Expression of Antibody Fragments with a Controlled N-Glycosylation Pattern and Induction of Endoplasmic Reticulum-Derived Vesicles in Seeds of Arabidopsis

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Intracellular trafficking and subcellular deposition are critical factors influencing the accumulation and posttranslational modifications of proteins. In seeds, these processes are not yet fully understood. In this study, we set out to investigate the intracellular transport, final destination, N-glycosylation status, and stability of the fusion of recombinant single-chain variable fragments to the crystallizing fragment of an antibody (scFv-Fc) of two antiviral monoclonal antibodies (2G12 and HA78). The scFv-Fcs were expressed in Arabidopsis (Arabidopsis thaliana) seeds and leaves both as secretory molecules and tagged with an endoplasmic reticulum (ER) retention signal. We demonstrate differential proteolytic degradation of scFv-Fcs in leaves versus seeds, with higher degradation in the latter organ. In seeds, we show that secretory versions of HA78 scFv-Fcs are targeted to the extracellular space but are deposited in newly formed ER-derived vesicles upon KDEL tagging. These results are in accordance with the obtained N-glycosylation profiles: complex-type and ER-typical oligomannosidic N-glycans, respectively. HA78 scFv-Fcs, expressed in seeds of an Arabidopsis glycosylation mutant lacking plant-specific N-glycans, exhibit custom-made human-type N-glycosylation. In contrast, 2G12 scFv-Fcs carry exclusively ER-typical oligomannosidic N-glycans and were deposited in newly formed ER-derived vesicles irrespective of the targeting signals. HA78 scFv-Fcs exhibited efficient virus neutralization activity, while 2G12 scFv-Fcs were inactive. We demonstrate the efficient generation of scFv-Fcs with a controlled N-glycosylation pattern. However, our results also reveal aberrant subcellular deposition and, as a consequence, unexpected N-glycosylation profiles. Our attempts to elucidate intracellular protein transport in seeds contributes to a better understanding of this basic cell biological mechanism and is a step toward the versatile use of Arabidopsis seeds as an alternative expression platform for pharmaceutically relevant proteins.

Recombinant monoclonal antibodies (mAbs) are of high therapeutic potential and have thus become a major product of the pharmaceutical industry (Aggarwal, 2009). In addition to full-length mAbs, antibodies are being engineered to alter their size, pharmacokinetics, specificity, valency, effector functions, etc. in order to better suit the intended applications (for review, see Filpula, 2007; Harmsen and De Haard, 2007). Of particular interest among these engineered fragments are single-chain variable fragments (scFvs), fusions of variable heavy and variable light domains that retain an antigen-binding function. Due to their smaller size as compared with their full-length counterparts, these molecules penetrate target tissues better and can even bind to intracellular targets. Furthermore, multimerization via disulfide bonds and/or multimerization domains allows for the production of divalent or higher order antibody-like molecules, where increased avidity

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has positive effects on the target-binding efficacy. Very promising products in this respect are so-called scFv-Fcs (i.e., fusions of a scFv to the crystallizing fragment of an antibody [Fc; hinge-C1\_h2-C1\_h3]). These molecules spontaneously fold and dimerize to give a structure similar to a full-length IgG (Zhang et al., 2007; Cao et al., 2009). Despite their small size, Fc-mediated effector functions are maintained and finally lead to the elimination of the antigen-antibody complex from the blood stream (Powers et al., 2001). An additional benefit of such molecules is their composition: one scFv-Fc consists of two polypeptides from one gene. Thus, homodimerization but not heterotetramerization is necessary for correct folding, which could allow for more efficient expression. In general, scFv-Fcs exhibit characteristics equivalent to their parent IgG (Shu et al., 1993; Powers et al., 2001; Cao et al., 2009) and have been tested for similar applications (Li et al., 2000; Yuan et al., 2006; Mori and Kim, 2008; De Lorenzo and D’Alessio, 2009; Olafsen et al., 2009; Riccio et al., 2009).

Plant seeds are increasingly becoming an attractive alternative platform for the production of recombinant proteins. In particular, low production costs, high accumulation of recombinant proteins in the small volume of the seed, and product stability over years allows the separation of cultivation from the purification process, thereby making this system an economically feasible proposition (for review, see Boothe et al., 2010). Notably, with the model plant Arabidopsis (Arabidopsis thaliana), very high accumulation levels have been reported, with recombinant polypeptides even exceeding 35% of the total soluble protein, which corresponds to over 70 g of recombinant protein per kg of dry seeds (De Jaeger et al., 2002). Regulatory elements from highly expressed seed storage proteins enable these elevated accumulation levels to be reached and have already been used for the expression of a scFv-Fc (Van Droogenbroeck et al., 2007). However, the high accumulation of recombinant proteins in seeds, including mAbs and scFv-Fcs, not only depends

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**Figure 1.** Schematic overview of the T-DNA region of the expression constructs generated in this study. wt-35SG12scSEC, wt-35SG12scKDEL, and wt-35SHA78scSEC were cloned into the vector pPhasGW (F. Morandini, B. Van Droogenbroeck, and A. Depicker, unpublished data) for transformation into wild-type and TKO plants. wt-35SG12scKDEL and wt-35SHA78scSEC were additionally cloned into the binary expression vector pPT2 (Strasser et al., 2005). LB, Left border; 3\_ocs, 3\_end of the octopine synthase gene; nptII, neomycin phosphotransferase II; Pnos, nopaline synthase promoter; Pphas, \( \beta \)-phaseolin promoter (1–1,470; GenBank accession no. J01263); attB1, attB2, attP1, attP2, attL1, attL2, attR1, and attR2, recombination sites for Gateway cloning (Invitrogen, 2003); CmR-ccdB, Gateway positive/negative selection cassette; 3\_arc5-I, approximately 4,000 bp of 3\_flanking region of the arceline 5I gene (part of GenBank accession no. Z50202); RB, right border; 2S2, signal peptide of the Arabidopsis 2S2 seed storage protein (Krebbers et al., 1988); KDEL, ER retrieval motif; Tnos, nopaline synthase terminator; P35S, cauliflower mosaic virus 35S promoter; g7T, 200 bp of transcript 7 3\_region (bp 398–598, Dhaese et al., 1983).
upon the level of synthesis but also on their stability after deposition within a suitable compartment. In this respect, a frequently reported approach is KDEL-mediated retention within the endoplasmic reticulum (ER), which seems to allow for increased protein accumulation (Wandelt et al., 1992; Fiedler et al., 1997; Ko et al., 2005; Petruccelli et al., 2006; Laguı́-Becher et al., 2010).

The deposition of recombinant proteins in a specific subcellular compartment has identified peculiarities of the seed secretory system, which obviously differs from the commonly accepted default pathway. The most obvious difference is the absence of a lytic vacuole, in terms of size of the dominant organelle of most plant cells, and instead the presence of protein storage vacuoles (PSVs), where the endogenous seed storage proteins are deposited (for review, see Müntz, 1998; Robinson et al., 2005). However, the targeting of recombinant proteins in seeds is not yet fully understood. For example, KDEL-tagged as well as secretory proteins have been detected in protein storage vacuoles, in the cell wall, in ER-derived compartments, or in a combination of the three (Herman et al., 1990; Philip et al., 1998, 2001; Wright et al., 2001; Reggi et al., 2005; Downing et al., 2006; Petruccelli et al., 2006; Van Droogenbroeck et al., 2007; Abranches et al., 2008; Schmidt and Herman, 2008; Loos et al., 2011). As a consequence of this atypical targeting and deposition, seed-derived recombinant products may carry unusual N-glycosylation profiles (Van Droogenbroeck et al., 2007; Rademacher et al., 2008; Ramesar et al., 2008). Since the glycosylation status of many therapeutic proteins, including mAbs and scFv-Fcs, has a profound effect on their biological activity, it is of great importance to fully understand the molecular mechanisms that underlie targeting along the secretory pathway and final deposition.

In this study, we set out to explore further the potential of plant seeds as a versatile expression platform for valuable recombinant proteins. To this end, a series of scFv-Fc versions of the two antiviral mAbs 2G12 and HA78 were placed under the control of seed-specific regulatory sequences. Secretory as well as KDEL-tagged recombinant products can carry unusual N-glycosylation profiles (Van Droogenbroeck et al., 2007; Rademacher et al., 2008; Ramesar et al., 2008). Since the glycosylation status of many therapeutic proteins, including mAbs and scFv-Fcs, has a profound effect on their biological activity, it is of great importance to fully understand the molecular mechanisms that underlie targeting along the secretory pathway and final deposition.

RESULTS
Cloning and Plant Transformation
Secreter and KDEL-tagged scFv-Fc versions of two different model antibodies (2G12 against Human immunodeficiency virus [HIV] and HA78 against Hepatitis A virus; Trkola et al., 1996; Cao et al., 2008) were constructed and cloned into the pPhasGW destination

<table>
<thead>
<tr>
<th>Name</th>
<th>scFv-Fc Based on mAb</th>
<th>Targeting Signals</th>
<th>Expression Vector</th>
<th>Promoter</th>
<th>Genetic Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-Ph2G12scSEC</td>
<td>2G12</td>
<td>For secretion</td>
<td>pPhasGW</td>
<td>β-Phaseolin</td>
<td>Wild type</td>
</tr>
<tr>
<td>wt-Ph2G12scKDEL</td>
<td>2G12</td>
<td>For ER retention</td>
<td>pPhasGW</td>
<td>β-Phaseolin</td>
<td>Wild type</td>
</tr>
<tr>
<td>TKO-Ph2G12scSEC</td>
<td>2G12</td>
<td>For secretion</td>
<td>pPT2</td>
<td>35S</td>
<td>Wild type</td>
</tr>
<tr>
<td>wt-35S2G12scSEC</td>
<td>2G12</td>
<td>For secretion</td>
<td>pPhasGW</td>
<td>β-Phaseolin</td>
<td>Wild type</td>
</tr>
<tr>
<td>wt-PhHA78scSEC</td>
<td>HA78</td>
<td>For secretion</td>
<td>pPhasGW</td>
<td>β-Phaseolin</td>
<td>Wild type</td>
</tr>
<tr>
<td>TKO-PhHA78scSEC</td>
<td>HA78</td>
<td>For secretion</td>
<td>pPT2</td>
<td>35S</td>
<td>Wild type</td>
</tr>
<tr>
<td>wt-35SHA78scSEC</td>
<td>HA78</td>
<td>For secretion</td>
<td>pPhasGW</td>
<td>β-Phaseolin</td>
<td>Wild type</td>
</tr>
</tbody>
</table>

Figure 2. Immunodetection of β-phaseolin-driven scFv-Fcs extracted from seeds. One microliter of crude seed extracts (corresponding to 10 μg of seeds) was separated by SDS-PAGE, blotted on a nitrocellulose membrane, and the scFv-Fcs were detected with goat anti-human IgG (H+L) HRP conjugate (Promega; W403B) and a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Pierce). After 3 s of film exposure, all constructs could be visualized. Arrowheads indicate degradation fragments, and bars indicate the height of marker bands (in kD).
vector (F. Morandini, B. Van Droogenbroeck, and A. Depicker, unpublished data; Fig. 1) and were expressed under the control of the seed-specific phaseolin promoter from Phaseolus vulgaris (De Jaeger et al., 2002; Van Droogenbroeck et al., 2007). The vectors were used to transform Arabidopsis Columbia wild-type (wt) plants and glycosylation mutants lacking plant-specific core α-1,3-fucosylation and β-1,2-xylsosylation, referred to as “triple knockout” (TKO; Strasser et al., 2004). In addition, the secretory versions were fused to the constitutive cauliflower mosaic virus 35S promoter in the vector pPT2 (Strasser et al., 2005) and used to transform Arabidopsis wild-type plants. In total, eight different combinations of construct and genetic background were established (Table I).

Expression of scFv-Fc Molecules

At least 25 primary transformants of each series of transformants were screened by ELISA and immunoblotting (Fig. 2; Table II). Based on expression levels, two lines each were selected and selfed to obtain homozygous plants. Maximal expression levels as determined by ELISA ranged from 0.8 to 9.4 mg g⁻¹ dry seed weight (Table II). Generally, the HA78 constructs accumulated to a higher level than the 2G12 constructs and KDEL tagging did not lead to an increased accumulation. These results are in accordance with the recently expressed full-length versions of 2G12 and HA78 mAbs in Arabidopsis seeds (Loos et al., 2011). Expression levels of the 35S-driven constructs were not analyzed in detail. However, when compared with the β-phaseolin-driven constructs, they were significantly lower (as deduced from immunoblotting).

Seed extracts from transformed plants were subjected to immunoblotting and revealed strong signals consisting of double bands at approximately 60 kD (Fig. 2). The smaller, less intense band represents the nonglycosylated fraction, as previously shown by Van Droogenbroeck et al. (2007). Additionally, degradation products are visible at around 28 to 34 kD. These fragments are derived from heavy chain domains as determined by mass spectrometry (MS) analysis of tryptic peptides (data not shown). KDEL-tagged versions exhibited a slightly increased mass of the intact molecule as well as of their approximately 30-kD degradation products (Fig. 2), most likely due to the KDEL tag and a different N-glycosylation pattern. Although the KDEL tag does not lead to an increased accumulation of scFv-Fcs, it seems to confer enhanced stability, which is especially pronounced for the HA78 scFv-Fcs (Fig. 2). The degradation pattern of the 35S-driven constructs (wt-35S2G12scSEC and wt-35SHA78scSEC) extracted from leaves clearly differs from the degradation pattern of the scFv-Fcs extracted from seeds (Figs. 2 and 3), indicating the presence of different proteases in these two tissues. Surprisingly, 35S-driven scFv-Fcs (wt-35S2G12scSEC and wt-35SHA78scSEC) accumulated a higher proportion of intact protein in leaves than in seeds (Figs. 2 and 3; Supplemental Fig. S5).

N-Glycosylation

All constructs carry one N-glycosylation site in the C_H2 domain. The N-glycosylation status of Protein A-purified scFv-Fcs was analyzed by liquid-chromatography (LC)-MS (Fig. 4). KDEL-tagged versions of 2G12 and HA78 (wt-PhHA78scKDEL and wt-Ph2G12scKDEL) exhibited as expected exclusively oligomannosidic structures, mainly Man7 and Man8. Seed-produced secretory HA78 constructs (wt-PhHA78scSEC and TKO-PhHA78scSEC) carry complex-type glycans, GnGnXF and GnGn, respectively, depending on the expression host, Columbia wild type or TKO. In addition, substantial amounts of oligomannosidic structures are present. This ER-typical glycosylation is completely absent when secretory HA78 is expressed in leaves:

Table II. Maximal expression levels of scFv-Fcs in Arabidopsis seeds

<table>
<thead>
<tr>
<th>Name</th>
<th>Primary Transformants Screened</th>
<th>Maximum Expression Level μg mg⁻¹ dry seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-2G12scSEC</td>
<td>43</td>
<td>0.8</td>
</tr>
<tr>
<td>wt-2G12scKDEL</td>
<td>40</td>
<td>0.8</td>
</tr>
<tr>
<td>TKO-2G12scSEC</td>
<td>25</td>
<td>3.5</td>
</tr>
<tr>
<td>wt-35S2G12scSEC</td>
<td>20</td>
<td>n.d.</td>
</tr>
<tr>
<td>wt-HA78scSEC</td>
<td>30</td>
<td>8.0</td>
</tr>
<tr>
<td>wt-HA78scKDEL</td>
<td>40</td>
<td>3.9</td>
</tr>
<tr>
<td>TKO-HA78scSEC</td>
<td>40</td>
<td>9.4</td>
</tr>
<tr>
<td>wt-35SHA78scSEC</td>
<td>30</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Figure 3. Immunodetection of 35S-driven scFv-Fcs extracted from seeds and leaves. Nine microliters of crude seed extract and 5 μL of crude leaf extract (corresponding to 90 μg of seeds and 375 μg of leaf material) were separated by SDS-PAGE, blotted on a nitrocellulose membrane, and detected with goat anti-human IgG (H+L) HRP conjugate (Promega; W403B) and a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate; Pierce). Film exposure for 30 s reveals scFv-Fc bands as well as degradation products. Bars indicate the height of marker bands (in kD).
Figure 4. N-Glycosylation patterns of different seed- and leaf-produced scFv-Fcs. N-Glycan analysis was carried out by LS-ESI-MS of tryptic peptides. Due to incomplete tryptic digest, each glycan structure is represented by two glycopeptides assigned -1 and -2. All 2G12 constructs as well as wt-PhHA78sc KDEL carry exclusively oligomannosidic N-glycans. wt-PhHA78sc SEC carries xylosylated and fucosylated complex-type N-glycans as well as oligomannosidic structures, while TKO-PhHA78sc SEC carries nonxylosylated and nonfucosylated complex-type N-glycans as well as oligomannosidic structures. wt-35SHA78sc SEC extracted from leaves carries xylosylated and fucosylated complex-type N-glycans. The depicted glycoforms show one of the possible isoforms. For N-glycan nomenclature, see www.proglycan.com [See online article for color version of this figure.]
Man7 Isomer Distribution

Another interesting observation was the presence of a relatively high proportion of Man7 structures in seed-extracted scFv-Fcs, even in constructs designed for secretion (i.e. wt-Ph2G12scSEC, TKO-Ph2G12scSEC, wt-35S2G12scSEC, and wt-35SHA78scSEC). As known from other studies, this Man7 structure occurs in at least three isomers, Man7.1, Man7.2, and Man7.7 (Neeser et al., 1985; Tomiya et al., 1991). There is some evidence that Man7.1 isolates are substrates for the endoplasmic reticulum-associated protein degradation (ERAD) pathway, which directs misfolded proteins from the ER for ubiquitination and subsequent proteasomal degradation (Islam et al., 1991; Zeng et al., 2003; Rademacher et al., 2009). There were also studies indicating that Man7.1 isomer results from the elongation of the Man7.2 species (Neeser et al., 1985; Tomiya et al., 1991).

Subcellular Localization

In order to reveal the stations of intracellular transport and the final destination of the recombinant scFv-Fcs, IEM was carried out on mature and developing seeds. The final deposition status of the target proteins can be determined in mature seeds; however, more organelles are visible in developing seeds, therefore enabling a more detailed investigation of intracellular trafficking. Plants that were transformed with scFv-Fcs driven by the seed-specific phaseolin promoters (i.e. wt-Ph2G12scSEC, wt-35S2G12scKDEL, wt-35SHA78scSEC, and wt-35SHA78scKDEL) were analyzed. The results for mature seeds are shown in Supplemental Figures S1 to S4 and in Supplemental Results S1.

Intense labeling of the extracellular space was obtained in seeds expressing wt-PhHA78scSEC, showing the efficient secretion of the scFv-Fc to that compartment (Fig. 6A). In addition, dense vesicles were intensely labeled (Fig. 6B), but minor amounts of gold particles were also detected in the Golgi stack itself (Fig. 6C). This labeling pattern is reminiscent of the expression of the secretory full-length antibody versions of 2G12 and HA78 in Arabidopsis seeds, which also localize to the same structures (Loos et al., 2011). wt-PhHA78scKDEL accumulated in globular, membrane-delimited structures of around 200 to 400 nm diameter (Fig. 7). These structures were partially studded with ribosomes, indicating their ER origin, and are thus called endoplasmic reticulum-derived vesicles (ERVs). The PSVs were consistently only slightly labeled (Fig. 7C). However, none of the other compartments, like the Golgi apparatus (Fig. 7A), putative multivesicular bodies (Fig. 7B), or the extracellular space (data not shown), was labeled. Mature seeds expressing wt-PhHA78scSEC also showed gold particles in the extracellular space; however, in contrast to developing seeds, ERVs were additionally present in the cytoplasm and labeled (Supplemental Fig. S3). Mature seeds expressing wt-PhHA78scKDEL exhibited labeling exclusively in ERVs and dilated nuclear envelope (Supplemental Fig. S2).

Surprisingly, IEM studies in developing seeds expressing wt-Ph2G12scSEC (Fig. 8) exhibited a similar labeling pattern as obtained for wt-PhHA78scKDEL (Fig. 7). Gold label was found exclusively in globular, partly ribosome-studded ER-derived vesicles of around 200 to 400 nm. In addition, the nuclear envelope was partially dilated and labeled (Fig. 8A), which was not observed for the HA78 scFv-Fc constructs. Golgi stacks, the extracellular space, as well as the PSV were devoid of label (Fig. 8, B, D, and E). Such an unexpected labeling pattern was also detected in mature seeds (Supplemental Fig. S3). These findings were surprising, as the protein was designed for secretion; however, they were consistent with the observed ER-typical oligomannosidic N-glycan profile. For wt-Ph2G12scKDEL, IEM was conducted on mature seeds only (Supplemental Fig. S4) and gave a very similar result as for wt-Ph2G12scSEC, with intensive labeling of ERVs. Notably, ERVs were not detected in nontransformed Arabidopsis seeds (data not shown).

Antigen-Binding Activity and in Vitro Virus Neutralization

Functional activities of seed-produced scFv-Fcs were also determined. First, the antigen-binding capacities of purified wt-Ph2G12scKDEL, wt-PhHA78scSEC, and wt-PhHA78scKDEL, as well as wt-35SHA78scSEC, were determined by ELISA. Whereas all HA78 scFv-Fc variants
exhibited binding comparable to a Chinese hamster ovary (CHO)-produced full-length mAb (Fig. 9), none of the 2G12 constructs was able to bind the respective antigen (data not shown). In vitro virus neutralization assays were conducted with the same scFv-Fcs. Again, the three HA78 scFv-Fc variants exhibited a hepatitis A virus neutralization efficiency similar to CHO- and plant-produced full-length mAbs (Table III), while wt-Ph2G12scKDEL did not show any neutralizing activity (Table IV).

DISCUSSION

In this study, we focused on the expression, stability, intracellular trafficking, deposition pattern, and N-glycosylation status of recombinantly expressed scFv-Fc versions of two antiviral antibodies. We have exploited the capability of plant seeds to produce high amounts of these complex, potentially therapeutic proteins. The accumulation levels of the scFv-Fcs expressed in Arabidopsis seeds lie between 0.8 and 9.4 \( \mu \text{g mg}^{-1} \) dry seeds (Table II). This is in the same range as recently published for the respective full-length IgGs (Loos et al., 2011) but clearly lower than previously reported for scFvs and scFv-Fcs (74 \( \mu \text{g mg}^{-1} \) dry seeds [De Jaeger et al., 2002] and 26 \( \mu \text{g mg}^{-1} \) dry seeds [Van Droogenbroeck et al., 2007], respectively). The reason for the lower accumulation level...
is not clear but may lie in sequence elements with unfavorable codon usage. Thus, codon optimization might further increase expression levels, as has been frequently shown (e.g. Geyer et al., 2010).

Besides the intact protein, secretory versions of both 2G12 and HA78 scFv-Fcs exhibit a substantial amount of degradation products. This degraded fraction is more pronounced for the scFv-Fc constructs than for the recently reported respective IgGs (Loos et al., 2011), indicating a reduced stability of the engineered scFv-Fc compared with the full-length counterparts. Surprisingly, seed-produced scFv-Fcs seem to be more stable when expressed to a higher level: under the control of the stronger, seed-specific β-phaseolin promoter, the ratio of intact scFv-Fcs versus degraded peptides is higher than for the 35S-driven constructs (compare the ratio of intact scFv-Fcs versus degraded polypeptides in Fig. 2, lane 4, and Fig. 3, lane 2). One possible explanation is that proteases responsible for degradation are overloaded by the vast amount of scFv-Fc produced by the β-phaseolin promoter but still cope with the smaller amount produced by the 35S promoter. Alternatively, a different temporal and spatial control of expression might be responsible for the observed differences in degradation (Takaiwa et al., 1995).

When comparing seed- and leaf-produced scFv-Fcs, a clear difference in the degradation pattern is visible (Figs. 2 and 3). This indicates that a different set of proteases is active in the different tissues or might represent differences in the secretory pathways of these two tissues. The higher ratio of intact scFv-Fcs versus degraded peptides in leaves compared with seeds suggests that 35S-driven scFv-Fcs are more stable in leaves (Fig. 3). This contrasts with the prevailing notion that seeds possess a lower lytic potential and are thus especially well suited for recombinant protein production (Fiedler and Conrad, 1995; Stoger et al., 2002). An alternative suggestion to increase the protein stability is retention in the ER via a

![Figure 7](image_url)

Figure 7. Subcellular localization of \(\text{wt-PhHA78scKDEL}\) in developing Arabidopsis seeds by IEM. A, Gold label was nearly exclusively found in globular structures that were partially ribosome studded (arrowheads), indicating an ER origin (ERV). The nuclear envelope was neither swollen nor labeled. B, A putative multivesicular body was not labeled. C, ER-derived globular structures were strongly labeled, the PSV was slightly labeled, and oil bodies were free of labeling. N, Nucleus; NE, nuclear envelope; MVB, multivesicular body; OB, oil body. Bars = 200 nm.

![Figure 8](image_url)

Figure 8. Subcellular localization of \(\text{wt-Ph2G12scSEC}\) in developing Arabidopsis seeds by IEM. A, Gold label was mainly found in ERVs. The nuclear envelope was partially swollen and labeled in a density similar to the ERVs. B, The ERVs were partially ribosome studded (arrowheads), indicating ER origin. Gold label was not found in the Golgi apparatus. C, ERVs were abundant structures. Some ERVs are indicated by arrows. D, The PSV was not labeled, but a Golgi stack was slightly labeled. E, The extracellular space was devoid of labeling. cl, Chloroplast; CW, cell wall; EC, extracellular space; G, Golgi apparatus; N, nucleus; NE, nuclear envelope; OB, oil body; SNE, swollen nuclear envelope. Bars = 200 nm (A, B, D, and E) and 1,000 nm (C).
subcellular deposition are not clear yet but could be due to improper folding. The complete absence of antigen-binding and virus-neutralization activity indicates an improper conformation of the polypeptide. A detailed structural analysis of 2G12 IgG had revealed an unusual conformation of this antibody (Calarese et al., 2003), with the \( V_H \) domain of one heavy chain pairing with the \( V_L \) domain of the opposing light chain. We hypothesize that the correct three-dimensional assembly of this unusual structure is impeded in the scFv-Fc, leading to the observed findings. The 2G12 scFv-Fcs seem to be recognized by the ER-based quality-control systems, and their passage farther down the secretory pathway is prevented. The increased presence of Man7.1 on 2G12 scFv-Fcs further corroborates our hypothesis, as this isoform has been reported to act as a signal for ERAD, an intracellular control mechanism for the elimination of incorrectly folded proteins (Clerc et al., 2009; Liebminger et al., 2010). In contrast, Man7 of secretory HA78 scFv-Fcs consists mainly of the Man7.2 isomer, which does not seem to be involved in the ERAD pathway (Clerc et al., 2009). Surprisingly, also the KDEL-tagged HA78 scFv-Fc has an elevated fraction of Man7.1 isomers. Thus, it seems unlikely that Man7.1 structures alone are a mandatory signal for ERAD in Arabidopsis.

KDEL-tagged HA78 scFv-Fc was detected in newly formed ER-derived vesicles. This deposition pattern is in accordance with the N-glycosylation pattern. However, it is not in full agreement with a similar KDEL-tagged HA78 scFv-Fc construct, which was previously expressed in Arabidopsis seeds and had accumulated to a large extent in the periplasmic space between the plasma membrane and the cell wall (Van Droogenbroeck et al., 2007). Such a periplasmic space was not detected in this study. Yet, another subcellular localization was found for a KDEL-tagged full-length version of 2G12 recently expressed in Arabidopsis seeds. This protein was targeted nearly exclusively to protein storage vacuoles, and neither formation of ERVs nor a periplasmic space was detected (Loos et al., 2011). The factors responsible for the different deposition patterns of these related KDEL-tagged polypeptides are

![Figure 9. Antigen-binding activity of seed-produced HA78 scFv-Fc variants. All seed-produced HA78 scFv-Fc variants exhibit the same antigen-binding activity as a CHO-produced full-length HA78 IgG. OD, Optical density.](image-url)
so far unknown. Possible reasons could be differences in the protein sequences and/or differences in expression level. However, despite their different subcellular depositions (between plasma membrane and cell wall, in ERVs, and PSVs), all these KDEL-tagged proteins carry comparable oligomannosidic N-glycosylation profiles, indicating identical trafficking routes along the early secretory pathway.

In contrast to the 2G12 constructs, the secretory versions of HA78 scFv-Fcs (wt-PhHA78scSEC, TKO-PhHA78scSEC, and wt-35SHA78scSEC purified from leaves) carry the expected complex N-glycans, indicating efficient secretion via the Golgi apparatus, where these modifications occur. Intensive labeling of the extracellular space and the Golgi found in our IEM studies confirms this assumption. The complete absence of plant-specific β-1,2-Xyl and α-1,2-Fuc on the HA78 scFv-Fc expressed in the N-glycosylation mutant TKO (TKO-PhHA78scSEC) confirms the suitability of this expression host for the production of glycoproteins with a humanized N-glycosylation profile. This is in accordance with our previous studies, where glyco-engineered Arabidopsis served as an expression system for the generation of mAbs with a humanized N-glycosylation pattern (Strasser et al., 2004; Schähs et al., 2007; Loos et al., 2011). Glycan structures as obtained in these studies (i.e. GnGn structures) have been shown to enhance Fc-mediated effector functions of mAbs and thus may contribute to the development of pharmaceuticals with enhanced in vivo efficacy (Forthal et al., 2010).

An unexpected observation was the presence of substantial portions of oligomannosidic N-glycans on β-phaseolin-driven secretory HA78 scFv-Fcs. Such ER-typical glycans were hardly found on leaf-produced HA78 scFv-Fc (wt-35SHA78scSEC purified from leaf). This observation seems to be due to the special characteristics of the seed secretory system rather than a consequence of the substantially increased accumulation level of the β-phaseolin-driven constructs, as degradation products of 35S-driven secretory HA78 scFv-Fcs (wt-35SHA78scSEC) purified from seeds also carry a substantial portion of oligomannosidic structures besides complex N-glycans (Supplemental Figs. S5 and S6; Supplemental Table S1). However, this increased fraction of ER-typical, oligomannosidic N-glycans is in line with the deposition pattern in mature seeds, where wt-PhHA78scSEC is secreted to the extracellular space but is also partially deposited intracellularly in ER-derived structures (Supplemental Fig. S51). This partial retention, which is not found in developing seeds, might occur in an advanced developmental stage. Overloading of the translocation machinery, the onset of senescence, and, related to that, decreased translocation activity or a development-related switch in pathways can all be envisaged to explain this unusual phenomenon. The same difference between immature and mature seeds (i.e. a stronger tendency toward ER-derived structures in later stages of maturation) was also observed in maize (Zea mays) seeds expressing a recombinant protein (Arcalis et al., 2010).

Recently, full-length versions of 2G12 expressed in maize seeds and tobacco (Nicotiana tabacum) seeds have been shown to carry a substantial fraction of single GlcNAc residues (Rademacher et al., 2008; Ramesar et al., 2008; Floss et al., 2009). With the exception of some degradation products of 35S-driven HA78 scFv-Fc, this specific glycoform has not been found in this study or in related studies on the expression of antibodies or fragments in Arabidopsis seeds (Van Droogenbroeck et al., 2007; Loos et al., 2011), nor has it been reported upon expression of other mAbs in other plant seeds.

To our knowledge, our results represent the first study that examines in a systematic way the stability, subcellular targeting, and deposition of recombinantly expressed proteins in combination with a detailed monitoring of the N-glycosylation status. This important posttranslational modification occurs along the secretory pathway and allows conclusions on the subcellular trafficking of the target protein. In summary, we demonstrate the efficient expression of recombinant proteins in Arabidopsis seeds, the targeted subcellular deposition, and the generation of customized N-glycosylation patterns. These results emphasize the high potential of plant seeds as reliable and versatile expression hosts for the production of complex mammalian glycoproteins. However, we also reveal some unusual phenomena within the secretory pathway of transgenic seeds, like the formation of ER-derived vesicles upon the expression of recombinant proteins. The exact mechanism that initiates the formation of such vesicles is not yet fully understood. Further studies are required to elucidate intracellular trafficking mechanisms in seeds, which are of particular importance for the production of proteins that need special posttranslational modifications, like glycosylation, to obtain full biological activity. So far, this elucidation remains a case-to-case study.

### MATERIALS AND METHODS

#### Cloning and Plant Transformation

2G12 V_{1} and V_{1} were amplified with forward primers containing SfiI and ApuLI sites, respectively, and reverse primers containing XhoI and NolI sites, respectively. The fragments were cloned into the pHEN2 phagemid vector.
of the 35S Promoter

Antigen-Binding Activity of Purified scFv-Fcs

Virus Neutralization Assays

Purification of scFv-Fcs Expressed under the Control of the 35S Promoter

Protein Extraction from Leaves

Protein Extraction from Seeds and Antibody Purification

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IEM on mature seeds was performed as described previously (Loos et al., 2011). Briefly, developing embryos were high-pressure frozen, freeze substituted with acetone, and infiltrated with HM20. Polymerization was conducted with UV light at ~35°C. Sections were labeled with γ-chain-specific antibodies and secondary gold-coupled antibodies. After postcontrasting, the samples were analyzed with a JEOL JEM-1400 transmission electron microscope at 80 kV. IEM on mature seeds was performed as described in Supplemental Materials and Methods S1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Subcellular localization of wt-PhHA78scSEC in mature Arabidopsis seeds by immunogold electron microscopy.

Supplemental Figure S2. Subcellular localization of wt-PhHA78scKDEL in mature Arabidopsis seeds by immunogold electron microscopy.

Supplemental Figure S3. Subcellular localization of wt-2G12scSEC in mature Arabidopsis seeds by immunogold electron microscopy.

Supplemental Figure S4. Subcellular localization of wt-2G12scKDEL in mature Arabidopsis seeds by immunogold electron microscopy.

Supplemental Figure S5. Coomassie-stained SDS-PAGE of wt-PhHA78scSEC purified from seeds.

Supplemental Figure S6. N-glycosylation pattern of the degradation products of seed-produced wt-PhHA78scSEC.

Supplemental Table S1. Relative distribution of N-glycans of the degraded fragments of seed-extracted wt-PhHA78scSEC and 2G12-PhHA78scSEC.

Supplemental Materials and Methods S1. IEM of mature seeds.

Supplemental Results S1. Subcellular localization in mature seeds and N-glycosylation of degradation products.

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LITERATURE CITED


