Chapter 6

Vacuolar Targeting and Characterization of Recombinant Antibodies

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Abstract

Plant-based platforms are extensively use for the expression of recombinant proteins, including monoclonal antibodies (mAbs). Generally, immunoglobulins (Igs) are sorted to the apoplast, which is often afflicted with intense proteolysis. Here, we describe methods to transiently express mAbs sorted to central vacuole in *Nicotiana benthamiana* leaves and to characterize the obtained IgG. Central vacuole is an appropriate compartment for the efficient production of Abs, consequently vacuolar sorting should be considered as an alternative strategy to obtain high protein yields.

Key words Immunoglobulin, N-glycosylation, Vacuolar sorting signals, Secretory pathway, Vacuolar transport, Molecular farming

1 Introduction

The plant vacuole is a multifunctional and dynamic organelle essential for the regulation and maintenance of plant cell growth and development, which share some of their properties with the lysosomes in animal cells [1]. Vacuoles can be divided into two main groups in terms of its functions: protein storage vacuoles (PSV) and lytic vacuoles (LV) [2]. Protein storage vacuoles (PSV) are special compartments found in seeds and specialized tissues, in which large amounts of foreign proteins can be accumulated for long periods in stable form [3]. In contrast, lytic vacuoles (LVs) are specialized in breaking down cellular material destined for degradation [2]. In vegetative tissues, deposition of recombinant proteins in central vacuoles has not been considered adequate for the hydrolytic characteristic expected for this compartment [4]. Nevertheless, several proteins such as avidin, cellulolytic enzymes, endolysin, human transglutaminase, human collagen, glucocerebrosidase, α1-proteinase inhibitor, complement-5a, interleukin-6, and immunoglobulins stably accumulated at high yields in vacuoles of leaves and/or suspension culture cells [5].

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Proteins destined to vacuoles are introduced into the plant secretory pathway by mean of a signal peptide and then they can follow different trafficking routes. The conventional transport pathway involves endoplasmic reticulum (ER) export via coat protein complex II (COPII) vesicles followed by Golgi and post-Golgi transport [6]. In addition, a direct transport from the ER to the vacuole, independent of COPII vesicles has also been described [7]. These different trafficking routes will have an impact in foreign protein N-glycosylation pattern [8].

Vacuolar transport requires of positive targeting information since the default pathway is the secretion [9]. Different vacuolar sorting signals (VSSs) have been described: sequence specific (ss) signals consist in the NPIXL or NPIR motif and work independent of its position in the protein sequence. On the other hand, hydrophobic C-terminal signals (Ct) do not have e consensus motif but are always located in the carboxyl-terminus of the protein [2, 10]. Stable accumulation of foreign proteins sorted to vacuole has been obtained by means of both types of VSSs [5]. For example, when the heavy chain of mAb14D9 was fused to the Ct VSS (KISIA) and the ssVSS (NIFRGF) accumulations levels of vacuolar antibodies was 10–15-fold higher than secretory's mAb version [8].

Immunoglobulins (Igs) are usually sorted to the apoplast, in which intense proteolysis often occurs [11–13]. Thus, protein accumulation in other cellular organelles, such as vacuoles, is an alternative strategy used to optimize protein yields, though glycosylation pattern will be dependent of the vacuolar trafficking route [8]. Protein N-glycosylation starts in the ER with the transfer of the oligosaccharide precursor Glc3Man9GlcNAc2 to specific asparagine residues of the nascent polypeptide, this process is follow by a limited glycan trimming in both the ER and Golgi. A subsequent addition of monosaccharides occurs as the protein travel through the Golgi apparatus, to form complex N-glycans, usually GlcNAc2 Man3XylFucGlcNAc2 (GnGnXF) structures [14]. Further modifications include the addition of galactose β-1,3 and fucose α-1,4 linked to the terminal GlcNAc forming the called Lewis A (Le^a) epitope for apoplast proteins [15, 16]. In vacuoles, removal of terminal GlcNAc residues from complex N-glycans results in the formation of paucimannosidic Man3XylFucGlcNAc2 (MMXF) structures [17, 18], however, we have shown that vacuolar sorted immunoglobulin carried mainly oligomannosidic (Man 7-9) followed by GnGnXF forms [8]. In contrast, Ig fused to the ssVSS (NPIRL) of sporamin at N terminus synthesized in transgenic tobacco BY2 cells, showed mainly paucimannosidic MMXF as main N-glycan structure [19]. Therefore N-glycosylation of vacuolar antibodies should be study to be sure that is adequate for the intended application.

This chapter describes optimized protocols for transient expression and purification of vacuolar targeting mAbs in *Nicotiana*

benthamiana leaves. IgG localization, quantification and antigen binding determination, integrity and glycan profile analysis methods are described as well.

2 Materials

2.1 Biological Material

1. *Agrobacterium tumefaciens* GV3101 possess integrated to its chromosome a Rifampicin resistance gen and also harbors a deleted version of pTiC58 called pMP90, from which the entire T-DNA region has been deleted and replaced with a gene conferring resistance to Gentamicin (*see* **Note 1**).
2. *Nicotiana benthamiana*: plants grown for 6–8 weeks in a growth chamber at 22 °C in a 16-h-light/8-h-dark cycle.

2.2 Buffers, Solutions and Reagents

All solutions are prepared using *Milli-Q ultrapure water* and analytical grade reagents. Unless indicated otherwise, all reagents are at RT.

1. *Bacteria medium*: Yeast Extract–Beef extract, YEB (1 L): 5 g of beef extract, 1 g of yeast extract, 5 g of tryptone, 5 g sucrose, 2 mL $MgSO_4$ 1 M. Sterilize by autoclave.
2. Kanamycin sulfate: Dissolve 50 mg in 1 mL of sterile H_2O to obtain a 1000× stock solution. Store at −20 °C.
3. Gentamicin: Dissolve 50 mg in 1 mL of sterile H_2O to obtain a 1000× stock solution. Store at −20 °C.
4. Infiltration buffer: 10 mM $MgCl_2$, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 200 μM acetosyringone. Adjust to pH 5.6 with NaOH 1 N.
5. Extraction buffer: 1.5 M NaCl, 1 mM EDTA, 45 mM Tris, 40 mM ascorbic acid, pH 7.4. Prepare a fivefold extraction buffer without ascorbic acid and store at 4 °C. Dilute to 1× and add ascorbic acid prior to use, adjust to pH 7.4 with NaOH (*see* **Note 2**).

2.3 SDS-PAGE

1. Sodium dodecyl sulfate (SDS) solution: SDS prepared a 10% (w/v) stock solution. Stored at room temperature (RT).
2. 4× Stacking Gel Buffer: 0.5 M Tris–HCl (pH 6.8), 0.4% SDS. Store at 4 °C.
3. 4× Separating Gel Buffer: 1.496 M Tris–HCl (pH 8.9), 0.4% SDS. Store at 4 °C.
4. 4× SDS-PAGE Sample Buffer: 1 M Tris–HCl (pH 8), 2% SDS, 50% (v/v) glycerol, 0.15% (w/v) bromophenol blue, 5% β-mercaptoethanol. Prepare the buffer without β-mercaptoethanol and store at 4 °C. Add β-mercaptoethanol prior to use.

5. 5× SDS-PAGE Running Buffer: 125 mM Tris–HCl, 0.95 M Glycine, 1% SDS. Adjust to pH 8.3. Store at 4 °C.
6. Ammonium persulfate: Prepare a 10% (w/v) stock solution. Fractionate 500 μL aliquots and store at −20 °C.
7. Polyacrylamide solution: Prepare a 30% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide solution. Filtrated through 0.45 μm filter. Handle with care, since both components are neurotoxins. Store at 4 °C in a bottle wrapped with aluminum foil (*see* **Note 3**).
8. *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED): Store at 4 °C.
9. Gel Staining Solution: 0.1% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) Methanol and 16% (v/v) glacial acetic acid in H_2O. Filtrate. Store at RT.
10. Destain Solution: 25% (v/v) Ethanol (*see* **Note 4**) and 10% (v/v) glacial acetic acid in H_2O. Store at RT.

2.4 In-Gel Digestion

1. Gel destaining: Prepare a 50% (v/v) Acetonitrile solution.
2. Neat acetonitrile.
3. 50 mM ammonium bicarbonate buffer.
4. 100 mM ammonium bicarbonate buffer.
5. 10 mM dithiothreitol (DTT) solution: Dissolve DTT in 100 mM ammonium bicarbonate buffer.
6. 55 mM iodoacetamide solution: Dissolve iodoacetamide in 100 mM ammonium bicarbonate buffer.
7. Trypsin solution: 1:6 dilution of 100 ng/μL trypsin in 25 mM NH_4HCO_3 solution.
8. 5% (v/v) Formic acid.

2.5 LC-ESI-MS Measurement

1. Solvent A: 65 mM ammonium formate buffer. Adjust to pH 3.0 with 25% NH_4 solution. Degas either by vacuum or by treatment in an ultrasonic bath (at least 10 min).
2. Solvent B: HPLC-grade acetonitrile. Degas in an ultrasonic bath (at least 10 min).

2.6 Western Blot

1. 5× FastBlot Buffer: 125 mM Tris–HCl (pH 8.3), 0.75 M glycine, 20% (v/v) methanol. Prepare the solution without methanol. Store at 4 °C. Dilute to 1× and add the methanol prior to use.
2. 10× Phospate Buffered Saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 10 mM KH_2PO_4. Adjust to pH 7.4 with HCl. Store at 4 °C.

Table 1
Antibodies and dilutions used in our laboratory

Assay	Primary antibody	Secondary antibody
RFP fusion	Rabbit anti-RFP antibody (1/1000)	Biotinylated goat anti-rabbit antibody (1/20,000)
Antibody chains integrity	Biotinylated goat anti-mouse antibody (1/3000)	–

3. Ponceau S Red: 0.2% (w/v) Ponceau S Red, 5% (v/v) acetic acid (*see* **Note 5**) in H_2O.
4. Blocking Buffer: PBS supplemented with 5% (w/v) nonfat dry milk.
5. Antibody Dilution Buffer: PBS supplemented with 1% (w/v) nonfat dry milk.
6. Antibodies: Dilutions used for each assay are given in Table 1.
7. Luminol: 0.2 mg Luminol/5 µL DMSO. Prepare the solution in the moment. Store at −20 °C (*see* **Note 6**).
8. p-Coumaric acid: 0.15 mg p-coumaric acid/10 µL DMSO. Prepare the solution in the moment. Store at −20 °C (*see* **Note 7**).
9. Chemiluminescent solution A: 5 µL Luminol, 2.2 µL p-coumaric acid, 33 µL 5× separation gel buffer, and 470 µL H_2O. Prepare prior to use.
10. Chemiluminescent solution B: 3.3 µL H_2O_2, 33 µL 5× separation gel buffer, and 470 µL H_2O. Prepare prior to use.
11. Antibodies dilutions commonly used are described in Table 1.

2.7 Enzyme-Linked Immunosorbent Assay (ELISA)

1. Blocking Buffer: PBS supplemented with 3% (w/v) nonfat dry milk.
2. Antibody Dilution Buffer: PBS supplemented with 1% (w/v) nonfat dry milk.
3. Antibodies: goat anti-mouse antibody specific to LC and to HC.
4. Tetramethylbenzidine (TMB) peroxidase substrate.

2.8 Microsome Insolation

1. 1.5× High Density Extraction Buffer: 150 mM Tris–HCl, 37.5% (w/w) sucrose, 5.7% (v/v) glycerol, 15 mM EDTA, 15 mM EGTA, 5.7 mM KCl, and 1.5 mM DTT. Adjust to pH 7.5. Store at −20 °C.
2. Wash Buffer: 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, and 5 mM EGTA.

2.9 Special Equipment

1. Greenhouse or plant growth chamber with controlled temperature, photoperiod, and humidity.
2. Incubator (28 or 37 °C) with horizontal shaker.
3. Spectrometer and plate lector.
4. Vacuum concentrator.
5. Reversed-phase LC column: BioBasic C18, 5 μm particle size, 0.32 × 150 mm (ThermoScientific).
6. LC-ESI-MS system: Q-TOF Ultima Global mass spectrometer (Waters Corporation, MA, USA).
7. Centrifuge: Refrigerate Hermle z326k centrifuge with 1.5-mL tube and 15- or 50-mL Falcon tube rotor.

3 Methods

3.1 Agroinfiltration

1. Inoculate YEB medium (*see* **Notes 8** and **9**) with the agrobacterium carrying LC, HC; LC-FP and HC-FP constructs (Fig. 1), ER-GFP and silencer suppressor P19. Add the corresponding antibiotics to the liquid medium. Incubate overnight at 28 °C and 200 rpm.
2. Harvest bacterial cells by centrifugation at 5000 × *g* for 5 min at RT.
3. Carefully discard supernatant and resuspend the pellet in 1–3 mL of infiltration buffer by carefully pipetting up and down.
4. Measure the optical density at 600 nm (OD_{600}).

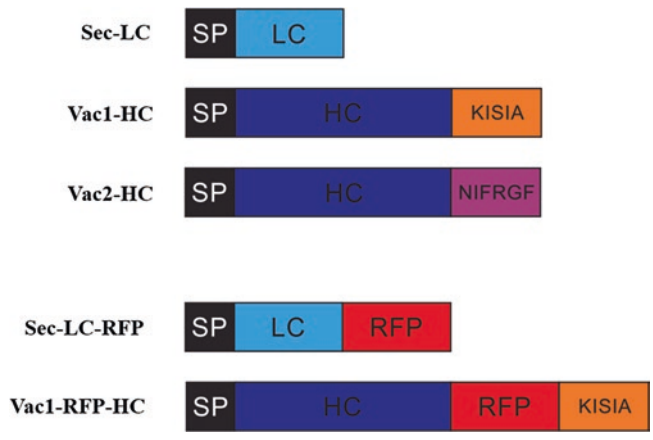

Fig. 1 Schematic representation of the 14D9 antibody constructions used for *Agrobacterium tumefaciens*-mediated transient expression in *Nicotiana benthamiana* leaves. *SP* murine signal peptide, *LC* light chain, *HC* high chain, *RFP* red fluorescent protein, *KISIA* Ct-VSS, *NIFRGF* ss-VSS

Table 2
Amount of agrobacteria used in leaf infiltration

Assay	Construct	OD_{600}
Antibody purification Microsome isolation	Sec-LC	0.6
	Vac1-HC	0.6
	Vac2-HC	0.6
Subcellular localization	Sec-LC-RFP	0.1
	Sec-LC	0.1
	Vac1-HC	0.3
	Vac2-HC	0.3
	Vac1-HC-RFP	0.3

5. Mix the necessary volumes of resuspended agrobacteria to obtain the desired OD_{600} for each construction and fill up with infiltration buffer to the final volume needed. The values used in our laboratory for each assay are given in Table 2.
6. Infiltrate onto the lower epidermal surface of leaves using a 1 mL needleless plastic syringe applying gentle pressure (*see* **Note 10**).
7. Grown plants for 5 days before harvest (*see* **Note 11**).
8. Proceed to section 3.2 for immunoglobulin purification for glycan analysis, to section 3.5 for antibody quantification and hapten recognition assays, and to section 3.4 for confocal microscopy analysis.

3.2 Immunoglobulin Purification

All the steps were performed at 4 °C unless indicated otherwise.

1. If needed, weight the leaf material and frozen at −80 °C or proceed immediately.
2. Ground 10 g of agroinfiltrated leaves until a fine powder with mortar and pestle in liquid N_2.
3. Extract the soluble proteins with extraction buffer for 15 min at 4 °C with agitation in a 1 mL of buffer per 1 g of fresh leaf.
4. Centrifuge the extracts three times at 10,000 × *g* for 10 min at 4 °C (*see* **Note 12**).
5. Incubate the supernatant with 2 μL of Protein G Sepharose with gentle agitation for 1 h 30 min at 4 °C.
6. Pass the extract through a Micro Bio-Spin column (*see* **Note 13**).
7. Wash the column three times with 800 μL of extraction buffer without ascorbic acid or PBS.

8. Elute adding 50 μL of SDS-PAGE sample buffer and heating at 95 °C 5 min (*see* **Note 14**). Centrifuge at 500 × *g* 1 min.
9. Store at −20 °C or use immediately. Proceed to Subheading 3.6.

3.3 Microsome Isolation

All steps were performed at 4 °C unless indicated otherwise.

1. Ground 200 mg (~20 leaves discs) with ice-cold 200 μL of 1.5× high-density extraction buffer (*see* **Note 15**) and a tip of spatula of polyvinylpolypyrrolidone (PVPP).
2. Centrifuge at 600 × *g* for 5 min. Collect the supernatant in a new tube.
3. Reextract the pellet with 100 μL of ice-cold 1.1× high-density extraction buffer.
4. Repeat **step 2**.
5. Reextract the pellet with 67 μL of ice-cold 1× high-density extraction buffer.
6. Repeat **step 2**.
7. Put all the supernatant together and centrifuge at 600 × *g*.
8. Calculate the final sucrose concentration in the homogenate and dilute to a 12% (w/v) sucrose concentration.
9. Fractionate the sample in 200 μL aliquots (*see* **Note 16**) and centrifuge at 21,000 × *g* for 2 h.
10. Keep the supernatant as the soluble fraction.
11. Wash the pellet with 150 μL of wash buffer and centrifuge at 21,000 × *g* for 45 min.
12. Resuspend the pellet in 100 μL of SDS-PAGE sample buffer.
13. Store at −20 °C or use immediately. Proceed to Subheading 3.6.

3.4 Confocal Microscopy

1. Cut out a small square (0.5 × 0.5 cm) from the agroinfiltrated region of the leaf.
2. Place the square in a microscope slip with the lower epidermis facing upward.
3. Mount the leaf square with water or perfluorodecalin (*see* **Note 17**). Put a cover slip on top of the leaf.
4. Follow standard confocal microscopy procedure. The parameters used for our laboratory are described in Table 3.
5. Agroinfiltrated leaves could be store at −80 °C or used immediately. Proceed to Subheading 3.5 for fusion integrity analysis.

3.5 Total Protein Extraction

All steps were performed at 4 °C unless indicate otherwise.

1. Ground leaves in extraction buffer. Extract for 15 min.
2. Centrifuge at 10,000 × *g* for 20 min.

Table 3
Confocal microscopy parameters

Fluorophore	Laser	Laser Intensity	Excitation (nm)	Detection (nm)
RFP	HeNe 1.5 mW	54%	543	570–630
GFP	Argon 100 mW	24%	480	496–532

3. Measure soluble protein concentration in the supernatant using Bradford assay (*see* **Note 18**).
4. Store at −20 °C or use immediately. Proceed to Subheading 3.6 to fusion integrity analysis or to Subheading 3.12 to immunoglobulin quantification and Subheading 3.13 to hapten recognition assay.

3.6 SDS-PAGE

1. Clean the glasses.
2. Assemble the mini-gel system according to your model. Make sure it stands on a flat surface.
3. Prepare the monomer solution for a 12.5% gel by combining the following volumes in a 15 mL Falcon tube: 2.5 mL 5× separating gel buffer; 4.1 mL polyacrylamide solution; 50 µL persulfate solution; 10 µL TEMED; and 3.4 mL H_2O. Mix well.
4. Immediately pour the monomer solution between the glasses (*see* **Note 19**).
5. Prevent exposure to oxygen by adding 70% (v/v) ethanol on top of the monomer mix.
6. Allow gel to polymerization for about 30 min (*see* **Note 20**).
7. Remove ethanol.
8. Prepare the stacking gel solution in a 15 mL Falcon tube: 650 µL 4× stacking buffer; 0.4 mL polyacrylamide solution; 20 µL persulfate solution; 5 µL TEMED; and 1.5 mL H_2O. Mix well.
9. Immediately pour the stacking solution on top of the polymerized running gel.
10. Allow polymerization for about 30 min.
11. After removing the gel sandwich from the casting stand, install the gel according to the instructions, specific to your model. Remove the comb carefully.
12. Fill the lower and upper buffer chambers with 1× running buffer.
13. Load molecular weight markers and your samples into the lanes (*see* **Note 21**).

14. Run the gels using 15 mA constant current per gel in the stacking zone and 25 mA in the running zone. The run is complete when the blue dye front reaches the bottom of the gel.
15. Disassemble the gel sandwich and remove the stacking gel. Remove the top right corner of the running gel for orientation purposes.
16. Proceed to the Subheadings 3.7 or 3.10 to western blot analysis.

3.7 Gel Staining

1. Put the running gel in staining buffer for 1 h (*see* **Note 22**).
2. Detain using detaining buffer for 1 h.
3. Conserve the gel or proceed to the Subheading 3.8.

3.8 In-Gel Digestion

All procedures are carried out at RT unless otherwise specified.

1. Excise band of interest from the gel and cut into small pieces (*see* **Note 23**).
2. Transfer the gel pieces to a tube with H_2O (the pieces of gel in water can be stored at 4 °C for at least 1 week).
3. Throw away the water and add 50 μL of 50% acetonitrile, incubate for 5 min at RT (*see* **Note 24**), and discard liquid.
4. Repeat **step 3** once.
5. Add 50 μL of 100% acetonitrile, shake for a few seconds, gel pieces should get white, and discard liquid.
6. Add 30 μL of 100 mM ammonium bicarbonate buffer and incubate for 5 min.
7. Add 30 μL of 100% acetonitrile and incubate for 15 min.
8. Discard liquid and dry gel pieces in a vacuum concentrator for 15 min.
9. Add 50 μL of 10 mM DTT and incubate at 56 °C for 45 min. Submerge sample complete in buffer.
10. Cool down to RT and discard liquid.
11. Add 50 μL of 55 mM iodoacetamide solution and incubate in dark for 30 min at RT.
12. Discard liquid and dry gel pieces in a vacuum concentrator for 15 min.
13. Repeat **steps 3–8** once.
14. Add 20 μL of Trypsin solution. Incubate overnight at 37 °C. Submerge sample complete in buffer.
15. Add 50 μL of 50 mM ammonium bicarbonate buffer and shake for min on a vertical shaker.
16. Add 50 μL of 100% acetonitrile and shake for another 15 min.
17. Transfer the supernatant (contains the peptides) to a fresh vial.

18. Add 50 μL of 5% formic acid in H2O, shake for 15 min, and transfer the supernatant to this fresh vial.
19. Repeat **step 18** once.
20. Dry the peptides in a vacuum concentrator.
21. Peptides are now ready for analysis or can be stored at −20 °C.
22. Before analysis, redissolve peptides in a volume of H_2O appropriate for at least two injections.

3.9 LC-ESI-MS Measurement

1. Redissolve peptides in 10–40 μL of H_2O.
2. Inject a tryptic digest of bovine serum albumin to verify proper performance of the analytic system.
3. Prior to every queue, calibrate the instrument to ensure best mass accuracy possible.
4. Use 1% solvent B to equilibrate chromatographic system.
5. Inject sample to LC-ESI-MS system.
6. Elute glycopeptide (*see* **Note 25**) using a flow rate of 6 μL/min. After a 5 min hold time, develop a gradient from 1% B to 35% B in 45 min, followed by a 15-min ramp to 80% B.
7. Turn the instrument for the mass region of 800–1600 amu while measuring from 500–900 amu.
8. Perform the analysis with data-dependent acquisition (DDA).
9. Prepare a spreadsheet listing likely glycoforms with different charge states.
10. Identify eluting glycopeptide. In these conditions the sample generates one glycopeptide EEQFNSTFR [M+H]1+: 1157.52 Da.
11. In a not too narrow zone around the glycopeptide signal, sum up the spectra.
12. Deconvolute the obtained spectra to simplify the glycopeptide profile.
13. Assign glycan compositions to the multiple peaks in the deconvoluted profile (*see* **Note 26**).
14. Perform relative quantization by listing heights of the peaks of different glycoforms.

3.10 Gel Transference

1. Run the samples in a SDS-PAGE.
2. Place the running gel in ice-cold 1× FastBlot buffer with methanol. Do not left the gel in buffer more than 5 min.
3. Measure the dimensions of the running gel obtained. Cut four pieces of blotting paper and one piece of nitrocellulose membrane of the same dimensions. Remove the top right corner of the membrane for orientation purposes.

4. Put the nitrocellulose membrane and the blotting papers in ice-cold 1× FastBlot buffer with methanol.
5. Transfer two pieces of blotting paper to the bottom of the plate of the semidry transfer system. With a Pasteur's pipette smooth out any bubbles.
6. Put the nitrocellulose membrane in top of the blotting paper stack.
7. Take out the gel from the FastBlot buffer and lay it on top of the stack. Roll a Pasteur's pipette to smooth out any bubbles as before.
8. Repeat **step 5** placing the blotting papers in top of the gel.
9. Place the lid on the transfer system.
10. Apply a 5 mA/cm^2 for about 1 h (*see* **Note 27**).
11. Remove the lid and gently disassemble the transfer stack.
12. Stain the membrane with Ponceau S for about 1 min. Remove the dye and wash the membrane with water until the protein bands are visible. Gently mark the marker band and the position of the lanes with a pencil (*see* **Note 28**).
13. Proceed to the Subheading 3.11.

3.11 Immunolabeling

1. Place the membrane in blocking buffer for 1 h at 37 °C or overnight at 4 °C (*see* **Note 28**) to block nonspecific binding sites.
2. Discard the blocking buffer and wash the membrane three times with 1× PBS for 10 min.
3. Incubate the membrane with the primary antibody 1 h at 37 °C on a rocking platform or overnight at 4 °C (*see* **Note 29**).
4. Wash the membrane three times with 1× PBS for 10 min.
5. If appropriate, incubate the membrane with the secondary antibody for 1 h at 37 °C on a rocking platform.
6. Repeat **step 4**.
7. Incubate the membrane with streptavidin fused to horseradish peroxidase (HRP) for 45 min at 37 °C on a rocking platform.
8. Repeat **step 4**.
9. In a dark room, place the membrane between two acetate films.
10. Mix 500 μL of solution A and B and wet the membrane with it. Dry the excess of solution.
11. Put an X-ray film on top of the acetate and place the stack between two magnetic sheets. Fold the bottom right of the plate to orientation purpose.

12. After 10–20 min (*see* **Note 30**), transfer the film to developer solution. When the protein bands become visible, wash the film with water.
13. Transfer the film to fixer solution. Wash with water.

3.12 Sandwich ELISA

1. Coat wells with 1 μg/mL of goat anti-mouse antibody specific to LC overnight at 4 °C.
2. Block nonspecific binding sites with blocking buffer for 1 h at 37 °C.
3. Wash three times with 1× PBS 10 min at RT.
4. Incubate the plates with 100 μg of soluble protein leaf extract at 4 °C overnight.
5. Repeat **step 3**.
6. Incubate plates with 1 μg/mL biotinylated goat anti-mouse antibody specific to HC overnight at 4 °C.
7. Repeat **step 3**.
8. Incubate plates with high sensitive HRP conjugated streptavidin for 30 min at 37 °C.
9. Repeat **step 3**.
10. Incubate the plates with TMB peroxidase substrate for 20 min at 37 °C in dark. Measure OD at 650 nm (*see* **Note 31**).

3.13 Indirect ELISA

1. Coat the wells by passive absorption with 1 μg/mL of BSA couple 14D9 enol ether hapten and incubate overnight at 4 °C.
2. Follow **steps 2–10** from Subheading 3.12.

4 Notes

1. This strain develops spontaneous tetracycline resistance and should be used with care. Fast growing colonies after transformation are false. True transformants appear 1–2 days after fast growing colonies.
2. Ascorbic acid is not stable in solution. Do not store 1× extraction buffer.
3. For a 100 mL solution, weight 30 g of acrylamide and 0.2 g of bis-acrylamide, transfer to a 100 mL graduated tube with 40 mL of H_2O and make up to 100 mL.
4. The destain solution could also be prepared with methanol in the same proportion.
5. Do not freeze. Alternative recipe for stain solution 0.2% (w/v) Ponceau in 3% (v/v) acetic acid.

6. This solution should not be stored for more than 2 weeks.
7. This solution should not be stored for more than a month.
8. The volume of the liquid culture depends on the OD and final infiltration volumes needed.
9. Different agrobacterium could grow at different rates. We recommend to plate the bacteria from the cryo-stock onto a plate with the correspondent antibiotics 2 or 3 days before agroinfiltration and use these plates to inoculate the liquid cultures. The plates can be stored at 4 °C for 3, 4 weeks.
10. For microsome isolation, ELISA and subcellular localization experiment we used at least five plants per construct and three biological replicates. Starting with the youngest mature leaf, leaves were numbered from the top-down (Fig. 1) and each construct was infiltrated in leaves located at different positions (leaf 3–5, or counting from the top).
11. Maximal expression levels were obtained between day 5 and 8 post-infiltration. The leaves can be harvested between these days.
12. The extract can be filtrated with a Miracloth filter prior centrifugation.
13. The column can be centrifuged at low revolutions for less than 30 s.
14. If antibody functionality is needed, this elution method should be avoided. Elution can be performed using an elution with a pH 2.4 buffer.
15. A concentrated high-density extraction buffer is used to take into consideration tissue water content.
16. Whole supernatant can be centrifuged, but a minimum amount of extract is used to expose the whole sample to the maximum RFC possible.
17. Perfluorodecalin enhance in vivo microscopy resolution of mesophyll and significantly improves the optical qualities of the leaf compared with water. In addition, mounting the specimen in perfluorodecalin has minimal physiological impact when compared to water.
18. Do not use Bicichoninic Acid protein assay (BCA), since ascorbic acid interfere with the redox reaction of this assay.
19. Nonpolymerized polyacrylamide is a neurotoxin, handle with care.
20. Polymerization time is temperature dependent.
21. When working with total soluble protein extracts, we load the same amount of protein in each lane, normally, 20 μg.
22. Do not leave the gel staining for more than an hour. This is really important for glycan analysis, since particular care needs

to be taken in order to avoid unintended modifications of the analyte.

23. Higher extraction yields are reached if the gel pieces are small, however, too small pieces could cause problems during extraction.
24. The volume used depends on the size of the bands since all pieces need to be covered with liquid. Normally, 50 μL are sufficient.
25. The N-glycan composition of a protein can be analyzed by LC-ESI-MS after glycans release with PNGase A treatment. However, site-specific analysis of glycosylation is performed by mass spectrometric analysis of the generated glycopeptides. This approach has several advantages over analysis of release glycans: (a) It is sure that the detected glycoforms are linked to the protein of interest and do not belong to a highly glycosylated impurity. (b) It can be assumed that the different glycoforms of a peptide have similar detection sensitivity, since ionization efficiency is dominated by the peptide. (c) It can be used to analyze several glycosylation sites in a protein, which is of particular interest in the case of antibodies, since glycosylation of the Fc region has relevant functional implications.
26. The different variants of the glycopeptide elute essentially at the same time.
27. The transfer time depends on the sample.
28. It is no needed to completely destain the membrane; the blocking solution will take a pinkish color, which does not affect the outcome of the experiment.
29. In our experience, better sensibility and less background noise is obtained when incubate overnight at 4 °C, but the other option can also be used.
30. Exposition time depends on protein level obtained.
31. OD can also be measured at 450 nm after stopping the reaction with 200 μL of 0.16 M sulfuric acid.

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References

1. Marty F (1999) Plant vacuoles. Plant Cell 11:587–599
2. Vitale A, Raikhel NV (1999) What do proteins need to reach different vacuoles? Trends Plant Sci 4:149–155
3. Herman EM, Larkins BA (1999) Protein storage bodies and vacuoles. Plant Cell 11:601–613
4. Tamura K, Shimada T, Ono E, Tanaka Y, Nagatani A, Higashi S-i, Watanabe M, Nishimura M, Hara-Nishimura I (2003) Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. Plant J 35:545–555
5. Marin Viegas VS, Ocampo CG, Petruccelli S (2017) Vacuolar deposition of recombinant proteins in plant vegetative organs as a strategy to increase yields. Bioengineered 8:203–211
6. Xiang L, Etxeberria E, Van Den Ende W (2013) Vacuolar protein sorting mechanisms in plants. FEBS J 280:979–993
7. De Marchis F, Bellucci M, Pompa A (2013) Traffic of human alpha-mannosidase in plant cells suggests the presence of a new endoplasmic reticulum-to-vacuole pathway without involving the Golgi complex. Plant Physiol 161:1769–1782
8. Ocampo CG, Lareu F, Marin Viegas VS, Mangano S, Loos A, Steinkellner H, Petruccelli S (2016) Vacuolar targeting of recombinant antibodies in Nicotiana benthamiana. Plant Biotechnol J 14:2265–2275. https://doi.org/10.1111/pbi.12580
9. Denecke J, Botterman J, Deblaere R (1990) Protein secretion in plant cells can occur via a default pathway. Plant Cell 2:51–59
10. Matsuoka K, Neuhaus JM (1999) Cis-elements of protein transport to the plant vacuoles. J Exp Bot 50:165–174
11. Goulet C, Khalf M, Sainsbury F, D'Aoust MA, Michaud D (2012) A protease activity-depleted environment for heterologous proteins migrating towards the leaf cell apoplast. Plant Biotechnol J 10:83–94
12. Benchabane M, Goulet C, Rivard D, Faye L, Gomord V, Michaud D (2008) Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol J 6:633–648
13. Niemer M, Mehofer U, Torres Acosta JA, Verdianz M, Henkel T, Loos A, Strasser R, Maresch D, Rademacher T, Steinkellner H et al (2014) The human anti-HIV antibodies 2F5, 2G12, and PG9 differ in their susceptibility to proteolytic degradation: down-regulation of endogenous serine and cysteine proteinase activities could improve antibody production in plant-based expression platforms. Biotechnol J 9:493–500
14. Castilho A, Steinkellner H (2012) Glyco-engineering in plants to produce human-like N-glycan structures. Biotechnol J 7:1088–1098
15. Fitchette AC, Cabanes-Macheteau M, Marvin L, Martin B, Satiat-Jeunemaitre B, Gomord V, Crooks K, Lerouge P, Faye L, Hawes C (1999) Biosynthesis and immunolocalization of Lewis a-containing N-glycans in the plant cell. Plant Physiol 121:333–344
16. Strasser R (2016) Plant protein glycosylation. Glycobiology 26:926–939
17. Gomord V, Fitchette A, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 8:564–587
18. Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Lainé AC, Gomord V, Faye L (1998) N-glycoprotein biosynthesis in plants: recent developments and future trends. Plant Mol Biol 38:31–48
19. Misaki R, Sakai Y, Omasa T, Fujiyama K, Seki T (2011) N-terminal vacuolar sorting signal at the mouse antibody alters the N-linked glycosylation pattern in suspension-cultured tobacco BY2 cells. J Biosci Bioeng 112(5):476–484