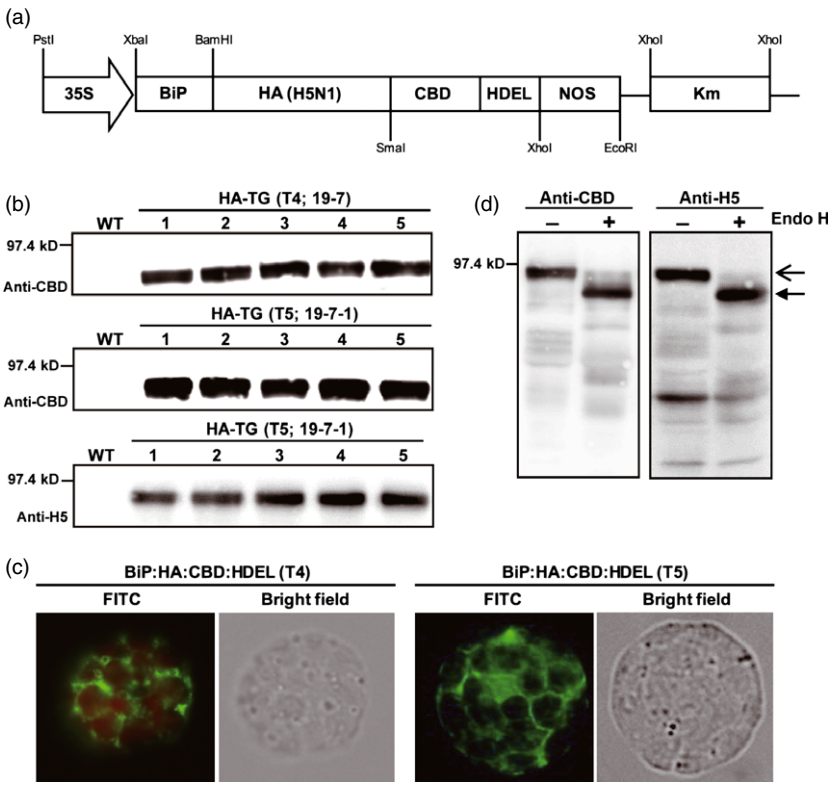

Figure 1 HA fusion proteins targeted to the ER of *Arabidopsis* shows a high-level accumulation and *N*-glycosylation. (a) Schematic representation of the *BiP:HA:CBD:HDEL* construct. 35S, cauliflower mosaic virus 35S promoter; BiP, the leader sequence of BiP; HA (H5N1), haemagglutinin of Influenza A virus [A/Hong Kong/213/2003 (H5N1)]; CBD, cellulose-binding domain; HDEL, ER retention signal; NOS, nos-terminator; Km, Kanamycin-resistance gene. Restriction endonuclease sites used for generating the construct are indicated. (b) Expression levels of BiP:HA:CBD:HDEL. Leaf extracts from five individual plants of T4 or T5 generation of a transgenic line 19 (HA-TG) and WT plants (WT) were analysed by Western blotting using anti-CBD and anti-H5 antibodies. (c) Localization of BiP:HA:CBD:HDEL in the ER. Protoplasts from leaf tissues of transgenic plants (T4 or T5 generation) were immunostained with anti-CBD antibody followed by FITC-labelled anti-rabbit IgG. (d) *N*-glycosylation of BiP:HA:CBD:HDEL. Protein extracts from leaf tissues were incubated for 1 h with (+) or without (–) Endo H and analysed by Western blotting using anti-CBD and anti-H5 antibodies. Open arrow, glycosylated form; filled arrow, deglycosylated form.

was *N*-glycosylated and the oligosaccharide sidechains are unprocessed.

To assess whether and to what extent the ER-localized antigen can withstand the harsh conditions of the gastrointestinal tract, we examined how much of BiP:HA:CBD:HDEL in freeze-dried leaf powders of HA-TG remains after treating with pepsin. Suspensions of freeze-dried HA-TG leaf powders and purified HA (derived from HA-TG) with or without freeze-dried WT leaf powders were incubated with pepsin at 37 °C for 1 h and analysed by Western blotting using anti-CBD and anti-H5 antibodies (Figure 2a). The majority (over 60%) of BiP:HA:CBD:HDEL in the suspension of freeze-dried HA-TG leaf powders remained intact, while purified HA at both conditions was almost undetectable, indicating that the ER-localized proteins are protected against pepsin. Next, we examined stability of the ER-localized BiP:HA:CBD:HDEL at room temperature (RT) to assess the long-term stability. After keeping freeze-dried leaf powders of HA-TG at RT for 1, 2, 4, 8 and 12 months, extracts from these powders were analysed by Western blotting using anti-CBD and anti-H5 antibodies (Figure 2b). The BiP:HA:CBD:HDEL level was maintained nearly at the same level for 1 year at RT, indicating that BiP:HA:CBD:HDEL proteins are stable in the freeze-dried leaf powders of HA-TG for a long time at RT.

Humoral immune responses induced by orally administrated HA-TG plants depend on the type of adjuvants, antigen doses and immunization schedule

To design an optimal edible vaccination protocol, we initially screened adjuvants for the induction of an HA-specific immune response. Cholera toxin (CT), flagellin and saponin are considered to be potent mucosal adjuvants (Lycke and Holmgren, 1986; Pickering *et al.*, 2006; Skountzou *et al.*, 2010). Accordingly, mice were orally administered with 22 mg HA-TG (containing 15 μg of BiP:HA:CBD:HDEL) together with an adjuvant, CT, recombinant flagellin (recFLA) or saponin. The amount of BiP:HA:CBD:HDEL was determined based on a previous study showing that 20 μg of HA is optimal for oral vaccination (Barackman *et al.*, 2001). As

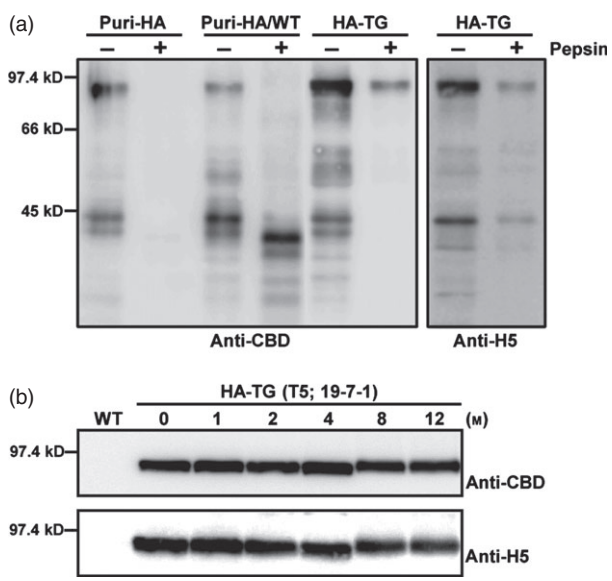

Figure 2 BiP:HA:CBD:HDEL localized in the ER shows a higher degree of resistance to pepsin treatment and long-term stability. (a) Resistance to pepsin treatment. Suspensions of freeze-dried leaf powders of HA-TG plants (HA-TG) and purified BiP:HA:CBD:HDEL (Puri-HA) with (Puri-HA/WT) or without freeze-dried WT leaf powders were incubated with (+) or without (–) pepsin for 1 h at 37 °C and analysed by Western blotting using anti-CBD and anti-H5 antibodies. (b) Long-term stability of BiP:HA:CBD:HDEL. Protein extracts from TG-HA plants (T5 generation) stored for the indicated period of time at RT were analysed by Western blotting using anti-CBD and anti-H5 antibodies. WT plants were included as a negative control. M, month.

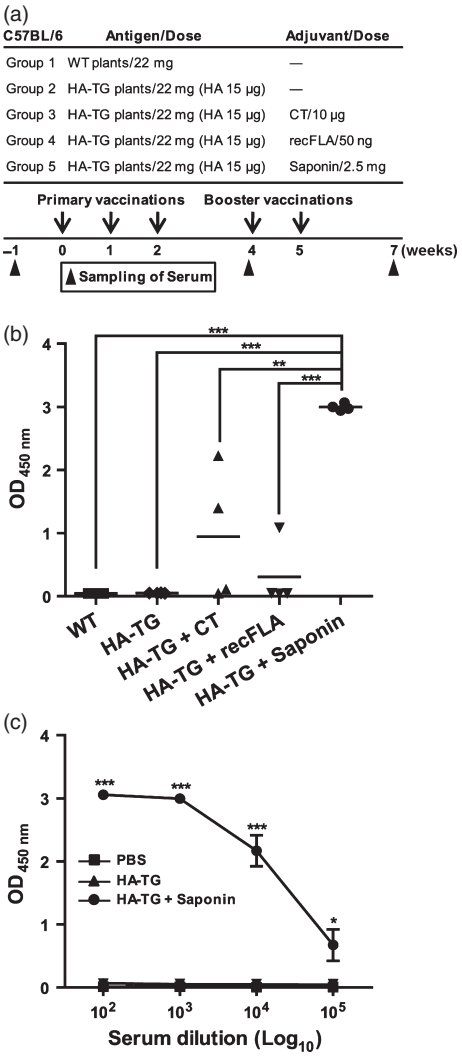

Figure 3 HA-specific antibody response induced by orally administered HA-TG plants in mice shows strong dependency on the type of adjuvants. (a) Oral immunization protocol. The types of plants and the amount of antigens are indicated. In addition, the types of adjuvant and the amount of adjuvants are indicated. HA-TG, transgenic *Arabidopsis* plants; WT, wild-type plants; CT, cholera toxin; recFLA, recombinant flagellin. HA, BiP:HA:CBD:HDEL. W, week. (b) Induction of HA-specific IgG in mice. Mice were orally immunized with HA-TG plant (22 mg) together with indicated adjuvants as in (a). Sera were collected from mice 2 weeks after final immunization (7W) and HA-specific IgG levels were measured by ELISA. Each symbol represents a single mouse and horizontal lines indicate the mean values ($n = 4$). (c) HA-specific IgG levels in serum. HA-specific IgG levels in serum were measured by ELISA at the indicated dilution points. Error bars represent the SEM ($n = 4$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Results shown in this figure are representative of two independent experiments.

negative controls, mice were given HA-TG without adjuvant or WT plants (Figure 3a). Mice were immunized five times in a total, and sera were collected at three different time points to examine HA-specific antibody responses (Figure 3a). Among the immunized animals, the highest HA-specific IgG response was induced in the mice fed with HA-TG plus saponin (Figure 3b and c). In addition, oral administration of HA-TG plus CT- or recFLA-induced low levels of HA-specific IgG responses, corresponding to 31.6% and 10.2% of that of the saponin group, respectively. However, neither HA-TG plants nor WT plants alone did not develop any detectable levels of HA-specific antibody responses (Figure 3b and c). These results indicate that saponin is most effective in the induction of the immune response with orally administered HA-TG plants.

These results (Figure 3) prompted us to define the most efficient immunization schedule. We designed three different immunization schedules, Protocol I to III (Figure S1a). Among these three protocols we tested, Protocol II involving four times of priming at weekly intervals followed by a one-week break and two times of boosting at weekly intervals induced the strongest HA-specific responses (Figure S1b). Next, we determined the optimal dose of the antigen to elicit the immune responses. Mice were orally immunized with 12.5, 25 or 50 mg of freeze-dried leaf powders of HA-TG which contain 10, 20 or 40 μg of BiP:HA:CBD:HDEL, respectively, together with 2.5 mg of saponin according to Protocol II. Regardless of the amount of HA-TG, all mice developed HA-specific IgG antibody responses (Figure 4b). Of the three different doses we used, 20 μg HA induced the highest antibody response (Figure 4b). The HA-specific IgG levels were maintained 2 months after immunization, indicating the long persistence time of the immune response (Figure 4c). These results suggest that edible vaccination for AI can be developed using HA expressed in the ER of plants. To further confirm this, we examined the immune response in mice fed with WT plants together with saponin and found no HA-specific antibody

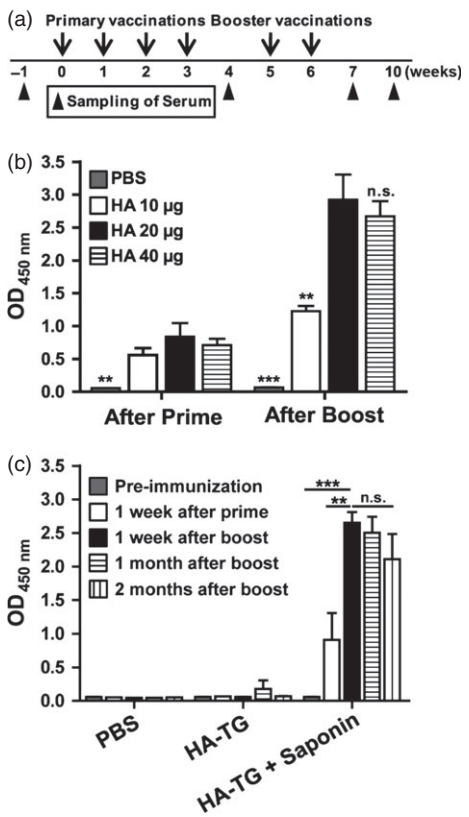

Figure 4 Induction of HA-specific antibody strongly depends on the amount of orally administered HA-TG plants. (a) Oral immunization protocol. W, week. (b) Induction of HA-specific antibody. Mice were orally immunized with 12.5, 25 or 50 mg of HA-TG (containing 10, 20 and 40 μg BiP:HA:CBD:HDEL, respectively) together with saponin (2.5 mg). As a negative control, mice were administered with PBS. Sera were collected 1 week after priming or 1 week after boosting immunization, and HA-specific IgG levels were measured by ELISA. HA, BiP:HA:CBD:HDEL. (c) Time course of HA-specific IgG induction. Sera were collected from mice fed with PBS, HA-TG alone or HA-TG plus saponin at the indicated time points. HA-specific IgG levels in serum were measured by ELISA. Results displayed are mean ± SEM ($n = 4$). $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. not significant. Data shown in this figure are from one experiment and are representative of two independent experiments.

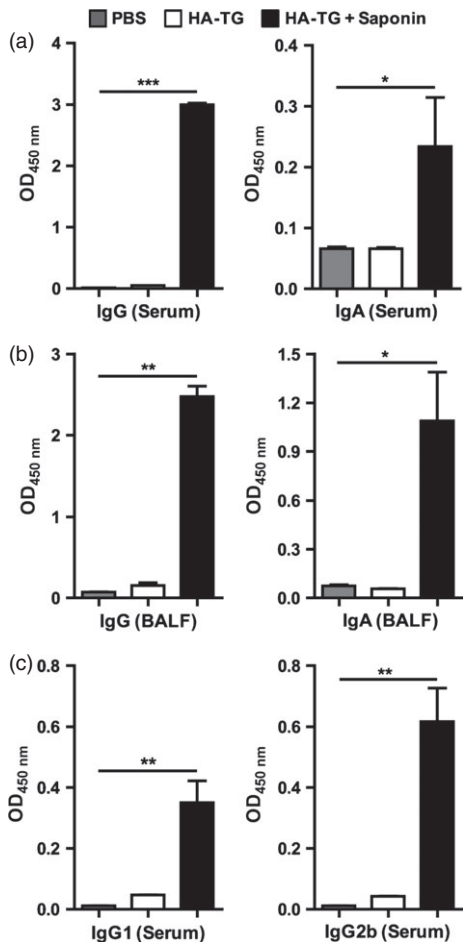

Figure 5 Oral immunization of HA-TG plants together with saponin induces both IgG and IgA antibody responses. (a, b) HA-specific IgG and IgA levels in sera and BALF. Sera and BALF were collected from mice fed with PBS, HA-TG alone or HA-TG together with saponin. HA-specific IgG and IgA antibody titres in serum (a) and BALF (b) were measured by ELISA. (c) HA-specific IgG1 and IgG2b antibody levels. HA-specific IgG1 and IgG2b titres were determined by ELISA. Results represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Pooled results from three independent experiments are shown, each of them obtained from five mice per group.

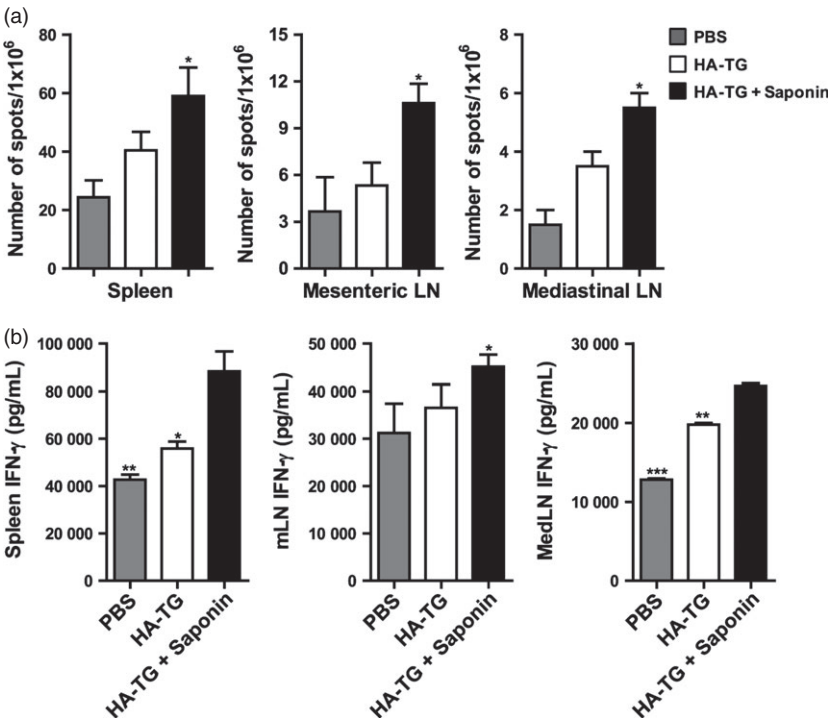

Figure 6 Immunized mice show HA-specific T-cell responses in the spleen, mesenteric lymph node and mediastinal lymph node. (a) T-cell responses in immunized mice. Mice were orally administered with PBS, HA-TG alone or HA-TG together with saponin. T cells from spleen, mesenteric lymph nodes (LN) or mediastinal LN were restimulated *in vitro* with recombinant haemaggulutinin (HK213-HA) for 36 h (37 °C, 5% CO_2) and IFN-γ-secreting T cells were calculated by ELISPOT. The number of IFN-γ-secreting T cells is shown as the average number of spot-forming cells/1 × 10^6 cells. (b) IFN-γ production in T cells. T cells were restimulated *in vitro* with anti-CD3/CD28 antibodies for 48 h (37 °C, 5% CO_2). Supernatants were collected and assayed for IFN-γ production by ELISA. Results represent mean ± SEM (n = 5). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. mLN, mesenteric lymph node; MedLN, mediastinal LN. Data shown are from one experiment and are representative of two independent experiments.

responses (Figure S2a and b), confirming that the HA-specific responses are induced by HA expressed in transgenic *Arabidopsis* plants.

Oral immunization of HA-TG plants together with saponin elicits both systemic and mucosal immune responses

To evaluate the nature of immune responses induced by orally administered HA-TG, the titres of IgG and IgA in serum and bronchoalveolar lavage (BAL) fluids were analysed 1 week after the final immunization. HA-specific IgG and IgA antibody titres in the sera (Figure 5a) and BAL fluids (Figure 5b) were significantly higher with HA-TG plus saponin than with PBS or HA-TG alone. Thus, oral immunization of HA-TG plus saponin strongly induced both systemic and mucosal immune responses.

To further characterize the immune response elicited by HA-TG together with saponin, the amount of the IgG subclass, IgG1 and IgG2b, was determined. Both antibody isotypes were greatly increased in the mice fed with HA-TG together with saponin compared to mice fed with PBS or HA-TG plants alone (Figure 5c), indicating that a mixed IgG1/IgG2b subtype response was induced by oral immunization of HA-TG together with saponin. Furthermore, oral administration of HA-TG together with saponin did not elicit the IgE response in mice (Figure S3), suggesting the low risk of inducing allergic response by the treatment.

We further determined the T-cell response in the spleen, mesenteric LNs and mediastinal LNs. Mice administered orally with HA-TG plus saponin showed an increase in the number of IFN-γ-producing T cells in response to *in vitro* HA antigen restimulation (Figure 6a) and IFN-γ production in response to anti-CD3/28 antibodies stimulation in all lymphoid tissues we examined (Figure 6b). These results indicate that oral administration of HA-TG together with saponin elicits strong HA-specific antibody and T-cell responses.

Oral immunization of HA-TG plus saponin induces protective immunity against influenza virus challenge

We examined whether the HA-specific antibodies induced by the oral administration of HA-TG plus saponin confers a protective immunity against influenza virus infection. Mice immunized with HA-TG together with or without saponin, WT plants together with or without saponin, saponin alone or PBS alone were intranasally infected with a lethal dose of H5N2 avian influenza virus 1 week after the final immunization (Figure 7a and Figure S4a). Mice were daily monitored for their mortality and weight loss for 4 weeks (Figure 7b and c). The mice fed with PBS or HA-TG alone showed a rapid weight loss and all mice succumbed within 10 (PBS) or at 12 days (HA-TG alone) after the challenge. In addition, mice fed with saponin alone and WT plants with or without saponin died in comparable ratio to that of PBS and HA-TG alone groups (Figure S4b). By contrast, the majority (72%) of mice fed with HA-TG plus saponin survived. Moreover, after initial loss of body weight up to 25% until 10 days after viral challenge, the body weight gradually recovered and reached the prechallenge level by 28 days of postchallenge, indicating that the protection of mice from lethal influenza infection conferred by oral immunization of HA-TG plus saponin is resulted from the induction of immunity against HA. Taken together, these results suggest that oral vaccination of mice using HA-TG together with saponin provides for strong protection against a lethal H5N2 avian influenza virus infection.

Oral immunization using HA-TG plants together with saponin attenuates pulmonary inflammation caused by H5N2 infection

To further evaluate the vaccination efficacy, we examined lung inflammation by analysing viral RNA levels in lung, cellular

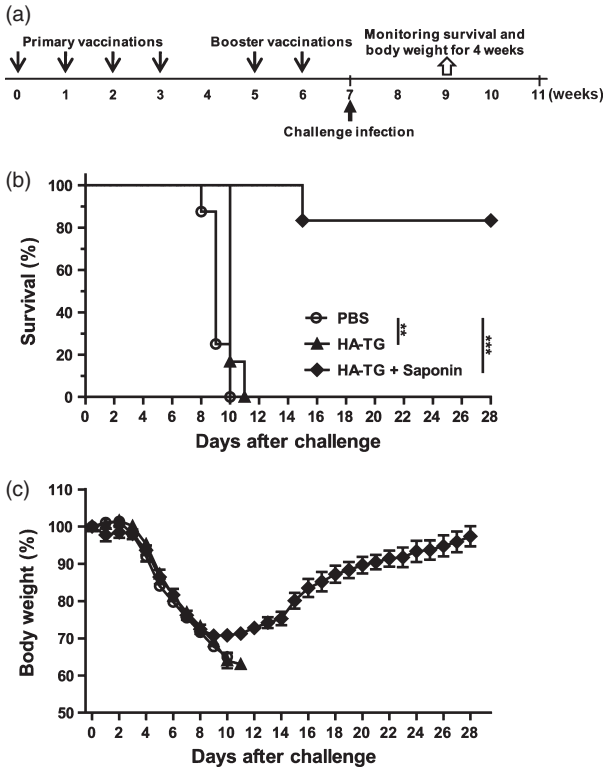

Figure 7 Oral immunization of HA-TG plants together with saponin confers protective immunity against a lethal dose of H5N2 avian influenza virus infection. (a) Experimental scheme. W, week. (b, c) Survival rate and body weight change after H5N2 challenge. Mice immunized with PBS, HA-TG plants or HA-TG plus saponin were infected with H5N2 avian influenza virus (A/Korea/w81/2005) 1 week after final immunization. Survival rate (b) and change in the body weight (c) were monitored daily for 28 days after the challenge. Body weights of the examined day were presented as relative to that prior to challenge. Results shown are as mean ± SEM. $^{**}P < 0.01$; $^{***}P < 0.001$ by log-rank test. Pooled results from two independent experiments are shown, each of them obtained from eight mice.

infiltration into lung and pro-inflammatory cytokines in BAL fluids in the mice at 6 days postchallenge (Figure 8a). Viral load, determined by the expression level of H5N2 viral RNA in the lung, was significantly lower with HA-TG plus saponin than with PBS or HA-TG alone (Figure 8b). In particular, the viral RNA was not detected in surviving mice by 28 days postchallenge, indicating the complete clearance of influenza virus in the mice (Figure 8b). BAL cell count (Figure 8c) showed that an HPAI H5N2 infection caused severe neutrophilic infiltration into the lung. In addition, in mice immunized with HA-TG plus saponin, the cellular infiltration especially, neutrophilic infiltration, was significantly decreased (Figure 8c). Furthermore, the levels of pro-inflammatory cytokines such as IFN-γ, IL-6 and MCP-1 that were induced by H5N2 infection were significantly reduced in mice immunized orally with HA-TG plus saponin (Figure 8d). On the other hand, the production of anti-inflammatory cytokine, IL-10, was comparable in all immunized mice (Figure 8d). These results indicate that oral vaccination of mice with HA-TG plus saponin leads to amelioration of the pulmonary inflammation and protection of mice from lethal influenza infection.

Discussion

In this study, we generated transgenic *Arabidopsis* plants expressing HA of HPAI H5N1 in the ER and examined the possibility as to whether they can be used as an edible vaccine. Important prerequisites for the successful vaccination included a high-level accumulation, proper glycosylation and protection of the antigen proteins (Borges *et al.*, 2010). It has been proposed that plant cell walls provide protection of proteins against proteases, which is termed bio-encapsulation. However, this does not provide full protection to proteins against proteolytic degradation before the uptake into the mucosal immune system. An enhanced resistance to proteolytic degradation can be obtained when proteins are incorporated into biodegradable particles such as protein bodies which have a surrounding membrane (Nochi *et al.*, 2007). We used the ER as a storage place for antigens for the following reasons: the antigen can be *N*-glycosylated and the ER membrane may provide additional protection to proteolytic degradation. Consistent with our assumption, over 60% of the chimeric HA proteins expressed in the ER were protected against the pepsin treatment, while the same protein purified from the transgenic *Arabidopsis* was completely digested. In addition, HA proteins in the ER of transgenic *Arabidopsis* were *N*-glycosylated. Plant-expressed glycosylated proteins exhibit higher immunogenicity as compared to proteins expressed in mammalian cell cultures (Gomord *et al.*, 2010). Previously, it has been suggested that subcellular targeting of foreign proteins to appropriate organelles is a strategy to increase the protein yield in transgenic plants (Streatfield *et al.*, 2003). Indeed, high-protein expression levels are successfully achieved by targeting proteins to ER, chloroplasts or apoplasts (137–300 μg/g in fresh leaf biomass) (Kalthoff *et al.*, 2010; Maclean *et al.*, 2007). In this study, we achieved the HA expression level of approximately 700 μg/g (dry weight) or 140 μg/g (fresh leaf) by ER targeting.

Several studies reported the potential efficacy of plant-based influenza vaccine systems. Among them, HA virus-like particles made in *Nicotiana benthamiana* are of promising, as shown by the positive results of Phase II clinical trial by the conventional injection method (D'Aoust *et al.*, 2008). In addition, recombinant influenza haemagglutinin produced in *Nicotiana benthamiana* by rapid transient expression technology was tested for vaccinating mice, rabbits and ferrets by intramuscular (i.m.) injections in preclinical studies (Shoji *et al.*, 2011). Both groups showed the great potential for the plant-based system in influenza vaccine. Here, we add another option for the plant-based influenza vaccination by oral administration of TG plants.

In this study, we used HA without the transmembrane domain and designed it to be accumulated in the ER at high levels. Even though the transmembrane domain is essential for the trimerization, which is in turn crucial for the antigenicity (Phan *et al.*, 2013), we successfully demonstrated that HA without the transmembrane domain can induce HA-specific immune responses in mice. In previous studies, HA ectodomain fused with carrier proteins can elicit immune response (Musiychuk *et al.*, 2007; Shoji *et al.*, 2009). Thus, it is possible that the high level of HA in the plant ER may compensate the loss of the transmembrane domain which is required for HA trimerization.

In vaccination, an immunoenhancer called adjuvant is essential for inducing high levels of immune responses. Interestingly, of the three different types of adjuvants (CT, flagellin and saponin) that

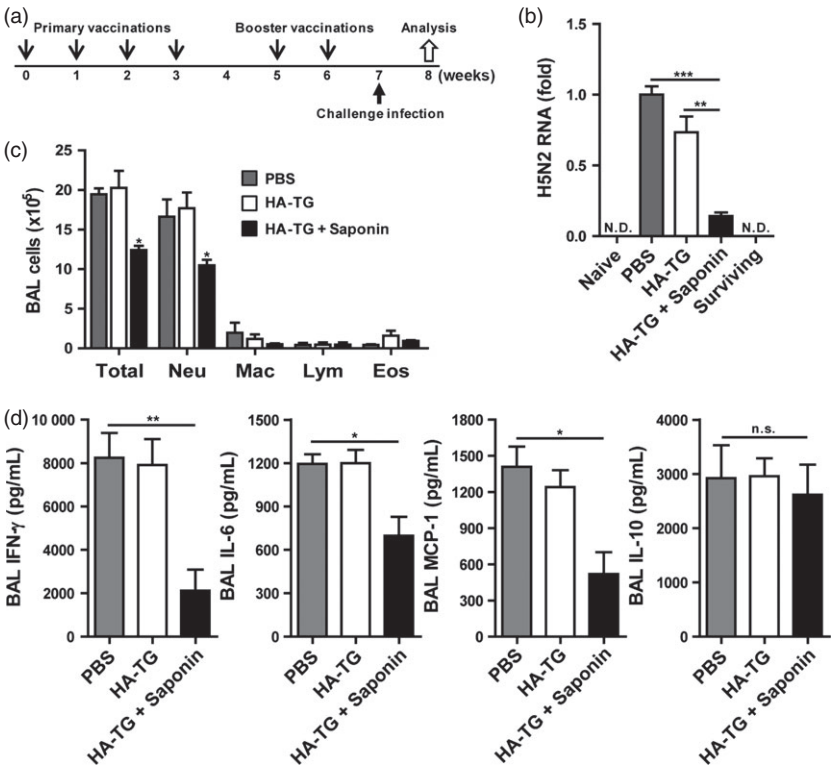

Figure 8 Oral immunization of HA-TG plants plus saponin attenuates lung inflammation after H5N2 avian influenza virus infection. (a) Experimental scheme. Mice were intranasally challenged with H5N2 influenza virus 1 week after the final immunization. Lungs and BALF were collected at 6 days postchallenge. (b) Viral mRNA level after H5N2 challenge infection. Viral RNA was detected by real-time reverse transcription-PCR. Viral RNA levels were normalized with the endogenous *GAPDH* transcript level and expressed as a fold change compared with control (PBS-fed mice). Each sample was analysed in duplicate. Naive, noninfected mice; Surviving, surviving mice 28 days after challenge; N.D. not detected. (c) Cell populations in the BALF. The number of total cells (Total), neutrophils (Neu), macrophages (Mac), lymphocytes (Lym) and eosinophils (Eos) in the BALF are shown. (d) The concentrations of cytokines. IFN-γ, IL-6, MCP-1 and IL-10 in the BALF were measured by ELISA. Results represent mean ± SEM ($n = 5$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s. not significant; N.D. not detected. Data shown in this figure are representative of two independent experiments.

we used, the strongest immune response was induced with saponin in our edible vaccine system. CT is one of the most frequently used potent mucosal adjuvants (Vajdy and Lycke, 1992) and a bacterial flagellin, a ligand of toll-like receptor 5, has a strong mucosal adjuvant activity (McSorley *et al.*, 2002). It is possible that saponin increases the permeability of intestinal epithelial cells, which may allow more efficient antigen uptake (Pickering *et al.*, 2006; Sjolander and Cox, 1998). Indeed, recent studies have shown that orally administered saponin enhances induction of cytokines, in particular, Th1 cytokines (IFN-γ) and antibodies of IgG2a/b/c isotypes, which represent Th1 response in mice (Sun *et al.*, 2009).

Production of HA-specific IgG antibodies alone can confer a protection against Influenza infection by neutralizing and destroying the virus in circulation (Bai *et al.*, 2011; Tamura and Kurata, 2004). Our vaccination protocol led to induction of HA-specific IgG1 and IgG2b. The different isotypes of IgG antibodies have different roles in preventing viral infection. IgG1 antibody, a Th2-type antibody, mainly contributes to viral neutralization (Gerhard *et al.*, 1997; Moran *et al.*, 1999). On the other hand, the Th1-type antibody IgG2b contributes more to clearance of virus from the host but is less effective in neutralization (Nimmerjahn and Ravetch, 2005). The mice fed with HA-TG plus saponin showed strong Th1 responses together with IgG2b production, which may mediate an important part of the vaccine-induced protection from influenza infection. IgG antibodies can be transferred into the mucosal space by FcRn-mediated transcytosis (Kim *et al.*, 2004; Nimmerjahn and Ravetch, 2005). Indeed, we detected high levels of HA-specific IgG antibody in both serum and BALF from mice immunized with HA-TG plus saponin. In addition to IgG antibodies, HA-TG plus saponin-fed mice also showed a strong IgA response. IgA is secreted into the mucosal space, thus contributing to inhibition of virus entry into host cells (Mbawuike *et al.*, 1999; Muramatsu *et al.*, 2014). Importantly, IgA could be detected in BALF where it encounters influenza virus first. Furthermore, the vaccination did not evoke IgE responses which potentially cause anaphylaxis in the host, which suggests that oral administration of HA-expressing plants with saponin may be a safer way of immunization.

The primary cause of death by influenza infection is the strong lung inflammation (Brandes *et al.*, 2013; Van Reeth *et al.*, 2002). The HA-TG plus saponin vaccination reduced lung inflammation after influenza challenge; lower cellular infiltration into lung and pro-inflammatory cytokine production. Concomitant with this observation, virus mRNA levels were low in the lung of mice fed with HA-TG plus saponin. As a result, the vaccinated mice are protected from death.

Birds such as waterfowl are considered as natural reservoir hosts for most influenza A viruses. Because of their natural behaviour, it is extremely difficult to prevent the avian host from spreading the influenza viruses (Webster and Govorkova, 2006). Previous studies demonstrated that immunization of plant-derived HA could protect chickens (Kalthoff *et al.*, 2010) and ferrets (Shoji *et al.*, 2009) against challenge infection when these animals were immunized i.m. or subcutaneously (s.c.) with HA purified from plants. In 2006, first plant-made Newcastle disease virus vaccine for chickens was approved by the US Department of Agriculture (Ling *et al.*, 2010). It is a purified injectable vaccine produced from a transgenic tobacco cell-line. Although this opened the way to the practical use of plant-made vaccine for poultry, immunizing the huge numbers of animals one by one in poultry farms or as free-living birds is practically impossible. Thus, edible vaccination using plants may be a strategy of choice for the large-scale immunization of animals such as chickens. Both HA-TG plants and saponin can be easily formulated as chicken feed. However, further studies are necessary to show that the results

obtained with mice are applicable to birds or poultry that has different digestive systems from mice.

In summary, we have demonstrated that oral administration of HA-expressing transgenic *Arabidopsis* plants with saponin induces neutralizing antibodies and cellular immune responses, which can confer protection against a lethal viral infection. In addition, the mucosal antibody and cellular immune responses were rapidly expanded upon viral challenge infection, which results in an inhibition of viral replication and lung inflammatory cytokine production. Accordingly, we propose that the edible vaccination employing oral administration of antigen-expressing plants together with saponin as adjuvant is an attractive method of future vaccination.

Experimental procedures

Construction of plasmid DNAs

The DNA fragments encoding HA (amino acid positions from 14 to 530) and the cellulose- binding domain (CBD) were PCR-amplified from the A/Hong Kong/213/03 (H5N1) influenza virus strain or *Clostridium stercorarium*, respectively. The sequences of primers used in this study are listed in Table S1. The ER retention signal HDEL was included in the primer for CBD amplification. The PCR products of *HA* and CBD with HDEL were sequentially ligated into the 326-BiP vector, which contains the leader sequence of binding protein (BiP) of *Arabidopsis* (Kim *et al.*, 2001). To generate plant transformation vector *pBI121-BiP:HA:CBD:HDEL*, *BiP:HA:CBD:HDEL* was ligated into the binary vector pBI121 (Clontech). All PCR products were sequenced to confirm product accuracy.

Generation of transgenic plants

To generate stable transgenic plants, the binary construct, *pBI121-BiP:HA:CBD:HDEL*, was transformed into *Arabidopsis* plants (ecotype Columbia-0) by the floral dipping method (Clough and Bent, 1998). Transgenic plants were screened on 1/2 Murashige and Skoog medium supplemented with kanamycin (50 mg/L). Homozygous lines were isolated at T3 generation.

Growth of plants

Transgenic plants were cultivated on Gamborg B5 plates supplemented with 1% sucrose at 20–22 °C in a growth chamber with 70% relative humidity under a 16/8-h light/dark cycle. Leaf or whole tissues were harvested from 2- to 3-week-old plants and used immediately for protoplast isolation or protein extraction. For oral immunization, transgenic plants were grown on soil and healthy leaves harvested from 4-week-old transgenic *Arabidopsis* were freeze-dried for 24 h using Freeze Dry Systems (Labconco, Kansas City, MO). Dried leaf materials were ground to fine powder using a mortar and pestle and stored at room temperature.

Immunocytochemical analysis

To determine the localization of BiP:HA:CBD:HDEL, protoplasts from T4 or T5 transgenic *Arabidopsis* leaf tissues were immunostained with anti-CBD antibody followed by fluorescein isothiocyanate (FITC)-conjugated anti-Rabbit IgG as described previously (Park *et al.*, 2005). Images were captured using a Zeiss LSM 510 META laser scanning confocal microscope in a multitrack mode. Excitation/emission wavelengths were 488/505–530 nm for FITC. The data were processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA), and the images were rendered in pseudocolour (Jin *et al.*, 2001).

Western blot analysis

Leaf tissues were homogenized by adding denaturation buffer [1% SDS, 2% (v/v) β-mercaptoethanol], and the extracts were separated by 10% SDS-PAGE. Western blot analysis was performed using rabbit anti-CBD (a 1 : 5000 dilution, prepared in our laboratory) and rabbit anti-H5N1 haemagglutinin polyclonal antibodies (a 1 : 1000 dilution, Bioss, Inc.) as described previously (Kim *et al.*, 2014). The blot was developed with the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ), and images were captured using the LAS3000 system (Fujifilm, Tokyo, Japan).

Quantification of HA protein levels in transgenic *Arabidopsis* plants

Protein extracts were prepared from the fresh-leaf tissues or fine power of dried leaf tissues using extraction buffer [100 mM NaCl, 10 mM EDTA, 200 mM Tris–HCl, pH 8.0, 0.1% SDS, 200 mM Sucrose, 0.05% Tween20, 14 mM β-mercaptoethanol and a protease inhibitor cocktail]. The homogenates were centrifuged at 19 000 *g* for 20 min at 4 °C, and the resulting supernatants (total soluble protein, TSP) were collected and stored at −20 °C until analysed. The TSP was quantified by Bio-Rad protein assay with BSA standards following the manufacturer's protocol. To determine the expression level of HA fusion proteins, protein extracts from HA-TG together with various amount of purified GFP/CBD fusion proteins (10, 50 and 100 ng) as reference standard were analysed by Western blotting using anti-CBD antibody. The signal intensity of bands was measured using Multi Gauge (V3.0) software (Fujifilm) as described previously (Liu *et al.*, 2014; Verma *et al.*, 2008).

Purification of HA from transgenic *Arabidopsis*

Fresh leaves harvested from 3-week-old transgenic *Arabidopsis* were homogenized in buffer (10 mM HEPES, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 5 mM DTT, 0.2% triton X-100). The homogenates were centrifuged at 19 000 *g* for 10 min at 4 °C and the resulting supernatants were loaded on a column prepared with cellulose (Sigma type 20) and washed with washing buffer (10 mM HEPES, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA, 5 mM DTT). Cellulose-bound BiP:HA:CBD:HDEL proteins were eluted with buffer (1% cellobiose, 10 mM HEPES, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 5 mM EGTA, 5 mM EDTA). Purified proteins were analysed by Western blotting using anti-CBD antibody.

Endo H and pepsin treatments

The protein extracts were incubated with Endo H (Roche) for 1 h at 37 °C and analysed by Western blotting using anti-CBD and anti-H5 antibodies (Lee *et al.*, 2011). The pepsin treatment was performed as described previously (Nochi *et al.*, 2007).

Oral immunization and sample collection

Wild-type C57BL/6 (B6) mice (Jackson Laboratories, Bar Harbor, ME) were maintained under pathogen-free conditions. All experiments were performed in accordance with the guideline of the Institutional Animal Care Committee of POSTECH. For all experiments, 6–8 weeks old female B6 mice were used.

Mice were orally administered with freeze-dried plant tissues [suspended in PBS (22 mg/350 μL) of different volume depending on the vaccine protocols] with or without adjuvants [cholera toxin (List Biological Laboratories, Inc., Campbell, CA), 10 μg; recFLA

(Invivogen, San Diego, CA), 50 ng; purified saponin from *Quillaja Bark* (Sapogenin content, 20–35%) (Sigma, St. Louis, MO), 2.5 mg] in a total of six times at one-week interval using feeding needle (Fuchigami). As controls, mice were given WT plants with or without saponin, saponin alone or PBS alone.

Bronchoalveolar lavage fluids (BALF) were obtained 1 week after the final immunizations (Kim *et al.*, 2007). Serum and BALF were centrifuged, and the supernatants were stored at −20 °C. Spleen, mesenteric lymph nodes and mediastinal lymph nodes were immediately removed after sacrifice and lymphocytes were collected for *in vitro* T-cell proliferation assay.

Intranasal challenge infection

For influenza challenge, 1 week after the final vaccination, mice were anesthetized by intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine hydrochloride (10 mg/kg) in PBS and intranasally challenged with two 50% lethal dose (LD50) of H5N2 avian influenza virus (A/Korea/w81/2005, H5N2 that had HA with 93% amino acid sequence identity to HA of H5N1 we used in this study) as previously described (Park *et al.*, 2009). Mice were daily monitored for death and weigh loss for 28 days. Sera and BALF were collected from survived mice at 28 days after challenge. Lungs were removed for viral RNA isolation. In some experiments, mice were euthanized on day 3 or 6 postchellenge and sera, BALF and lungs were collected.

ELISA

HA-specific antibody responses in sera and BALF were measured by direct enzyme-linked immunosorbent assay (ELISA) (Park *et al.*, 2009). Purified Influenza A Virus H5N1 HA (A/Hong Kong/213/03) protein (Sino Biological Inc., Beijing, China) was used to capture antigen. HRP-conjugated polyvalent goat anti-mouse IgG, IgG1, IgG2b and IgA (Southern Biotechnology, Birmingham, AL) were used as the detection antibodies. Total IgE concentration in serum was determined using the Mouse IgE ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions.

The levels of IFN-γ, IL-6, MCP-1 and IL-10 in BALF were measured by ELISA with mouse ELISA Ready-SET-Go for IFN-γ and IL-10 (eBioscience, San Diego, CA) and Duoset ELISA mouse kit for IL-6 and MCP-1 (R&D Systems, Minneapolis, MN), in accordance with manufacturer's instructions.

Measurement of cytokine production by T cells

The number of IFN-γ producing cells in response to *in vitro* HA antigen restimulation and IFN-γ production in response to anti-CD3/28 antibodies stimulation in spleens, mesenteric lymph nodes (mLNs) and mediastinal lymph nodes (MedLNs) were analysed by ELISPOT or ELISA assay, respectively. Immunized mice were sacrificed 1 week after the final vaccination and their spleens, mLNs and MedLNs were removed and homogenized into single-cell suspensions (Park *et al.*, 2009). Cells isolated from spleens, mLNs and MedLNs were resuspended in complete RPMI 1640 medium containing 10% FCS, HEPES (20 mM), penicillin (1000 U/mL), streptomycin (1000 μg/mL), L-Glutamate (2 mM). Red blood cells were lysed using ACK buffer. For ELISPOT assay, 1×10^6 cells were plated in Multiscreen plates (MSHAN 4510; Millipore, Burlington, MA) coated with purified rat anti-mouse IFN-γ antibody (BD Pharmingen, San Jose, CA) and cultured in the complete medium containing influenza A/Hong Kong/213/03 HA antigen (5 μg/mL) as stimulator, PMA (50 ng/mL)/ionomycine (750 ng/mL) as positive control or medium alone as negative control for 36 h. Spots were counted using a dissection microscope. For cytokine measurements, 2×10^5 cells were incubated with anti-CD3 (5 μg/mL) plus anti-CD28 (2 μg/mL) antibodies for 48 h at 37 °C in 5% CO_2. The levels of IFN-γ in cell culture supernatants were measured by Ready-SET-Go ELISA kit (eBioscience), according to the manufacturers' instructions.

BAL cell counts

The pellet of the centrifuged BAL fluid was resuspended in PBS, and the total number of inflammatory cells was counted with a hemocytometer. After Diff-Quick staining (Dade Behring, Inc., Newark, DE) of the BAL cells prepared by cytospin, the types of inflammatory cells including neutrophils, macrophages, lymphocytes or eosinophils, were classified by counting 300 cells and the percentages of each cell type were calculated.

Real-time reverse transcription-PCR for detection of virus RNA

Total RNA was extracted from lung tissues using Trizol (Life Technologies, Carlsbad, CA). cDNA was synthesized using random primers and GoScript Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. RT-PCR was conducted using specific primer pairs (TGCGGGAAAGCAGATAGTGG and TCAGTTAGGTAGCGCGAAGC) for H5N2 influenza virus. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal control. The PCR conditions were followed by Applied Biosystems 7500 FAST Real-Time PCR System.

Statistical analysis

The unpaired two-tailed Student's *t*-test or log-rank test was performed to analyse the significance in the difference between samples. All statistical analyses were done with Prism 5 software (GraphPad, San Diego, CA) and P values <0.05 were considered statistically significant.

Acknowledgements

This study was supported by grants from the National Research Foundation (2013070270), Ministry of Science, ICT and Future Planning (Korea) and Samsung Research Funding Center of Samsung Electronics (SRFC-MA1301-04) to IH, the BIO & Medical Technology Development Program of National Research Foundation of Korea (MEST, 2012M3A9C6049936) to JHK and Institute for Basic Science (IBS) to MHJ.

References

Abdel-Ghafar, A.N., Chotpitayasunondh, T., Gao, Z., Hayden, F.G., Nguyen, D.H., de Jong, M.D., Naghdaliyev, A., Peiris, J.S., Shindo, N., Soeroso, S. and Uyeki, T.M. (2008) Update on avian influenza A (H5N1) virus infection in humans. *N. Engl. J. Med.* **358**, 261–273.

Bai, Y., Ye, L., Tesar, D.B., Song, H., Zhao, D., Bjorkman, P.J., Roopenian, D.C. and Zhu, X. (2011) Intracellular neutralization of viral infection in polarized epithelial cells by neonatal Fc receptor (FcRn)-mediated IgG transport. *Proc. Natl Acad. Sci. USA*, **108**, 18406–18411.

Barackman, J.D., Ott, G., Pine, S. and O'Hagan, D.T. (2001) Oral administration of influenza vaccine in combination with the adjuvants LT-K63 and LT-R72 induces potent immune responses comparable to or stronger than traditional intramuscular immunization. *Clin. Diagn. Lab. Immunol.* **8**, 652–657.

Borges, O., Lebre, F., Bento, D., Borchard, G. and Junginger, H.E. (2010) Mucosal vaccines: recent progress in understanding the natural barriers. *Pharm. Res.* **27**, 211–223.

Brandes, M., Klauschen, F., Kuchen, S. and Germain, R.N. (2013) A systems analysis identifies a feedforward inflammatory circuit leading to lethal influenza infection. *Cell*, **154**, 197–212.

Chu, L. and Robinson, D.K. (2001) Industrial choices for protein production by large-scale cell culture. *Curr. Opin. Biotechnol.* **12**, 180–187.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Cox, R.J., Brokstad, K.A. and Ogra, P. (2004) Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. *Scand. J. Immunol.* **59**, 1–15.

D'Aoust, M.A., Lavoie, P.O., Couture, M.M., Trepanier, S., Guay, J.M., Dargis, M., Mongrand, S., Landry, N., Ward, B.J. and Vezina, L.P. (2008) Influenza virus-like particles produced by transient expression in Nicotiana benthamiana induce a protective immune response against a lethal viral challenge in mice. *Plant Biotechnol. J.* **6**, 930–940.

Das, S.R., Puigbo, P., Hensley, S.E., Hurt, D.E., Bennink, J.R. and Yewdell, J.W. (2010) Glycosylation focuses sequence variation in the influenza A virus H1 hemagglutinin globular domain. *PLoS Path.* **6**, e1001211.

Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A., Pappas, C., Alpuche-Aranda, C.M., Lopez-Gatell, H., Olivera, H., Lopez, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson, P.D. Jr, Boxrud, D., Sambol, A.R., Abid, S.H., St George, K., Bannerman, T., Moore, A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I. and Cox, N.J. (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science,* **325**, 197–201.

Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G. and Maiese, K. (1997) Role of the B-cell response in recovery of mice from primary influenza virus infection. *Immunol. Rev.* **159**, 95–103.

Gomord, V., Fitchette, A.C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D. and Faye, L. (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnol. J.* **8**, 564–587.

Guan, Z.J., Guo, B., Huo, Y.L., Guan, Z.P., Dai, J.K. and Wei, Y.H. (2013) Recent advances and safety issues of transgenic plant-derived vaccines. *Appl. Microbiol. Biotechnol.* **97**, 2817–2840.

Hodge, L.M., Marinaro, M., Jones, H.P., McGhee, J.R., Kiyono, H. and Simecka, J.W. (2001) Immunoglobulin A (IgA) responses and IgE-associated inflammation along the respiratory tract after mucosal but not systemic immunization. *Infect. Immun.* **69**, 2328–2338.

Holmgren, J. and Czerkinsky, C. (2005) Mucosal immunity and vaccines. *Nat. Med.* **11**, S45–S53.

Jin, J.B., Kim, Y.A., Kim, S.J., Lee, S.H., Kim, D.H., Cheong, G.W. and Hwang, I. (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in Arabidopsis. *Plant cell*, **13**, 1511–1526.

Kalthoff, D., Giritch, A., Geisler, K., Bettmann, U., Klimyuk, V., Hehnen, H.R., Gleba, Y. and Beer, M. (2010) Immunization with plant-expressed hemagglutinin protects chickens from lethal highly pathogenic avian influenza virus H5N1 challenge infection. *J. Virol.* **84**, 12002–12010.

Kim, D.H., Eu, Y.J., Yoo, C.M., Kim, Y.W., Pih, K.T., Jin, J.B., Kim, S.J., Stenmark, H. and Hwang, I. (2001) Trafficking of phosphatidylinositol 3-phosphate from the trans-Golgi network to the lumen of the central vacuole in plant cells. *Plant cell*, **13**, 287–301.

Kim, K.J., Fandy, T.E., Lee, V.H., Ann, D.K., Borok, Z. and Crandall, E.D. (2004) Net absorption of IgG via FcRn-mediated transcytosis across rat alveolar epithelial cell monolayers. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L616–L622.

Kim, Y.K., Oh, S.Y., Jeon, S.G., Park, H.W., Lee, S.Y., Chun, E.Y., Bang, B., Lee, H.S., Oh, M.H., Kim, Y.S., Kim, J.H., Gho, Y.S., Cho, S.H., Min, K.U., Kim, Y.Y. and Zhu, Z. (2007) Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *J. Immunol.* **178**, 5375–5382.

Kim, Y., Lee, G., Jeon, E., Sohn, E.J., Lee, Y., Kang, H., Lee, D.W., Kim, D.H. and Hwang, I. (2014) The immediate upstream region of the 5′-UTR from the AUG start codon has a pronounced effect on the translational efficiency in Arabidopsis thaliana. *Nucleic Acids Res.* **42**, 485–498.

Lee, M.H., Jung, C., Lee, J., Kim, S.Y., Lee, Y. and Hwang, I. (2011) An Arabidopsis prenylated Rab acceptor 1 isoform, AtPRA1.B6, displays differential inhibitory effects on anterograde trafficking of proteins at the endoplasmic reticulum. *Plant Physiol.* **157**, 645–658.

Ling, H.Y., Pelosi, A. and Walmsley, A.M. (2010) Current status of plant-made vaccines for veterinary purposes. *Expert Rev. Vaccines*, **9**, 971–982.

Liu, Z., Li, T., Jiang, K., Huang, Q., Chen, Y. and Qian, F. (2014) Induction of chemoresistance by all-trans retinoic acid via a noncanonical signaling in multiple myeloma cells. *PLoS ONE*, **9**, e85571.

Lycke, N. (2012) Recent progress in mucosal vaccine development: potential and limitations. *Nat. Rev. Immunol.* **12**, 592–605.

Lycke, N. and Holmgren, J. (1986) Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology*, **59**, 301–308.

Ma, J.K., Drake, P.M. and Christou, P. (2003) The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* **4**, 794–805.

Maclean, J., Koekemoer, M., Olivier, A.J., Stewart, D., Hitzeroth, I.I., Rademacher, T., Fischer, R., Williamson, A.L. and Rybicki, E.P. (2007) Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J. Gen. Virol.* **88**, 1460–1469.

Mbawuike, I.N., Pacheco, S., Acuna, C.L., Switzer, K.C., Zhang, Y. and Harriman, G.R. (1999) Mucosal immunity to influenza without IgA: an IgA knockout mouse model. *J. Immunol.* **162**, 2530–2537.

McSorley, S.J., Ehst, B.D., Yu, Y. and Gewirtz, A.T. (2002) Bacterial flagellin is an effective adjuvant for CD4+ T cells in vivo. *J. Immunol.* **169**, 3914–3919.

Mitragotri, S. (2005) Immunization without needles. *Nat. Rev. Immunol.* **5**, 905–916.

Moran, T.M., Park, H., Fernandez-Sesma, A. and Schulman, J.L. (1999) Th2 responses to inactivated influenza virus can Be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. *J. Infect. Dis.* **180**, 579–585.

Munster, V.J. and Fouchier, R.A. (2009) Avian influenza virus: of virus and bird ecology. *Vaccine*, **27**, 6340–6344.

Muramatsu, M., Yoshida, R., Yokoyama, A., Miyamoto, H., Kajihara, M., Maruyama, J., Nao, N., Manzoor, R. and Takada, A. (2014) Comparison of antiviral activity between IgA and IgG specific to influenza virus hemagglutinin: increased potential of IgA for heterosubtypic immunity. *PLoS ONE*, **9**, e85582.

Musiychuk, K., Stephenson, N., Bi, H., Farrance, C.E., Orozovic, G., Brodelius, M., Brodelius, P., Horsey, A., Ugulava, N., Shamloul, A.M., Mett, V., Rabindran, S., Streatfield, S.J. and Yusibov, V. (2007) A launch vector for the production of vaccine antigens in plants. *Influenza Other Respir. Viruses*, **1**, 19–25.

Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R.T., Linder, T., Spyr, C. and Steffen, R. (2004) Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N. Engl. J. Med.* **350**, 896–903.

Nimmerjahn, F. and Ravetch, J.V. (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*, **310**, 1510–1512.

Nochi, T., Takagi, H., Yuki, Y., Yang, L., Masumura, T., Mejima, M., Nakanishi, U., Matsumura, A., Uozumi, A., Hiroi, T., Morita, S., Tanaka, K., Takaiwa, F. and Kiyono, H. (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl Acad. Sci. USA*, **104**, 10986–10991.

Ogra, P.L., Faden, H. and Welliver, R.C. (2001) Vaccination strategies for mucosal immune responses. *Clin. Microbiol. Rev.* **14**, 430–445.

Park, M., Lee, D., Lee, G.J. and Hwang, I. (2005) AtRMR1 functions as a cargo receptor for protein trafficking to the protein storage vacuole. *J. Cell Biol.* **170**, 757–767.

Park, K.S., Lee, J., Ahn, S.S., Byun, Y.H., Seong, B.L., Baek, Y.H., Song, M.S., Choi, Y.K., Na, Y.J., Hwang, I., Sung, Y.C. and Lee, C.G. (2009) Mucosal immunity induced by adenovirus-based H5N1 HPAI vaccine confers protection against a lethal H5N2 avian influenza virus challenge. *Virology*, **395**, 182–189.

Phan, H.T., Pohl, J., Floss, D.M., Rabenstein, F., Veits, J., Le, B.T., Chu, H.H., Hause, G., Mettenleiter, T. and Conrad, U. (2013) ELPylated haemagglutinins produced in tobacco plants induce potentially neutralizing antibodies against H5N1 viruses in mice. *Plant Biotechnol. J.* **11**, 582–593.

Pickering, R.J., Smith, S.D., Strugnell, R.A., Wesselingh, S.L. and Webster, D.E. (2006) Crude saponins improve the immune response to an oral plant-made measles vaccine. *Vaccine*, **24**, 144–150.

Shoji, Y., Bi, H., Musiychuk, K., Rhee, A., Horsey, A., Roy, G., Green, B., Shamloul, M., Farrance, C.E., Taggart, B., Mytle, N., Ugulava, N., Rabindran, S., Mett, V., Chichester, J.A. and Yusibov, V. (2009) Plant-derived hemagglutinin protects ferrets against challenge infection with the A/Indonesia/05/05 strain of avian influenza. *Vaccine*, **27**, 1087–1092.

Shoji, Y., Chichester, J.A., Jones, M., Manceva, S.D., Damon, E., Mett, V., Musiychuk, K., Bi, H., Farrance, C., Shamloul, M., Kushnir, N., Sharma, S. and Yusibov, V. (2011) Plant-based rapid production of recombinant subunit hemagglutinin vaccines targeting H1N1 and H5N1 influenza. *Hum. Vaccin.* **7** (Suppl), 41–50.

Sjolander, A. and Cox, J.C. (1998) Uptake and adjuvant activity of orally delivered saponin and ISCOM vaccines. *Adv. Drug Deliv. Rev.* **34**, 321–338.

Skountzou, I., Martin Mdel, P., Wang, B., Ye, L., Koutsonanos, D., Weldon, W., Jacob, J. and Compans, R.W. (2010) Salmonella flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. *Vaccine*, **28**, 4103–4112.

Streatfield, S.J. (2005) Delivery of plant-derived vaccines. *Expert. Opin. Drug. Deliv.* **2**, 719–728.

Streatfield, S.J., Jilka, J.M., Hood, E.E., Turner, D.D., Bailey, M.R., Mayor, J.M., Woodard, S.L., Beifuss, K.K., Horn, M.E., Delaney, D.E., Tizard, I.R. and Howard, J.A. (2001) Plant-based vaccines: unique advantages. *Vaccine*, **19**, 2742–2748.

Streatfield, S.J., Lane, J.R., Brooks, C.A., Barker, D.K., Poage, M.L., Mayor, J.M., Lamphear, B.J., Drees, C.F., Jilka, J.M., Hood, E.E. and Howard, J.A. (2003) Corn as a production system for human and animal vaccines. *Vaccine*, **21**, 812–815.

Sun, H.X., Xie, Y. and Ye, Y.P. (2009) Advances in saponin-based adjuvants. *Vaccine*, **27**, 1787–1796.

Swartz, J.R. (2001) Advances in Escherichia coli production of therapeutic proteins. *Curr. Opin. Biotechnol.* **12**, 195–201.

Tamura, S. and Kurata, T. (2004) Defense mechanisms against influenza virus infection in the respiratory tract mucosa. *Jpn. J. Infect. Dis.* **57**, 236–247.

Vajdy, M. and Lycke, N.Y. (1992) Cholera toxin adjuvant promotes long-term immunological memory in the gut mucosa to unrelated immunogens after oral immunization. *Immunology*, **75**, 488–492.

Van Reeth, K., Van Gucht, S. and Pensaert, M. (2002) Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs. *Viral Immunol.* **15**, 583–594.

Verma, D., Samson, N.P., Koya, V. and Daniell, H. (2008) A protocol for expression of foreign genes in chloroplasts. *Nat. Protoc.* **3**, 739–758.

Walmsley, A.M. and Arntzen, C.J. (2003) Plant cell factories and mucosal vaccines. *Curr. Opin. Biotechnol.* **14**, 145–150.

Wang, K., Holtz, K.M., Anderson, K., Chubet, R., Mahmoud, W. and Cox, M.M. (2006) Expression and purification of an influenza hemagglutinin–one step closer to a recombinant protein-based influenza vaccine. *Vaccine*, **24**, 2176–2185.

Webster, R.G. and Govorkova, E.A. (2006) H5N1 influenza–continuing evolution and spread. *N. Engl. J. Med.* **355**, 2174–2177.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y. (1992) Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179.

Webster, R.G., Peiris, M., Chen, H. and Guan, Y. (2006) H5N1 outbreaks and enzootic influenza. *Emerg. Infect. Dis.* **12**, 3–8.

Yusibov, V. and Rabindran, S. (2008) Recent progress in the development of plant derived vaccines. *Expert. Rev. Vaccines*, **7**, 1173–1183.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Comparison of three immunization schedules for induction of HA-specific antibody responses.
Figure S2 HA-specific antibody is produced only by HA-TG plants with saponin.
Figure S3 Oral immunization with HA-TG plants together with saponin does not induce IgE response.
Figure S4 Survival rate of mice immunized with WT plants with or without saponin after H5N2 challenge.
Table S1 Primer sequences used in this study.