Vacuolar targeting of recombinant antibodies in *Nicotiana benthamiana*

Carolina Gabriela Ocampo$^1$, Jorge Fabricio Lareu$^1$, Vanesa Soledad Marin Viegas$^1$, Silvina Mangano$^{1,†}$, Andreas Loos$^{2,†}$, Herta Steinkellner$^2$ and Silvana Petruccelli$^1$.

$^1$Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina

$^2$Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Vienna, Austria

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* Correspondence (Tel/Fax: +54-221-424-9287 | 425-4853 | 489-0741; email silvana@biol.unlp.edu.ar)

† Present address: Fundación Instituto Leloir, Av. Patricia Argentinas 435, Buenos Aires, Argentina.

‡ Present address: Aridis Pharmaceuticals Inc., 5941 Optical Court, San Jose, CA 95138, USA

**Summary**

Plant-based platforms are extensively used for the expression of recombinant proteins, including monoclonal antibodies. However, to harness the approach effectively and leverage it to its full potential, a better understanding of intracellular processes that affect protein properties is required. In this work, we examined vacuolar (vac) targeting and deposition of the monoclonal antibody (Ab) 14D9 in *Nicotiana benthamiana* leaves. Two distinct vacuolar targeting signals (KISIA and NIFRGF) were C-terminal fused to the heavy chain of 14D9 (vac-Ab) and compared with secreted and ER-retained variants (sec-Ab, ER-Ab, respectively). Accumulation of ER- and vac-Ab was 10- to 15-fold higher than sec-Ab. N-glycan profiling revealed the predominant presence of plant typical complex fucosylated and xylosylated GnGnXF structures on sec-Ab while vac-Ab carried mainly oligomannosidic (Man 7-9) next to GnGnXF forms. Paucimannosidic glycans (commonly assigned as typical vacuolar) were not detected. Confocal microscopy analysis using RFP fusions showed that sec-Ab-RFP localized in the apoplast while vac-Ab-RFP were exclusively detected in the central vacuole. The data suggest that vac-Ab reached the vacuole by two different pathways: direct transport from the ER bypassing the Golgi (Ab molecules containing Man structures) and trafficking through the Golgi (for Ab molecules containing complex N-glycans). Importantly, vac-Ab were correctly assembled and functionally active. Collectively, we show that the central vacuole is an appropriate compartment for the efficient production of Abs with appropriate post-translational modifications, but also point to a reconsideration of current concepts in plant glycan processing.

**Introduction**

The synthesis of pharmaceutical and industrial proteins in plants has become a reality with numerous products on the market and a variety of technologies available and facilities installed for large-scale production (Sack et al., 2015). Both, stable and transient expression systems, have been successfully used to express mAbs in different plant species, organs and subcellular compartments (De Muynck et al., 2010). Recombinant protein accumulation levels rely on numerous factors among them subcellular localization (Egelkrout et al., 2012). Immunoglobulins (Igs) are generally sorted to the apoplast which is, unfortunately, often afflicted with intense proteolysis (Benchabane et al., 2008; Niemer et al., 2014). Thus, alternative strategies to optimize protein accumulation in other cellular organelles are considered. Retention of Abs in the endoplasmic reticulum (ER) frequently results in increased yields (De Muynck et al., 2010). Although the plant central vacuole is considered a hostile environment for foreign protein accumulation, it has been shown that some proteins, such as human alpha-mannosidase (De Marchis et al., 2013), human complement factor C5a (Nausch et al., 2012), collagen (Stein et al., 2009) and human transglutaminase 2 (Marin Viegas et al., 2015), accumulate in this organelle. In contrast, in carrot suspension cell cultures, secretory versions of human IgG1 and G4 have higher yields than ER and vacuolar variants (Shaaltiel et al., 2012). Accumulation of full-length antibodies sorted to vacuoles in leaves has not been studied.

The plant secretory pathway is more complex than other euukaryotic organisms. Multiple distinct vacuolar compartments with either storage or degradative functions coexist in the same cell (Jaah et al., 1998; Paris et al., 1996). Small individual vacuoles fuse with each other to form larger vacuoles when different cellular processes take place (Zhang et al., 2014). It is well known that cargo proteins can be transported to vacuoles by different transport routes. The conventional vacuolar trafficking pathway involves ER export via coat protein complex II (COPII) vesicles and subsequent Golgi and post-Golgi transport (Xiang et al., 2012). In addition, a direct transport from the ER to the vacuole that is independent of COPII vesicles has also been described (De Marchis et al., 2013; Viotti et al., 2013). Proteins destined to vacuoles have different vacuolar sorting signals (VSSs) that can be either sequence specific (SS) (NPIXL or NPIR motif) and work independent of its location on the protein sequence or hydrophobic C-terminal signals (C1) (Matsuoka and Neuhaus, 1999; Vitale and Raikeh, 1999). These VSSs are specifically recognized by vacuolar sorting receptors (VSRS) that are located...
in Golgi and post-Golgi compartments, suggesting similar mechanisms to the clathrin-coated vesicles (CCV) pathway found in other eukaryotic cells (Hinz et al., 2007; Niemes et al., 2010). Nevertheless, it has been reported that interaction of vacuolar cargo with VSR is initiated in the ER (Niemes et al., 2011), which supports the conventional model for vacuolar protein sorting (Robinson and Pimpl, 2013; Xiang et al., 2012).

N-glycosylated proteins, such as immunoglobulins, are well suited molecules to study vacuolar targeting mechanisms as the N-glycosylation status allows conclusions on intracellular trafficking processes. Protein N-glycosylation starts in the ER with the transfer of the oligosaccharide precursor Glc₃Man₉GlcNAc₂ to specific asparagine (Asn) residues of the polypeptide followed of a limited trimming in both the ER and Golgi and sequential addition of monosaccharides, as the protein travel through the Golgi complex, to yield complex N-glycans, typically GlcNAc₂ManₓXylFucGlcNAc₂ (GnGnXF) structures (Castilho and Steinkeller, 2012). Further modifications of the oligosaccharides include the addition of galactose beta-1,3 and fucose alpha-1,4 linked to the terminal GlcNAc forming the Lewis A oligosaccharide for apoprot antibodies (Fitchette-Lainé et al., 1997; Strasser et al., 2007). In addition, paucimannosidic ManₓXyl-FucGlcNAc₂ (MMXF), which derives from the removal of terminal GlcNAc residues from complex N-glycans, is present. The formation of such plant typical structures has long been assigned to bevaculospatic (Comord et al., 2010; Lerouge et al., 1998); however, recent characterization of the enzymes responsible for the cleavage of terminal GlcNAc residues (i.e. hexosaminidases) (Liebming et al., 2011) and the presence of significant amounts of MMXF structures in the plant apoplastic fluid (Schneider et al., 2015) point to a broader distribution. Interestingly, the expression of a vacuum sorted IgG in tobacco BY2 cells showed an increased level of MMXF structures when both heavy chain (HC) and light chain (LC) are fused to sporamin ssVSS in comparison with when only HC is fused (Misaki et al., 2011).

In this work, we studied the impact of vacuolar deposition of an IgG antibody in Nicotiana benthamiana leaves. Thus, we fused two different VSSs derived from the amaranth 11S globulin (KISIA and the NIFRGF ss) to a mAb, to evaluate vacuolar accumulation as alternative production strategy. Further, we aimed to elucidate so far poorly understood mechanisms of vacuolar trafficking pathways and N-glycan processing in this subcellular compartment.

Results

Transient expression of the 14D9 mAb variants in Nicotiana benthamiana leaves

To study the impact of subcellular targeting strategies on the accumulation of a full-length IgG, the light chain (LC) carrying the native signal peptide (sec-LC) of the monoclonal antibody 14D9 was combined with different sorted versions of the heavy chain (HC), as is shown in Figure 1. The secretory (sec-HC) and the reticulum endoplasmic (ER-HC) versions of the HC, generated recently, were used as references (Petruccelli et al., 2006); HC of 14D9 was C-terminally fused to well-characterized vacuolar targeting signals, that is KISIA (vac1-HC) and NIFRGF (vac2-HC) derived from the amaranth 11S globulin (Petruccelli et al., 2007) (Figure 1). Transient expression experiments in Nicotiana benthamiana leaves were performed by infiltration of agrobacteria carrying sec-LC and the different HC variants: (i) sec-LC to produce secreted Ab (sec-Ab), (ii) ER-HC to generate ER-Ab and (iii) vac1-HC and vac2-HC to form vac1-Ab and vac2-Ab, respectively. Accumulation levels of assembled Abs were analysed by sandwich ELISA, using agroinfiltrated leaves from five different plants for each biological replicate and at least three independent experiments. Maximal expression levels were obtained between 5 and 8 days post infiltration (d.p.i.). ELISA data exhibited a similar expression level of ER- and vac-Ab (1.57% - 1.73% of TSP) while sec-Ab accumulation is 10- to 15-fold lower (0.13 ± 0.02%TSP). To test whether LC and HC variants were assembled into functional antibodies, the recognition of 14D9 to the corresponding antigen (i.e. BSA hapten) was evaluated by indirect ELISA. The four Ab variants were able to recognize the hapten (Figure 2b), and the obtained signal showed a good correlation with the accumulation levels of each Ab variant (Figure 2a).

Antibodies were purified from agroinfiltrated leaves using protein G affinity chromatography and subsequently analysed by immunoblotting using anti-mouse Ig serum for detection. Under reducing conditions, two bands of approximately 25 and 52 kDa were detected (Figure 3a) corresponding to LC and HC, respectively. Under non-reducing conditions, the four Ab variants gave only one high-molecular mass form at approximately 170 kDa (Figure 3b), confirming that the four variants of the HC were able to assemble with the sec-LC into heterotetramer and that assembled Abs can be purified from leaves.

N-linked glycosylation pattern of 14D9

N-glycan profiles of purified Abs were determined by LC-ESI-MS as described recently (Stadlmann et al., 2008). The mass spectrum for the glycopeptide EEQFNSTFR (1157.52 Da) which carries the single glycosite of 14D9 (Asn297 located in the Fc domain) from the different Ab variants is shown in Table 1. The sec-Ab...
significant difference by Tukey comparisons test: ****

Hapten indicating that the immunoglobulin fold properly. Statistically three independent ELISA experiments. The four Ab variants recognized the 650 nm per mg of protein. Data represent the mean value (SEM). ****Denotes statistically significant difference by Tukey comparisons test. Red fluorescence in central vacuole was detected in 76.6% ± 8.3 and 79.4% ± 11.5 of the epidermal cells expressing vac1-Ab and vac2-Ab, respectively (Figure S1), confirming that both proteins were efficiently transported to the vacuole. In addition, the presence of vacuolar red fluorescence in leaves infiltrated with agrobacteria carrying sec-Ab and ER-Ab constructs was analysed and in both cases it was <5%. Taking these results into account, it can be anticipated that the observed oligomannosidic structures of vac-Ab were not due to partial retention in the ER, but rather derive from vacuolar-located Abs.

Subcellular localization of the different sorted Ab variants

Different Ab fusions to RFP were generated (Figure 1), and protein deposition was monitored by confocal laser scanning microscopy (CLSM). To confirm that RFP fusions do not affect Ab sorting, expression of the different LC- and HC-RFP fusions was analysed. Figure 4a shows that sec-LC-RFP has an irregular red fluorescent pattern on the border of leaf epidermal cells typical of apoplast localization (red fluorescent channel showed in magenta colour). No colocalization with ER-GFP was observed. In contrast, sec-HC-RFP exhibited a typical network ER-pattern and colocalizes with ER-GFP (Figure 4b, white colour derives from the merge of RFP magenta and GFP green channels). This is in agreement with the proposed model for in vivo assembly of Ig stating that CH1 domain is unable to fold when LC is not present and therefore remains in the ER (Feige et al., 2009). When both sec-LC-RFP and sec-HC-RFP were coexpressed, an apoplast staining pattern was obtained (Figure 4c), confirming that the RFP fusion did not affect the final destination of sec-Ab-RFP. Similarly, sec-LC and sec-HC-RFP (Figure 4d) or sec-LC-RFP and sec-HC (Figure 4e) exhibited apoplast staining. When sec-LC-RFP was coexpressed with ER-HC (Figure 4f), a typical ER fluorescent pattern was obtained. Coexpression of sec-LC-RFP with vac1-HC (Figure 4g), vac2-HC (Figure 4h) and vac1-HC-RFP (Figure 4i) showed strong red fluorescent signals on central vacuoles, and no colocalization with ER-GFP was detected, not even around the nuclear envelope, where particularly strong signals were obtained (nuclear envelope has only green fluorescent). To estimate the efficiency of the vacuolar sorting, a quantitative analysis was performed. Red fluorescence in central vacuole was detected in 76.6% ± 8.3 and 79.4% ± 11.5 of the epidermal cells expressing vac1-Ab and vac2-Ab, respectively (Figure S1), confirming that both proteins were efficiently transported to the vacuole.

Vaciner targeting of antibodies

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To verify that red fluorescence signal correspond to intact LC-RFP and HC-RFP fusions, an immunoblot analysis with RFP-specific antibody was performed (Figure 5). Only bands of ~50 kDa and ~77 kDa corresponding to LC-RFP and HC-RFP, respectively, were detected for the different combinations of LC and HC (Figure 5), confirming the integrity of LC-RFP and HC-RFP fusions. In consequence, it can be anticipated that red fluorescent staining in Figure 4 corresponds to the intact Abs-RFP fusions.

As the strong red fluorescence in the central vacuole can overlay the ER pattern, microsomes were isolated from leaves infiltrated with the four Ab variants and the obtained fractions were analysed by immunoblot. Figure 6 shows that both vac1-Ab and vac2-Ab and also sec-Ab were only detected in the soluble fraction (SF). On the other hand, ER-Ab was found in both the microsomal pellet (MP) and the SF, similar to the results observed for other ER resident proteins such as BiP (Abas and Luschnig, 2010; Yamamoto et al., 2008). These biochemical data confirmed that only the ER-Ab was located in the ER while the other

Figure 2 Determination of 14D9 Expression Level and Antigen Binding by ELISA. (a) Accumulation of Abs in agroinfiltrated leaves. Nicotiana benthamiana leaves were infiltrated with Agrobacterium carrying sec-LC and (i) sec-HC to produce secreted Ab (sec-Ab), (ii) ER-HC to generate ER-Ab or (iii) vac1-HC and vac2-HC to form vac1-Ab or vac2-Ab, respectively. Ab amounts were quantified by ELISA of three biological replicates (each replicate containing five leaf discs of the infiltrated tissue from a different plant) and were expressed as % of total soluble protein (TSP). Error bars represent the standard error of the mean (SEM). ****Denotes statistically significant difference by Tukey comparisons test (P ≤ 0.0001). ER-Ab, vac1-Ab and vac2-Ab protein yields were 10- to 15-fold higher than sec-Ab ones. Yields of Abs retained in the ER or sorted to vacuoles did not differ significantly. B: Recognition of BSA-14D9 hapten. An indirect ELISA, using the same amount of soluble leaf protein per well, obtained as described above, was performed. The values were expressed as OD at 650 nm per mg of protein. Data represent the mean value (± SEM) of three independent ELISA experiments. The four Ab variants recognized the hapten indicating that the immunoglobulin fold properly. Statistically significant difference by Tukey comparisons test: ****P ≤ 0.0001; ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05.

exhibited predominantly complex N-glycans composed of GnGnXF, GnF and GnGn structures (73%) typical for Golgi-processed oligosaccharides (Table 1). Notably, 27% of oligomannosidic structures (Man 7-9) were detect as well. In contrast, vac1-Ab and vac2-Ab carried mainly oligomannosidic structures (75%) typical for ER-retained molecules (Table 1). In addition, vac-Abs displayed about 25% GnGnXF structures. No paucimannosidic glycan structures were detected on the vac-Ab variants.
Ab variants were absent in microsome fraction; therefore, oligomannosidic structures in vac-Abs were not due to ER retention.

**Discussion**

Currently, the most advanced platform for the production of IgG antibodies in plants is tobacco leaves using either transient or stable transformation systems (Ma et al., 2015; Mapp Biopharmaceuticals, 2015). In leaves, Abs are generally sorted to the apoplast, an environment that favours protein degradation (Benchabane et al., 2008; Niemer et al., 2014). Different strategies have been assayed to prevent apoplast degradation (Goulet et al., 2012; Jutras et al., 2015; Robert et al., 2013), but satisfying solutions are elusive. Thus, sorting of immunoglobulins to other subcellular compartments is a viable alternative. In this work, a depth analysis of the impact of vacuolar targeting of a full-length Abs was performed. We showed that vacuolar variants of the mAb14D9 accumulated at levels 10- to 15-folds higher than secretory forms. Although ER retention increased antibody yields in some cases (Juarez et al., 2013; Ko et al., 2005; Petruccelli et al., 2006; Schouten et al., 1996), accumulation of Ig sorted to central vacuole in leaves has not been previously reported. Only one report compared apoplastic and vacuolar accumulation of mAbs in carrot cell suspension culture, and the highest yield was obtained for secreted forms of human IgG 1 and IgG 4 (Shaaltiel et al., 2012). Even though proteolytic enzymes have been reported to reside in the vacuoles (Jaquinod et al., 2007; Muntz, 2007), protein degradation dependent on various factors such as environment conditions (e.g. pH) that influence proteolytic activity. For example, vacuolar GFP is prone to proteolysis as a consequence of light-dependent vacuole acidification but stable under dark conditions (Tamura et al., 2003). Here, we showed that a mouse IgG accumulated at increased yields in leaf central vacuole indicating low-Ab-related proteolytic activities in this compartment. Similar results were reported for human α-mannosidase (De Marchis et al., 2013), human complement factor C5a, (Nausch et al., 2012), collagen (Stein et al., 2009), human α1-proteinase inhibitor (Jha et al., 2012) and human transglutaminase 2 (Marin Viegas et al., 2015).

Notably, we demonstrate the robustness of FP fusions for the subcellular visualization of Abs, including the central vacuole. Notwithstanding, such fusions have to be performed with great care, as they might interfere with Ab properties. In mammalian cells, fusion of IgG to citrine (yellow fluorescent protein) but not GFP was produced without interfering in folding, secretion and functionality of the IgG (Haas et al., 2008). At difference of such observations, we showed that the mAb14D9-RFP fusions sorted to the assigned final destinations.

The predominant presence of complex N-glycan structures (GnGnXF, GnGn and GnGnF) on sec-Ab indicates its transport and final destinations. SDS-PAGE was performed under reducing (a) and nonreducing (b) conditions and detected by goat anti-mouse IgG serum. Abbreviation corresponds to Figure 2a. Black arrows indicate assembled IgG (170 kDa), HC (52 kDa) and LC (25 kDa).

Molecular masses are given in kilo-dalton.

**Figure 3** Immuno detection of purified Abs.

**Table 1** N-linked glycans on the 14D9 variants. N-glycosylation profile as determined by liquid chromatography–electrospray ionization–mass spectrometry (LS-ESI-MS). Numbers represent the presence of the different glycoforms in %

<table>
<thead>
<tr>
<th>N-glycan species</th>
<th>Sec-Ab (%)</th>
<th>Vac-Ab (%)</th>
</tr>
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<tbody>
<tr>
<td>Complex (total)</td>
<td>73</td>
<td>25</td>
</tr>
<tr>
<td>GnGnXF</td>
<td>43</td>
<td>25</td>
</tr>
<tr>
<td>GnF</td>
<td>14</td>
<td>–</td>
</tr>
<tr>
<td>GnGn</td>
<td>16</td>
<td>–</td>
</tr>
<tr>
<td>Oligomann. (Man7-9)</td>
<td>27</td>
<td>75</td>
</tr>
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</table>

Ab variants were absent in microsome fraction; therefore, oligomannosidic structures in vac-Abs were not due to ER retention.
Immunoblot analysis and antigen-binding studies demonstrated that vac-Abs were correctly assembled and functionally active. It is known that unfolded or misfolded proteins in the ER can activate ER-associated degradation (ERAD)-releasing proteins from the ER for proteasome degradation. Also autophagy that delivers unfolded or misfolded molecules to the vacuole for degradation has been reported (Yang et al., 2016). Taking into account that HC and LC are assembled into functional immunoglobulin in the ER (Feige et al., 2009), it can be anticipated that vac-Abs were transported to vacuoles as fully assembled molecules. Given that sec-Abs were found in the apoplast, it is very unlikely that the transport of vac-Abs to vacuoles is mediated by hidden VSSs in the protein sequence, as reported for a hybrid IgA/G HC (Hadlington et al., 2003).

Figure 4  Subcellular localization of Ab variants by confocal laser scanning microscopy (CLSM). Nicotiana benthamiana leaves were infiltrated with Agrobacterium carrying ER-GFP, and different combinations of HC- and LC-RFP fusions (see Figure 1) are as follow: sec-LC-RFP (a), sec-HC-RFP (b), sec-LC-RFP + sec-HC-RFP (c), sec-LC + sec-HC-RFP (d), sec-LC-RFP + sec-HC (e), sec-LC-RFP + ER-HC (f), sec-LC-RFP + vac1-HC (g), sec-LC-RFP + vac2-HC (h), sec-LC-RFP + vac1-HC-RFP (i). FP inspection in agroinfiltrated epidermal cells was performed 5 d.p.i. The images correspond to the merge channel resulting from the combination of RFP fusions (showed in magenta) and GFP (showed in green). Colocalization is shown in white colour. (a) sec-LC-RFP has an irregular red fluorescent pattern typical of apoplast localization. (b) sec-HC-RFP has a typical network ER pattern. (c) sec-LC-RFP + sec-HC-RFP showed an apoplast pattern. (d,e) sec-LC + sec-HC-RFP and sec-LC-RFP + sec-HC showed an apoplast staining pattern, respectively. (f) sec-LC-RFP + ER-HC exhibited typical ER staining. (g) sec-LC-RFP + vac1-HC, (h) sec-LC-RFP + vac2-HC, (i) sec-LC-RFP + vac1-HC-RFP. (g-i) showed a strong red fluorescence on central vacuoles, no colocalization with ER-GFP was detected around the nucleus. Scale bars 15 μm.
C-terminal VSSs are typical of seed storage proteins that are packed into dense vesicles that drop out of the cis-Golgi, while sequence-specific VSSs are characteristic of lytic enzymes found in post-Golgi compartments (Vitale and Hinz, 2005). Previous studies using nonglycosylated fluorescent proteins fused to VSS derived from storage or lytic proteins showed that these reporter proteins localized in the same prevacuolar compartments and interact to vacuolar sorting receptors in similar ways, supporting that both types of VSS sort cargo to vacuoles through the same transport pathway (Miao et al., 2008; Niemes et al., 2011). Here, using Abs as reporters that allow us to track the transit route followed via N-glycan modifications, our results suggest that cargos having Ct-VSS or ssVSS followed the same pathway although several trafficking mechanisms were initially inferred (Vitale and Hinz, 2005).

A direct transport route was described in pumpkin seeds where precursor-accumulating (PAC) vesicles carried storage proteins from the ER to protein storage vacuoles bypassing the Golgi (Mitsuhashi et al., 2001), but in leaves, few data support a direct transport of soluble cargo. The recognition of vacuolar cargo’s VSS by vacuolar sorting receptors in the ER (Niemes et al., 2011) and transport of human alpha-mannosidase in tobacco leaves (De Marchis et al., 2013) are other proofs of the ‘nonclassical model’ proposed recently (Robinson and Pimpl, 2013). Here, we demonstrated that 75% of immunoglobulin molecules followed this direct trafficking route. Why the same IgG protein is transported by two different pathways to the vacuole in leaves is unknown, but saturation of the transport machine as occurs when IgG are overexpressed in Chinese hamster ovary (Hasegawa et al., 2011) might trigger the direct transport to vacuoles in plants.

The N-glycosylation pattern of a reduced number of vacuolar glycoproteins, most of them from seeds, has been determined, and based on these studies, it was postulated that in the vacuole, the terminal GlcNac residues from GnGn oligosaccharides are removed to produce paucimannosidic structures (Gomord et al., 2010; Lerouge et al., 1998). In addition, in carrot cells, glucocerebrosidase fused to Ct-VSS from tobacco chitinase A has mainly paucimannosidic structures (Shaaltiel et al., 2007), supporting trimming of GlcNac residues in vacuoles. Also, mouse IgG fused to the sporamin NPIRL ssVSS expressed in tobacco BY2 cells had mainly MMXF oligosaccharide (Misaki et al., 2011). At differences of these reports, we did not detect paucimannosidic structures in vac-Abs produced in tobacco leaves. Similar to our findings, the secretory human IgA produced in Nicotiana benthamiana accumulated in intracellular compartments (protein...
bodies and vacuoles) but not in the apoplast and had predominantly high mannose but limited evidence for paucimannosidic structures (Paul et al., 2014). On the other side, significant portions of paucimannosidic structures are detected in the apoplastic fluid representing the secretome of a plant cell (Schneider et al., 2015) and recombinant proteins located in the apoplast (Castilho et al., 2014; Dirnberger et al., 2001). Collectively, these data suggest a reconsideration of referring paucimannosidic structures as vacuolar specific/typical.

In conclusion, we showed that antibody deposition inside the cell, either the ER or in the vacuoles, leads to increased yields compared to apoplast accumulation. Importantly, antibodies produced in different subcellular compartments are functionally active. Although N-glycosylation of antibodies intracellularly deposited may not suit therapeutic applications where complex structures are favoured, they might be useful for many research and biomedical applications as well as for productions of mAbs used in the downstream processing of many biologics. Other putative applications could be on synthesis of therapeutic aglycosylated antibodies with novel effector functions (Hristodorov et al., 2013).

Experimental procedures

Plants growth and maintenance

Nicotiana benthamiana plants were grown for 6–8 weeks in a growth chamber at 22 °C with a 16-h-light/8-h-dark cycle and used for transient expression. Infiltration was performed in the third and the fourth leaves counting top-down starting with the youngest mature leaf.

Construction of plant antibody expression binary vectors

Plasmids containing 14DL9C (GenBank Accession Number KU933514) and 14D9HC (KU933515) are described in Petruccelli et al. (2006). To obtain vac1-HC and vac2-HC, 14D9HC was deposited with LR clonase (Life Technologies SA) to obtain pGWB2-LC-RFP and pGWB2-HC-RFP. To fuse 14D9 antibody genes to fluorescent protein (FP) gene, an overlap extension polymerase chain reaction (OE-PCR) strategy was used. LC gene was amplified with F-SP and R-LC-

2 to produce LC-RFP and HC-RFP, respectively. Then, LC-RFP and HC-RFP PCR products were directionally cloned into pENTR-D-TOPO (Life Technologies SA). After that, LC-RFP or HC-RFP fragments, flanked by attL sites, were released from pENTR-HC-RFP or pENTR-LC-RFP by digestion MluI restriction enzyme (Promega, Fitchburg, WI), and these DNA fragments were used in the LR recombination reaction with pGW2 as destination vector (Nakagawa et al., 2007) LR clones (Life Technologies SA) to obtain pGWB2-LC-RFP and pGWB2-HC-RFP.

Agrobacterium-mediated transient protein expression

Agrobacterium tumefaciens strain GV3101 cultures carrying LC, HC; LF-SP and HF-SP constructs were grown in YEB media (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 2 mM MgSO₄) at 28 °C overnight. Cells were collected by centrifugation at 5000 g and resuspended in infiltration media, IM (100 mM MgCl₂, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.7, 200 μM acetylsyringone) adjusting agrobacterium OD₆₅₀ to 0.6 for LC and HC constructs and to 0.1 for P19 silencing suppressor of tomato bushy stunt virus (Baulcombe et al., 2002), followed with incubation at 28 °C for at least 3 h. The abaxial face of Nicotiana benthamiana leaves was pressure infiltrated with the agrobacterium suspension using a 1-mL syringe, and then, the plants were incubated 20 °C with 16 h light and 8 h dark. For CLSM, agrobacterium OD₆₅₀ was adjusted to 0.1 for LC-RFP or sec-LC, to 0.3 for HC-RFP, sec-HC, ER-HC, vac1-HC or vac2-HC, to 0.1 for GFP-HDEL (Brandizzi et al., 2003) and to 0.1 for P19 (Baulcombe et al., 2002). All infiltrations were performed with ER-GFP, P19 and different combinations of LC and HC: (a) sec-LC-RFP, (b) sec-HC-RFP, (c) sec-LC-RFP + sec-HC-RFP, (d) sec-LC + sec-HC-RFP, (e) sec-LC-RFP + sec-HC, (f) sec-LC-RFP + ER-HC, (g) sec-LC-RFP + vac1-HC, (h) sec-LC-RFP + vac2-HC, (i) sec-LC-RFP + vac1-HC-RFP. As control, infiltrations with agrobacteria carrying only ER-GFP, P19 or empty pGW2 vectors were also performed.

Enzyme-linked immunosorbent assay (ELISA)

To quantitatively evaluate the expression levels of the Ab variants, different sets of N. benthamiana plants were used for each transient expression independent experiment (three biological replicates). For each experiment, at least five plants per construct were used. Leaves were numbered from the top-down starting with the youngest mature leaf (no.1), and leaves no. 3, 4 and 5 were infiltrated. Each construct was infiltrated in leaves located at different positions (leaf 3, 4 or 5). Leaf samples were collected at 5–8 d.p.i and stored at ~80 °C until analysis. Each replicate contained five leaf pieces of the infiltrated tissue from different sets of plants that were grounded to a fine powder and suspended in extraction buffer (20 mM sodium phosphate, 0.5M sodium chloride, pH 7.5) for 15 min at 4 °C. After centrifugation at 10 000 g, total soluble protein concentration in the supernatant was measured by Bradford assay (Bradford, 1976) using bovine serum albumin as standard. Each replicate sample was analysed by sandwich ELISA in triplicate as previously described (Petruccelli et al., 2006). Briefly, plastic wells (Maxisorp, Nunc, Denmark) were coated with 1 μg/mL of goat anti-mouse antibody specific to LC in phosphate-buffered saline (PBS) at 4 °C overnight. Then nonspecific binding sites were subsequently blocked with 3% (w/v) nonfat milk solution in PBS for 1 h at 37 °C. After three washes, plates were incubated with 100 μg soluble protein leaf extract overnight at 4 °C. The plates were washed again and then incubated with biotinylated goat
anti-mouse antibody specific to HC (1 µg/mL) in 1% (w/v) nonfat milk, overnight at 4 °C, followed by incubation with high-sensitivity streptavidin-HRP conjugate for 30 min at 37 °C. Plates were washed five times before incubation with tetramethylbenzidine (TMB) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The optical density was measured at 650 nm. A purified mouse immunoglobulin (Sigma-Aldrich, St Louis, MO) was used as standard for the calibration curve.

To analyse the activity of the antibodies to interact with the antigen, an indirect ELISA was carried out. The mAb14DP is a catalytic antibody that recognized 14D9 enol ether hapten (Reymond et al., 1993). Plastic wells were coated with 14D9 hapten coupled to BSA (1 µg/mL) (provided by Richard Lerner, The Scripps Research Institute, La Jolla CA) by passive adsorption for 16 h at 4 °C. The plates were then blocked with 3% nonfat milk solution for 1 h at 37 °C, and subsequently, total leaf extracts were added to the plates and incubated overnight at 4 °C. Finally, incubations with biotinylated goat anti-mouse antibody, with streptavidin-HRP conjugate and TMB peroxidase substrate were performed as described above.

Total protein extraction and immunoblot analysis

*Nicotiana benthamiana* leaves were collected at 5–8 d.p.i., and total protein were extracted by grinding 16 leaf discs in 160 µL SDS-PAGE sample buffer (72 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, pH 7). The extracts were then boiled for 5 min and centrifuged at 15 000 g for 10 min. After that, approximately 20 µg of total leaf extracts were separated by SDS-PAGE followed by blotting to nitrocellulose membranes (Schleicher & Schuell Bioscience, Inc., Dassel, Germany). The membranes were stained with Ponceau S (Sigma-Aldrich) to ensure equal protein loading prior to immunodetection. Nitrocellulose membranes were first blocked with 5% (w/v) nonfat milk in TBS for 1 h at 37 °C. The membranes were incubated with a biotinylated goat anti-mouse antibody 1 : 3000 (#31802; Thermo Scientific Pierce, Rockford, IL), overnight at 4 °C, and with high-sensitivity streptavidin-HRP conjugate (# 21130; Thermo Scientific Pierce) 1 : 20 000 for 30 min at 37 °C. Finally, chemiluminescence was generated by addition of 1.25 mM luminol (#A8511; Sigma-Aldrich), 200 µM p-coumaric acid (#C9008; Sigma-Aldrich), 0.099% [v/v] H2O2, 0.072% [v/v] DMSO, 100 mM Tris-HCl pH 8.5 substrate, and luminescent signal was captured using X-ray film (Amersham Hyperfilm ECL, GE Healthcare Life Sciences Argentina SA). For RFP detection, a rabbit anti-RFP antibody (#R10367; Thermo Scientific Pierce) and goat anti-rabbit IgG (H+L) secondary antibody biotin conjugate (#R31820; Thermo Scientific Pierce) 1 : 2000 and 1 : 20 000, respectively, were used following the same procedure described above.

Purification of recombinant IgG from agroinfiltrated leaves

Agroinfiltrated *Nicotiana benthamiana* leaves (30 g for sec-Ab, 10 g for ER-Ab and vacs-Abs) were ground with mortar and pestle in liquid nitrogen until a fine powder. Then, the tissue powder was extracted with extraction buffer (1.5 mM NaCl, 45 mM Tris, 1 mM EDTA, 40 mM ascorbic acid, pH 7.5) for 15 min at 4 °C with agitation, using a ratio of 1 mL buffer per 1 g fresh leaf tissue. The leaf extracts were centrifuged three times at 10 000 g for 10 min at 4 °C. The supernatant was incubated with 20 µL of Protein G Sepharose (#17-0618-01GE Healthcare Life Science Argentina S.A.) for 1 h 30 min at 4 °C in gentle agitation.

Subsequently, antibodies bound to Protein G Sepharose were retained in Micro Bio-Spin columns (#732-6204; Bio-Rad, Hercules, CA), the column was washed three times with 800 µL of extraction buffer without ascorbic acid and finally elution was performed by addition of 50 µL of SDS-PAGE sample buffer followed of heating at 95 °C for 5 min. Extracted antibodies were used in N-glycan analysis as described below.

N-glycan analysis

The N-glycosylation profiles of Abs were determined by LC-ESI-MS as previously described by Stadlmann and colleagues (Stadlmann et al., 2008). In brief, purified IgG was separated by reducing SDS-PAGE, and bands corresponding to the HC were excised from the Coomassie-stained gel. Upon S-alkylation and tryptic or tryptic/GluC digestion, fragments were eluted from the gel with 50% acetonitrile and separated on a reversed-phase column (150 × 0.32 mm BioBasic-18, Thermo Scientific) using a gradient of 1–80% acetonitrile. This method generates one glycopeptide EEQFNSTFR [M+H]+: 1157.52 Da. Glycopeptides were analysed with a Q-TOF Ultima Global mass spectrometer (Waters, Milford, MA). Spectra were summed and deconvoluted for the identification of glycoforms. Glycans were annotated according to the proglycan nomenclature (www.proglycan.com).

Isolation of microsomal membrane fraction

Microsomal isolation was carried out mostly as described in Abas and Luschnig (2010). All steps were performed on ice and all centrifugations were performed at 4 °C. Leaves (50 mg for ER-Ab and vac-Abs and 200 mg for sec-Ab) were grounded with an extraction buffer EB (100 mM Tris–HCl, 25% [w/v] sucrose, 5% [v/v] glycerol, 10 mM EDTA, 10 mM EGTA, 5 mM KCl and 1 mM DTT, pH 7.5). About 1.0 µL EB/mg material, minimum volume 100 µL was used. The leaf extracts were centrifuged at 600 g for 3 min, and supernatant was kept. The pellet was re-extracted using half of the original volume and centrifuged at 600 g for 3 min, and the supernatant was collected and added to the first one. The leaf extracts were centrifuged at 600 g for 3 min, and supernatant was kept. The pellet was re-extracted with 1.1X EB and centrifuged at 600 g for 3 min, and the supernatant was collected and added to the first one. The combined supernatants were centrifuged at 600 g for 3 min. The resultant supernatant was kept aside as the cleared homogenate.

The samples were diluted to a final sucrose concentration of 12% w/v, divided into aliquots of 200 µL in 1.5 mL tubes and centrifuged at 21 000 g for 2 h. The supernatant was removed and stored as the soluble fraction (SF), and the microsomal pellet (MP) was washed with 150 µL of wash buffer (20 mM Tris–HCl [pH 7.5], 5 mM EDTA and 5 mM EGTA). Samples were re-centrifuged at 21 000 g for 45 min, and the wash buffer was discarded. The MP was resuspended in 100 µL SDS-PAGE sample buffer and boiled for 5 min.

Confocal laser scanning microscope analysis and image processing

*Nicotiana benthamiana* abaxial epidermal cells were observed at 5 d.p.i with a Leica TSC SP5 Confozl Laser Scanning Microscope (CLSM) (Advanced Microscopy Facility, FCE, UNLP, Argentina), using a 63X (NA 1.4) oil immersion objective. RFP was excited with a HeNe 1.5 mW laser (intensity 54%) at 543 nm and detected in the 570–630 nm range. GFP was excited with an Argon 100 mW laser (intensity 24%) and detected in the spectrum from 490 to 540 nm.
496–532 nm range. Simultaneous observation of RFP and GFP was performed by combining the settings described above in the sequential scanning facility of the microscope, as instructed by the manufacturer. All images were shown acquired using the same photomultiplier gain and offset settings. Postacquisition image processing was performed with ImageJ software (http://rsb.info.nih.gov/ij/).

To study the efficiency of vacuolar targeting, ten overview images with low magnification (30–35 cells per field) were taken per sample and the numbers of cells with vacuolar red fluorescence over the total number of transformed cells were counted. Data represent the average of three independent experiments performed with vac1-Ab-RFP (sec-LC-RFP + vac1-HC) and vac2-Ab-RFP (sec-LC-RFP + vac2-HC). As controls, also vacuolar red fluorescence of leaf infiltrated with sec-Ab-RFP (sec-LC-RFP + sec-HC) and ER-Ab-RFP (sec-LC-RFP + ER-HC) were also counted.

Statistical analysis

All statistical analyses were carried out using Prism 6 (GraphPad Software, GraphPad Inc., La Jolla, CA). One-way ANOVA test and Tukey’s multiple comparisons test were used to determine means with statistical differences. A P-value <0.05 was regarded as statistically significant.

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**Supporting information**

Additional Supporting information may be found online in the supporting information tab for this article:

**Figure S1** Vacuolar sorting efficiency.