

# Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic *Arabidopsis* seeds

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**Production of high-value recombinant proteins in transgenic seeds is an attractive and economically feasible alternative to conventional systems based on mammalian cells and bacteria. In contrast to leaves, seeds allow high-level accumulation of recombinant proteins in a relatively small volume and a stable environment. We demonstrate that single-chain variable fragment (scFv)-Fc antibodies, with N-terminal signal sequence and C-terminal KDEL tag, can accumulate to very high levels as bivalent IgG-like antibodies in *Arabidopsis thaliana* seeds and illustrate that a plant-produced anti-hepatitis A virus scFv-Fc has similar antigen-binding and *in vitro* neutralizing activities as the corresponding full-length IgG. As expected, most scFv-Fc produced in seeds contained only oligomannose-type N-glycans, but, unexpectedly, 35–40% was never glycosylated. A portion of the scFv-Fc was found in endoplasmic reticulum (ER)-derived compartments delimited by ribosome-associated membranes. Additionally, consistent with the glycosylation data, large amounts of the recombinant protein were deposited in the periplasmic space, implying a direct transport from the ER to the periplasmic space between the plasma membrane and the cell wall. Aberrant localization of the ER chaperones calreticulin and binding protein (BiP) and the endogenous seed storage protein cruciferin in the periplasmic space suggests that overproduction of recombinant scFv-Fc disturbs normal ER retention and protein-sorting mechanisms in the secretory pathway.**

glycosylation | molecular farming | recombinant antibody | subcellular localization

**T**ransgenic plants for the production of high-value recombinant proteins are a promising alternative to conventional recombinant protein production systems, such as bacteria, yeast, animal, and insect cell cultures (1). One of the most important factors driving research in this field is yield improvement, because of its significant impact on economic feasibility (2). Strategies to increase recombinant protein yield in plants include development of better expression cassettes, improvement of protein stability and accumulation by using specific subcellular targeting signals, and development of downstream processing technologies (3). In this perspective, seed-based platforms are particularly attractive because they allow recombinant proteins to stably accumulate at a relatively high concentration in a compact biomass, which is beneficial for extraction and downstream processing (4). By using a seed-specific expression cassette based on the regulatory signals of seed storage proteins of common bean (*Phaseolus vulgaris*), and by targeting the recombinant protein to the endoplasmic reticulum (ER), we obtained the highest yields of recombinant proteins in plants described so far: a single-chain variable fragment (scFv) accumulated to levels in excess of 36% of total soluble protein (TSP) in homozygous

*Arabidopsis* seeds, while retaining its antigen-binding activity and affinity (5).

For some applications, fusion of the scFv with the Fc domain is useful to restore antigen-binding avidity and antibody effector functions and to reach a prolonged serum half-life (6–8). In addition, scFv-Fc antibodies benefit from the Fc domain as a convenient affinity handle for purification and immunochemistry, eliminating the need for a proteolytically sensitive epitope and/or affinity tag (8). Moreover, a single cloning step between phagemid and scFv-Fc format allows high-throughput screening and accelerated validation of phage lead scFv candidates in a format mimicking the bivalent properties of IgG molecules (9, 10). Given these characteristics, together with the therapeutic potential of this recombinant antibody format (11, 12), the power of the seed-specific expression cassette was evaluated for the more complex scFv-Fc antibody format in *Arabidopsis*. The fusions are similar to those made in *Pichia pastoris*, mammalian, and insect cells (8, 12, 13) but instead expressed in plants. Because of the importance of subcellular localization (14) and N-glycosylation (15) on the stability, correct folding, and biological activity of recombinant antibodies, the N-terminal sequence, the N-glycan structures, and the intracellular localization of scFv-Fc antibodies expressed in *Arabidopsis* seeds were determined. Finally, we demonstrate the functionality of a seed-produced anti-hepatitis A scFv-Fc.

## Results

**scFv-Fc Production in *Arabidopsis* Seeds.** Four different scFv-Fc fusion proteins (see *Materials and Methods*) were cloned in the binary vector pPhas (Fig. 1A). Transgenic *Arabidopsis* plants were obtained by *Agrobacterium*-mediated floral dip transformation. For three of the four scFv-Fc constructs, i.e., MBP10, EHF34, and HA78, high-level accumulation of the recombinant protein was detected by SDS/PAGE in the protein extracts of T2-segregating seed stocks (Fig. 1B and Table 1). Levels of

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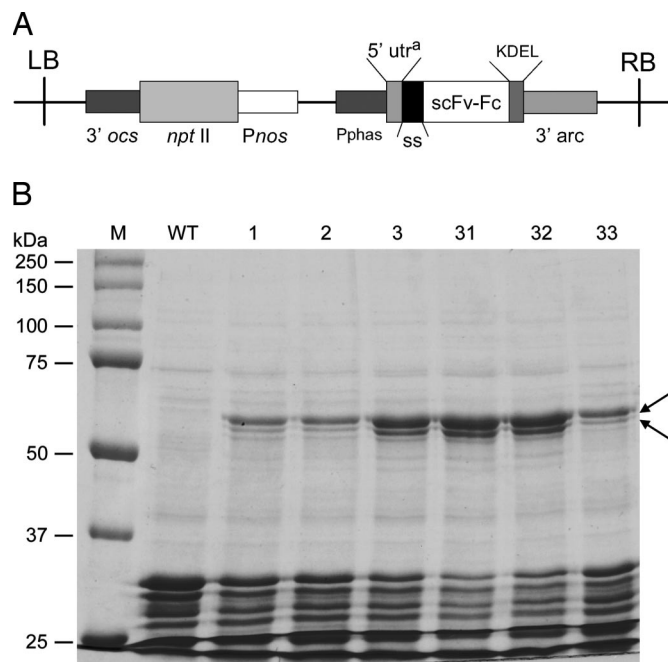
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Abbreviations: scFv, single-chain variable fragment; ER, endoplasmic reticulum; TSP, total soluble protein; PS, periplasmic space; PSV, protein storage vacuole; BiP, binding protein; ProtA, protein A; HAV, hepatitis A virus; HTNV, Hantaan virus; TCID<sub>50</sub>, tissue culture 50% infective dose.

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**Fig. 1.** Expression of scFv-Fc in transgenic *Arabidopsis* plants. (A) Schematic diagram of the T-DNA region in the binary vector pPhas. Pnos, nopaline synthase gene promoter; npt II, coding sequence of the neomycin phosphotransferase II gene; 3' ocs, 3' end of the octopine synthase gene; Pphas,  $\beta$ -phaseolin gene promoter (–1 to –1,470; GenBank accession no. J01263); 5' utr<sup>a</sup>, 5' UTR of *arc5-1* gene (13 bp; part of GenBank accession no. Z5020) (42); 3' arc, 3'-flanking regulatory sequences of the *arc5-1* genomic clone (3,900 bp; part of GenBank accession no. Z50202) (42); ss, signal peptide of the *Arabidopsis* 252 seed storage protein gene (43); scFv-Fc, scFv-Fc coding sequence; KDEL, ER retention signal (16); RB and LB, T-DNA right and left border, respectively. (B) Coomassie-stained, reducing (+DTT) SDS/PAGE gel on which 40  $\mu$ g was loaded of total seed protein extracts from wild-type (WT), and transgenic MBP10-producing *Arabidopsis* lines 1, 2, 3, 31, 32, and 33. The arrows indicate the doublet of scFv-Fc monomer bands. M, Precision Plus Protein Dual Color Standards (Bio-Rad).

HA16 were much lower and could only be detected by protein gel blots, in which multiple low-molecular-mass bands also were observed, indicating that HA16 probably was proteolytically degraded (data not shown). Therefore, HA16 lines were not included for further analysis. Under reducing conditions, scFv-Fc monomers migrated as doublets of major bands at the expected molecular mass of 55 kDa (Fig. 1B). Homodimers of

**Table 1.** Accumulation of scFv-Fc as a percentage of TSP content ( $\pm$ SD) in transgenic *Arabidopsis* seed stocks

Line	Seg T2*	Seg T3*	Ho T3†
MBP10-31	12.4 $\pm$ 1.9	7.4 $\pm$ 1.7	9.2 $\pm$ 0.3
		3.8 $\pm$ 0.6	8.8 $\pm$ 1.4
MBP10-39	11.6 $\pm$ 1.2	5.1 $\pm$ 3.4	9.4 $\pm$ 3.6
		5.4 $\pm$ 1.1	7.7 $\pm$ 0.7
HA78-6	8.4 $\pm$ 0.7	6.3 $\pm$ 1.8	12.5 $\pm$ 1.8
		4.8 $\pm$ 0.4	5.5 $\pm$ 0.7
HA78-8	9.0 $\pm$ 1.4	5.5 $\pm$ 0.7	13.1 $\pm$ 1.8
		4.8 $\pm$ 1.6	8.6 $\pm$ 0.6
EHF34-7	7.1 $\pm$ 0.6	7.0 $\pm$ 2.8	11.1 $\pm$ 0.6
		5.6 $\pm$ 0.9	9.6 $\pm$ 2.0
EHF34-10	6.9 $\pm$ 0.9	5.7 $\pm$ 1.0	13.9 $\pm$ 1.5
		5.0 $\pm$ 0.0	11.3 $\pm$ 3.5

\*Segregating seed stocks.

†Homozygous seed stocks.

**Table 2.** LC-ESI-MS analysis of the glycosylated Fc peptides EEQYN<sup>297</sup>STYR and TKPREEQYN<sup>297</sup>STYR from scFv-Fc-producing *Arabidopsis* lines

Glycoform	Relative abundance, * %					
	MBP10		HA78		EHF34	
	Upper	Lower	Upper	Lower	Upper	Lower
None	2.1	76.5	8.2	93.4	1.9	97.2
Man <sub>5</sub> GlcNAc <sub>2</sub>	9.0	2.6	6.4	0.0	6.4	1.4
Man <sub>6</sub> GlcNAc <sub>2</sub>	14.4	2.9	7.6	0.2	9.5	0.0
Man <sub>7</sub> GlcNAc <sub>2</sub>	27.6	5.5	21.9	2.0	21.0	0.0
Man <sub>8</sub> GlcNAc <sub>2</sub>	28.5	9.1	39.9	2.8	44.9	0.0
Man <sub>9</sub> GlcNAc <sub>2</sub>	0.7	0.3	3.2	0.0	0.0	0.0

\*Numbers are the sum of the relative abundances of a specific glycoform found on each of the two glycopeptides.

$\approx$ 2-fold the apparent molecular weight were detected under nonreducing conditions [supporting information (SI) Fig. 4], confirming that the scFv-Fc proteins assembled in the seeds as IgG-like dimers through disulfide bonds formed by cysteine residues present in the hinge region of the Fc. T2-segregating seeds stocks with the highest density of scFv-Fc bands on Coomassie-stained gels were selected out of 20 independent transformants and tested for single-locus T-DNA insertion. For each of the three constructs, the scFv-Fc concentration was estimated in the two best lines with a single T-DNA locus, and their segregating and homozygous offspring were determined by visual comparison with a standard on Coomassie-stained gels (SI Fig. 5). Accumulation levels of the three different constructs were in the same range, varying from 7.0% to 12.5% of TSP for EHF34 and MBP10 in T2-segregating seed stocks, respectively. In some lines, the expected dosage effects for T3 homozygous versus segregating seed stocks resulted in slightly higher expression levels in the homozygous seed stocks (Table 1). The scFv-Fc accumulation levels observed in these homozygous lines was retained over several subsequent generations (data not shown).

**MS Analysis of Tryptic Peptides and Glycopeptides.** scFv-Fc proteins have a conserved N-linked glycosylation site in the C<sub>H</sub>2 domain of the Fc. To verify whether the doublet of the scFv-Fc bands observed on SDS/PAGE gels under nonreducing conditions (Fig. 1B) was attributable to differential glycosylation, total seed protein extracts of a representative transgenic line for each scFv-Fc were treated with PNGase F, which releases all N-glycans devoid of a core  $\alpha$ (1,3)-fucosyl residue that are essentially oligomannosidic glycans in plants. Upon treatment with PNGase F, the doublet of recombinant bands had collapsed to a single band (SI Fig. 6), running at the same position as the lower band of the original doublet, indicating that the lower band represented a nonglycosylated scFv-Fc. The doublet bands were isolated separately from SDS/PAGE gels and subjected to a detailed glycoproteomic analysis by liquid chromatography/electrospray ionization MS (LC-ESI-MS). The lower scFv-Fc bands predominantly contained nonglycosylated peptides (Table 2 and SI Fig. 7A), confirming the results found with PNGase F. The respective peptides isolated from the upper bands were N-glycosylated to a large extent. The N-glycans were exclusively of the high-mannose type with five to nine mannose residues. The most abundant structures were Man<sub>8</sub>GlcNAc<sub>2</sub> and Man<sub>7</sub>GlcNAc<sub>2</sub> (Table 2 and SI Fig. 7B). As expected, no complex xylose-containing or  $\alpha$ (1,3)-fucose-containing N-glycans were detected in the mass spectra of the KDEL-tagged scFv-Fc proteins. When doublet bands were not excised separately from gel and analyzed as one sample, the unglycosylated isoform represented between 35% and 40% in each sample (data not



anti-HA78 IgG, purified from insect cells against HAV strain JN, was compared *in vitro* with a cell-culture assay. Neutralization efficiency of *Arabidopsis* HA78 was comparable to that of the full-length anti-HA78 IgG, showing a similar dose-dependent response, whereas anti-Hantaan virus (HTNV) IgG used as negative control did not neutralize HAV (Fig. 3B). These data demonstrate that the HAV-neutralizing activity of the *Arabidopsis*-produced HA78 is as high as that of the full-length IgG HA78 produced in insect cells.

## Discussion

As an alternative antibody format, scFv antibody fragments were fused to the Fc fragment of a human IgG. The production of scFv-Fc monomers, as single-gene products, results in correctly assembled IgG-like bivalent homodimers *in planta*. The use of regulatory sequences of common bean resulted in outstanding accumulation levels for three of the four scFv-Fc fusion proteins in T3-homozygous *Arabidopsis* seed stocks, ranging from 9.5% to 14.0% of TSP, equivalent to 19–28  $\mu\text{g}$  of scFv-Fc per mg of seed. For all three constructs, the variation in transgene expression was low, and high-accumulating lines could be identified in a small group of 20 screened independent transformants. These results confirm the scFv levels of 20% of TSP obtained previously in *Arabidopsis* seeds with the same  $\beta$ -phaseolin promoter construct (5).

Understanding how proteins are targeted intracellularly is important when endeavoring to exploit the plant secretory pathway for heterologous protein production. The EM localization study revealed that the KDEL-tagged MBP10 accumulated both in ER-derived spherical bodies and also, unexpectedly, in the PS between the plasma membrane and the cell wall, suggesting a direct transport of the MBP10 fusion proteins from the ER to the PS that bypasses the Golgi. These observations are consistent with the glycosylation analysis: glycosylated scFv-Fc proteins contained only high-mannose *N*-glycans and no complex glycans. The presence of  $\text{Man}_5\text{GlcNac}_2$  to  $\text{Man}_7\text{GlcNac}_2$  suggests that the *N*-glycans have been trimmed by  $\alpha$ -mannosidase I, an enzyme located in the *cis*-Golgi, and probably are sent back to the ER via retrograde transport mediated by the KDEL receptor (16). A similar *N*-glycan profile was found for a full-length IgG produced in tobacco (*Nicotiana tabacum*) leaves in which both heavy and light chains were fused via a long linker motif to the KDEL sequence to increase accessibility of the KDEL tag (17). Although undetected here, complex glycans were found on 10–20% of the full-length KDEL-tagged IgGs, produced in tobacco leaves (18, 19). Recently, a full-length antibody produced in leaves and seeds of tobacco has been characterized in detail (20). A SEKDEL tag was attached to both heavy and light chains. As expected, the leaf-produced IgG was efficiently retained in the ER and carried only high-mannose *N*-glycans, whereas, in contrast to our findings, the seed-produced SEKDEL-tagged antibody was found in both the PSV and the intercellular space, bearing complex glycans, suggesting a Golgi-dependent transport pathway in tobacco seeds. The same SEKDEL signal used in tobacco leaves did not prevent the transport of protein to late Golgi compartments in tobacco seeds (20). Similar tissue-dependent differences were observed when subcellular accumulation and glycosylation of a model glycoprotein were studied in rice (*Oryza sativa*). Although the recombinant protein was efficiently secreted from leaf cells, it was present in ER-derived prolamins bodies and PSVs within the endosperm. Consistent with the immunolocalization data, the endosperm-produced protein possessed oligomannose and vacuole-type *N*-glycans, whereas the leaf-produced protein contained predominantly secretion-type *N*-glycans (21). Our data indicate that the shorter KDEL tag is sufficient for efficient ER retention/*cis*-Golgi retrieval of scFv-Fc proteins produced in *Arabidopsis* seeds but that their high-level accumulation seems to overwhelm the ER storage capacity, inducing an export pathway of the recombinant protein to the PS. The

presence of calreticulin and BiP in the PS, as well as cruciferin that normally accumulates in the PSV (22), suggests that the overproduction of the recombinant scFv-Fc also disturbs the retention and sorting mechanisms of endogenous proteins. Given their strong biological and physical association (23), the colocalization of calreticulin and BiP in the PS is not surprising. Under normal circumstances, these ER-resident proteins are not found outside the plasma membrane (24). The localization of cruciferin in the PS indicates that the receptor-mediated transport of *Arabidopsis* seed-storage proteins to PSV (22) also is affected by the high scFv-Fc production level. Altogether, these findings suggest that, aside from accessibility and density of the KDEL sequence on the recombinant protein and tissue-specific differences in ER retention/retrieval efficiency, protein- and species-specific factors also influence the fate of a recombinant protein in the secretory pathway.

An explanation for the observation of a nonglycosylated scFv-Fc fraction might be that the plant ER translocation and glycosylation machinery is not able to hold all of the heterologous scFv-Fc proteins in a flexible condition. Nascent eukaryotic proteins are known to have to be in such a flexible state to be susceptible to oligosaccharyl transferase complex-induced conformational changes and subsequent attachment of the  $\text{Glc}_3\text{Man}_9\text{GlcNac}_2$  precursor (25). Saturation of the glycosylation machinery by the high rate of recombinant protein synthesis seems less likely because this hypothesis is in contrast with our observation that a fraction of unglycosylated scFv-Fc also is found in transgenic lines producing proteins at levels below 0.5% of TSP (B.V.D., A.D., and G.D.J., unpublished data), which we consider too low to be “saturating” for the glycosylation machinery. Alternatively, the underglycosylation could be related specifically to the scFv-Fc format because a fraction of nonglycosylated scFv-Fc occurred when scFv-Fc antibodies were produced in *Pichia* (8). Possibly, folding and coupling of two scFv-Fc monomers result in an Fc pocket smaller than that of the full-length IgGs. The presence of the large triantennary high-mannose-type *N*-glycans on the Fc domain of one glycosylated scFv-Fc monomer, attached both in *Pichia* (26) and plants, could prevent the pairing with another glycosylated scFv-Fc but only allow coupling to nonglycosylated scFv-Fc monomers. Such a process could explain that about the same ratio of glycosylated versus nonglycosylated scFv-Fc fusion protein is observed in all transgenic lines, independent of the expression levels. Future investigations will be necessary to resolve this phenomenon.

Finally, the binding assay of seed-produced HA78 and corresponding full-length anti-HA78 IgG, produced in insect cells, illustrated similar specificities in recognizing the HAV-purified antigen. These results confirm that neither the conversion of a scFv or full-length IgG into the scFv-Fc format (12, 27) nor the altered glycosylation on the  $\text{C}_\text{H}2$  domain of the plant-produced antibody affects the antigen-specific binding activity (18, 28). In addition, the seed-derived HA78 had an *in vitro* neutralizing activity against the Chinese HAV strain JN comparable to that of the full-length IgG produced in insect cells. Therefore, recombinant plant-produced HA78 could provide an alternative source of neutralizing antibody to be used in pre- and postexposure HAV prophylaxis (29). The importance of steric hindrance and valency in antibody-dependent virus neutralization has been demonstrated conclusively (30). Our results are consistent with those of other successful *in vitro* and *in vivo* virus neutralization based on the use of bivalent scFv-Fc fusion proteins (12). Altogether, we illustrate the value of a seed-specific expression platform for the safe and inexpensive production of antibodies in the scFv-Fc format, which are easier to produce and have the potential to replace IgGs in specific detection, purification, diagnostic, and even therapeutic applications.

## Materials and Methods

**scFv-Fc Construction.** Four scFv-Fc fusion proteins were used: MBP10 is based on an anti-MBP scFv, selected from a human synthetic scFv phage display library (9); HA16 and HA78 are based on two different human-derived, neutralizing Fab antibodies to HAV (31); and EHF34 is based on a human-derived Fab antibody to HTNV nucleocapsid protein (32). For the latter three, the  $V_H$  and  $V_L$  genes were amplified by PCR with forward and reverse primers containing NcoI and XhoI and ApaLI and NotI sites in the 5' and 3' ends, respectively, and aligned as scFv sequences in the pHEN2 phagemid vector. scFv-encoding sequences were cut from pHEN2-scFv by SfiI and NotI and fused to a human IgG1 Fc domain (hinge,  $C_H2$  and  $C_H3$ ) in pPICZ $\alpha$ Fc (9). scFv-Fc expression cassettes were cloned into pPphas (Fig. 1A).

**Plant Transformation.** *Arabidopsis thaliana* (L.) Heyhn., ecotype Columbia 0, was transformed by floral dip (33) with *Agrobacterium tumefaciens* strain C58C1Rif<sup>R</sup> (34). Transgenic T1 plants were selected on kanamycin-containing medium and maintained in soil. Single-locus transgenic lines and homozygous T3 seed stocks were identified as described in ref. 5.

**scFv-Fc Detection and Quantification.** *Arabidopsis* seeds were extracted, and TSP concentrations were determined as described in ref. 5. Essentially, soluble proteins were separated by SDS/PAGE, under reducing conditions (+DTT), and visualized by Coomassie-blue staining. Accumulation levels of scFv-Fc recombinant proteins in the best lines were estimated (as percentage of TSP) by comparing the intensity of the scFv-Fc band in the seed extract with that of different standards that contained different amounts of MBP10 proteins, produced in *Pichia* and purified with ProtA affinity chromatography (see below). To verify the *in vivo* dimerization of scFv-Fc monomers, soluble proteins were separated in NuPAGE Novex Bis-Tris 4–12% gradient gels (Invitrogen, Carlsbad, CA) under nonreducing conditions.

**MS Analysis of Tryptic Peptides and Glycopeptides.** PNGase F digests were performed on 10  $\mu$ g of total seed protein extract with 500 units of enzyme (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions. For MS analysis of *N*-glycans, Coomassie-stained bands were excised, destained, carbamidomethylated, digested with trypsin, extracted from gel pieces (35), and analyzed (36). First, the samples were analyzed in plain MS mode to facilitate detection of the sometimes less intense glycopeptide signals. Sequences of the *N*-terminal peptides were confirmed by tandem MS experiments. Data were analyzed with MassLynx 4.0 SP4 Software (Waters Micromass, Milford, MA). Possible glycopeptide masses were obtained from *in silico* tryptic digests by the addition of relevant glycan masses.

**EM Localization Study.** *Arabidopsis* seeds were imbibed with water for 20 h at 4°C. Seed coats were removed after seed incubation in hexadecene. Five to eight submerged seeds were mounted onto planchettes and frozen in a high-pressure freezer (HPF010; Bal-Tec, Balzers, Liechtenstein). Freeze substitution was carried out as described in ref. 37, except for an extra day of incubation at –85°C and two extra washing steps in 100% ethanol for 60 min before embedding in Lowicryl HM20 (Electron Microscopy Sciences, Hatfield, PA). To increase sectioning quality, the blocks were hardened with UV light for 3 days at room temperature. Ultrathin sections were cut on an Ultracut S (Leica, Bannockburn, IL) and incubated with antibodies against cruciferin (ProtA-purified serum at 1:50 dilution), affinity-purified BiP (at 1:25–1:50

dilution), and calreticulin (at 1:50–1:100 dilution) antibodies, followed by incubation with 10-nm gold-coupled secondary antibodies (GAR10; British Biocell International, Cardiff, U.K.) at a dilution of 1:50 in PBS supplemented with 1% BSA or directly with a ProtA-gold conjugate (EM.PAG10; British Biocell International). Sections were poststained with aqueous uranyl acetate/lead citrate and examined by transmission electron microscopy (CM10; Philips, Amsterdam, The Netherlands). For conventional fixation, imbibed, testa-free seeds were immersed in a phosphate-buffered 2% glutaraldehyde solution at room temperature for 2 h, washed in buffer, postfixed in 2% osmium tetroxide for 4 h at room temperature, washed again in buffer, and dehydrated in an acetone series. Specimens were embedded in Spurr's resin.

**Purification of scFv-Fc.** *Arabidopsis* seed protein extracts were filtered twice over a 0.45- $\mu$ m filter (Alltech, Nicholasville, KY) and diluted to 1 mg/ml TSP with 10% glycerol (VWR, West Chester, PA). In *Pichia*, production of scFv-Fc was as described in ref. 9. Basically, it was purified over a ProtA Sepharose 4 Fast Flow column (GE Healthcare, Fairfield, CT) according to the manufacturer's instructions, eluted with 0.1 M glycine-HCl (pH 3.0), and dialyzed overnight with 1 $\times$  PBS.

**HA78 Functional Assays.** HAV antigen was produced by infecting FRhK-4 cells with HAV Chinese JN strain, culturing the infected cells, and purifying the HAV particles by sucrose-gradient sedimentation (38). ELISA plates were coated overnight at 4°C with purified HAV antigens, blocked with 2% BSA in PBS for 1.5 h, and incubated with a 2-fold dilution series of *Arabidopsis* HA78, starting at 2.5  $\mu$ g/ml. Full-length anti-HA78 IgG, produced in insect cells (32), was included as positive control. After washing, bound scFv-Fc or IgG was detected by goat anti-human IgG (Fc-specific), conjugated to HRP (1:1,000 dilution; Sigma-Aldrich, St. Louis, MO). The ELISA was developed with 3,3',5,5'-tetramethylbenzidine as a substrate, and absorbance was measured at 405 nm.

HAV neutralization was assayed as described in ref. 39 with modifications. Chloroform-treated HAV JN strain was diluted in 10-fold steps from  $10^{-1}$  to  $10^{-5}$  to determine the tissue culture 50% infective dose (TCID<sub>50</sub>) in FRhK-4 cells (40). For the titer of infectious virus, the method of Reed and Muench (41) and 50 $\times$  TCID<sub>50</sub> of HAV were used. A 2-fold serial dilution of *Arabidopsis* HA78 antibody was incubated with HAV JN strain at 37°C for 2 h. The full-length anti-HA78 IgG, produced as described above, was included as positive control, and anti-HNTV IgG antibody AH100 (32) was included as negative control. Mixtures of antibody and virus were added to monolayers of FRhK-4 cells growing in 24-well plates. Mock-infected cells were included as negative control. Plates were incubated at 37°C for 1.5 h, the medium was removed, and 1.0 ml of fresh culture medium was added per well. Monolayers were incubated for 21 days at 33°C, with change of culture medium every 5–6 days. Cells were harvested after 21 days with 0.2 ml of PBS plus 0.05% Tween 20 and disrupted by three cycles of freeze-thawing. HAV titers were monitored by ELISA with human anti-HAV PcAb as coating antibody (1:1,000 dilution) and HRP-labeled human anti-HAV PcAb as detecting antibody (1:1,000 dilution). Neutralization efficiency was calculated according to the method of Reed and Muench (41).

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