Chapter 13

Isolation and Glycomic Analysis of *Trans*-Golgi Network Vesicles in Plants

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Abstract

The dynamic endomembrane system facilitates sorting and transport of diverse cargo. Therefore, it is crucial for plant growth and development. Vesicle proteomic studies have made substantial progress in recent years. In contrast, much less is known about the identity of vesicle compartments that mediate the transport of polysaccharides to and from the plasma membrane and the types of sugars they selectively transport. In this chapter, we provide a detailed description of the protocol used for the elucidation of the SYP61 vesicle population glycome. Our methodology can be easily adapted to perform glycomic studies of a broad variety of plant cell vesicle populations defined via subcellular markers or different treatments.

Key words Glycomics, Endomembrane trafficking, Immunoisolation, Vesicle isolation, SYP61, *Trans*-Golgi Network, Polysaccharides

1 Introduction

The plant endomembrane system is a complex and dynamic network of membranous compartments playing crucial roles in plant growth, development, and adaptation to the environment. It facilitates the transport of proteins and other cargoes and is pivotal for cell wall biosynthesis and assembly [1–5].

The cell wall is a complex structure made of polysaccharides, structural proteins and other molecules that surrounds and protects plant cells and is essential for their development. While many enzymes responsible for polysaccharide biosynthesis have been identified, our understanding of how polysaccharides are transported and assembled is still limited. Polysaccharides originate at distinct cellular locations; cellulose and callose are synthesized at the plasma membrane, whereas the synthesis of hemicellulose and pectin and the glycosylation of proteins take place in the Golgi

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apparatus and the *trans*-Golgi Network [6, 7]. Cell wall polysaccharides not synthesized *in muro*, associated enzymes and glycoproteins are carried to their specific cell wall deposition sites by vesicle transport pathways that remain elusive [1, 8–11]. The highly dynamic nature of the endomembrane system makes it challenging to assign unequivocal roles to specific vesicle populations in the transport of cell wall material and assembly of the cell wall.

The plant *trans*-Golgi Network/Early Endosome (TGN/EE) is a distinct compartment on the Golgi *trans*-side comprising diverse vesicle populations and serving as a hub of secretory and endocytic traffic [12–16]. The plant TGN/EE is unique in that it orchestrates the trafficking of cell wall polysaccharides from the Golgi, the point of their synthesis, to the plasma membrane, for cell wall deposition, assembly, and modification (Fig. 1a) [1, 11, 17].

Because polysaccharide composition determines the biological function of plant cell walls, the dynamics of polysaccharide traffic is a decisive factor in mechanisms that control cell wall deposition and assembly. Thus, the development of approaches to illuminate the glycome of plant vesicles is of great significance. Protocols for the isolation of specific plant vesicle populations have recently become available [18–20]. They are allowing us to identify the protein cargo of vesicles and to shed light into their biochemical properties, such as membrane lipid composition [13], for a better understanding of vesicle heterogeneity and vesicle functional compartmentalization.

Recent technical advances have made it possible to start piecing polysaccharides biosynthesis, assembly, and modification together. These include the application of chemical methods for compositional analysis of the plant cell wall [21] and the use of oligosaccharide mass profiling (OLIMP) to retrieve compositional data from preparations of Golgi-enriched fractions or isolated cell walls [22–24]. Additionally, the labeling and imaging of sugars modified via click chemistry [25–29] can provide kinetic details of cell wall formation. Cell wall glycan-directed antibodies are an elegant option for the identification of plant cell carbohydrates in diverse tissues and species [30–34]. During glycome profiling, antibody libraries are paired with an automated large scale enzyme-linked immunosorbent assay (ELISA), enabling the fingerprinting of plant cell wall glycan content with both high sensitivity and specificity [31].

To interrogate the glycome of intracellular vesicles, we optimized our vesicle isolation protocols for this specific application. In previous studies, we established a methodology for the isolation of the syntaxin of plants 61 (SYP61) TGN/EE vesicle subpopulation to high purity levels, suitable for subsequent proteomic studies [18, 19]. The SYP61 vesicle population has been implied in post-Golgi trafficking of the wall biosynthetic machinery, a notion supported by the analysis of the SYP61 proteome, which revealed

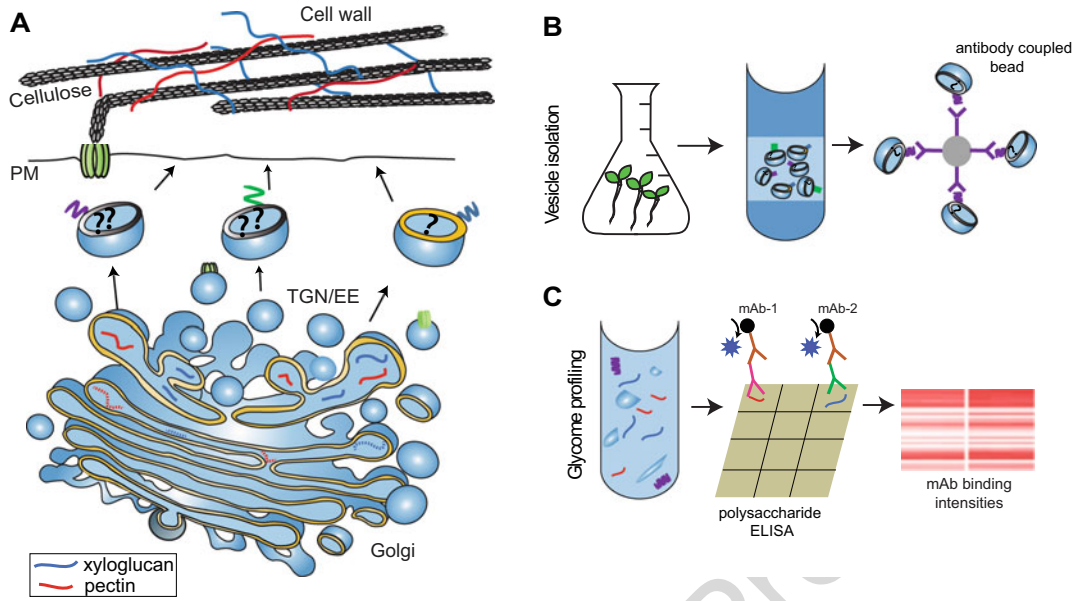

Fig. 1 Structural polysaccharide transport and deposition, and a hybrid methodology for vesicle glycomic analysis. (**a**) Schematic representation of structural polysaccharide synthesis, transport and deposition. The structural polysaccharides xyloglucan and pectin are synthesized in the Golgi and transported via *trans*-Golgi Network/Early Endosome (TGN/EE) vesicles to the apoplast. The type of vesicles carrying specific polysaccharide cargo to the cell wall is unknown. Three different vesicle subpopulations, indicated with magenta, green and blue vesicle protein markers are depicted to illustrate the heterogeneity of the TGN/EE. (**b**) Schematic representation of vesicle isolation. Plant extracts derived from liquid-grown plantlets are sucrose fractionated. A heterogeneous TGN/EE vesicle population is isolated from the Golgi/TGN/EE enriched sucrose fractions after which the specific vesicle subpopulation of interest (magenta surface protein marker) is purified with the aid of an antibody against the target protein. (**c**) Vesicle cargo release and glycome analysis. Vesicle cargo is released by sonication for glycome analysis. An ELISA-based method of glycome detection is used and the resulting data are summarized in a heat map for analysis. mAb, monoclonal antibody; PM, plasma membrane

several cellulose synthase subunits and cell wall modifying enzymes as cargo of the SYP61 vesicles [19, 35]. Such findings prompted the question whether not only cell wall biosynthetic and modifying enzymes but also cell wall structural polysaccharides are transported in this specific TGN/EE vesicle compartment. Such information could help mapping the intracellular transport of polysaccharides.

Toward answering this critical question, we designed an experimental approach combining an optimized protocol for SYP61 TGN/EE vesicle isolation with the large-scale profiling of TGN/EE vesicle through a polysaccharide carbohydrate antibody arraying technique investigating 155 carbohydrate epitopes [36]. The implementation of this hybrid approach revealed trafficking and sorting of diverse glycans of pectins, xyloglucans (XyGs), and structural cell wall glycoproteins through the SYP61 TGN/EE compartment in Arabidopsis [36]. Since TGN is a major intersection in post-Golgi trafficking, its comparison with the Golgi or

sub-Golgi glycome(s) [37, 38] can offer major insights into polysaccharide biosynthesis and transport within the secretory pathway.

This chapter describes in detail our (1) separation of vesicles by immunopurification, optimized for CFP-SYP61, combined with (2) large-scale automated carbohydrate antibody arraying methodology using an ELISA (Fig. 1b, c). Adopting this approach, different vesicle populations can be characterized, complementing our proteomic perspective of cellular pathways with glycomics.

2 Materials

2.1 Plant Material

1. 50–100 transgenic Arabidopsis seeds for each flask, expressing CFP-SYP61 [19] (*see* **Notes 1** and **2**).
2. Seed sterilization solution: 75% ethanol, 0.1% Triton X-100, 24.9% autoclaved deionized water.
3. Liquid Murashige and Skoog (MS) medium: Full strength 1× MS medium, 1% (w/v) sucrose. Dissolve 4.26 g of MS minimal media and 10 g of sucrose in 1000 mL deionized water. Aliquot 200 mL of media into a 500 mL Erlenmeyer flask and autoclave (*see* **Notes 3** and **4**).
4. Flask shaker placed in a temperature- and photoperiod-controlled environment (long day light cycle, 16 h of light at 22–24 °C) (*see* **Note 5**).

2.2 Vesicle Fractionation Components

1. Vesicle immunoprecipitation extraction buffer (VIB): 50 mM HEPES, pH 7.5, 0.45 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, 0.5% PVP (w/v), protease inhibitors (cOmplete™ Protease Inhibitor Cocktail, ROCHE).
2. Sucrose gradient solutions: 38% (w/v) sucrose (1.1 M), 33% (w/v) sucrose (0.96 M), and 8% (w/v) sucrose (0.23 M) in 50 mM HEPES pH 7.5 (*see* **Note 6**).
3. Mortar and pestle.
4. Razor blades.
5. Miracloth.
6. Small funnel.
7. Refrigerated benchtop centrifuge.
8. 50 mL conical centrifuge tubes.
9. Ultracentrifuge (e.g., Optima L-90 K Beckman Coulter, or equivalent) with rotors SW28 and 70Ti.
10. Centrifuge tubes for SW28 rotor (thickwall, polyallomer, 32 mL tubes).
11. Centrifuge tubes for 70Ti (polycarbonate aluminum bottle with cap assembly).

12. Disposable serological 5, 10, and 25 mL pipettes.
13. Pasteur pipettes.

2.3 Vesicle Immunoisolation Components

1. Resuspension buffer: 50 mM HEPES, pH 7.5, 0.25 M sucrose, 1.5 mM $MgCl_2$, 150 mM NaCl, protease inhibitors (*see* **Note 7**).
2. Wash buffer: 50 mM HEPES, pH 7.5, 0.25 M sucrose, 1.5 mM $MgCl_2$, 150 mM NaCl.
3. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4{\cdot}H_2O$, KH_2PO_4, pH 7.4.
4. Protein-A agarose beads.
5. Anti-GFP rabbit IgG (2 mg mL^{-1}) (e.g., Invitrogen A11122, anti-GFP IgG) (*see* **Note 8**). Alternatively, magnetic agarose beads covalently coupled to GFP antibodies (e.g., GFP-Trap®_MA ChromoTek) can be used for immunoisolation with non-coupled beads as controls.
6. Rabbit IgG (*see* **Note 9**).
7. Tube rotator.
8. Refrigerated benchtop centrifuge.

2.4 Material for Enzyme-Linked Immunosorbent Assay

1. Enzyme-linked immunosorbent assay (ELISA) acid-resistant 384 well flat bottom plates (e.g., Costar 3700 from Corning Life Sciences).
2. Automated platform mover (e.g., Orbitor RS Microplate Mover ORB2006 from Thermo Scientific).
3. Microplate sample processor (e.g., BioTek™ Precision™ XS from Biotek).
4. Washer Dispensers (e.g., MicroFlo™ and EL406™ Washer Dispensers from BioTek).
5. 0.1 M Tris-buffered saline (TBS), pH 7.6: 23.38 g of sodium chloride, 1.11 g of Tris-Base, 4.85 g of Tris–HCl, in 4 L of ultrapure water. Store at room temperature.
6. Blocking Buffer: 1.0% (w/v) milk in 0.1 M TBS, pH 7.6: 10 g of nonfat dry milk in 1 L of 0.1 M TBS. Store at 4 °C.
7. Wash Buffer: 0.1% (w/v) milk in 0.1 M TBS pH 7.6.
8. Primary antibodies: CCRC series of antibodies generated in mouse; JIM, MAC, and LM series of antibodies generated in rat. A web-accessible database listing most of the available plant cell wall glycan-directed mAbs and providing information about their characteristics and suppliers can be found at WallMabDB (http://www.wallmabdb.net). The three main suppliers of plant glycan-directed antibodies are CarboSource (http://www.carbosource.net), PlantProbes (http://www.plantprobes.net), and BioSupplies (http://www.biosupplies.com.au/).

9. Secondary antibody: anti-mouse or anti-rat IgG whole molecule goat antibody, conjugated with horseradish peroxidase diluted in wash buffer according to the manufacturer's instructions. Secondary antibody stocks are stored at −20 °C when not in use.
10. Substrate: TMB KPL 2-Component Microwell Peroxidase Substrate Kit (SeraCare Life Science Inc.).
11. Stop solution: 0.5 N sulfuric acid: 100 mL of 0.5 N sulfuric acid, 1 mL of 18 M sulfuric acid, 71 mL of deionized water.
12. Microplate spectrophotometer reader.
13. Probe sonicator, Branson 250–450 Sonifier or equivalent.
14. R-Console software.

3 Methods

3.1 Plant Preparation

1. Two weeks before vesicle isolation, sterilize seeds and stratify them at 4 °C overnight.
2. Grow 50–100 seeds in 200 mL liquid MS in each 500 mL Erlenmeyer flask while shaking at 150 rpm under a long day cycle at 22–24 °C for ~10 days. For the analysis of TGN compartments, more than 12 g of tissue are required. Plants should be grown ~10 days in liquid media to yield sufficient root tissue.

3.2 Golgi/TGN Fractionation by Sucrose Density Gradient Ultracentrifugation

Plant extracts from liquid grown plantlets are fractionated using discontinuous sucrose gradient centrifugation to enrich for SYP61 vesicles. During vesicle isolation, maintain all buffers and rotors at 4 ° C. All steps after harvesting tissues should be performed on ice. Figure 2 illustrates the sucrose fractionation procedure.

1. Rinse plants carefully in deionized water and pat-dry with paper towels in a large petri dish. Weigh plants.
2. Slice plants with a razor blade in the petri dish set on ice. Transfer the finely sliced tissues into a cold mortar on ice.
3. Add ice-cold VIB to a final v/w ratio of 2:1 (e.g., 2 mL of VIB buffer for 1 g of plant tissue) and grind the plant tissue as gently as possible to a rough pulp (*see* **Note 10**). Place funnel with Miracloth over a 50 mL conical centrifuge tube to filter the plant extract and centrifuge at 1000 × *g* at 4 °C for 20 min.
4. Meanwhile, using a 10 mL pipette, add 8 mL of 38% sucrose to a thickwall 32 mL centrifuge tube. Load gently the supernatant from **step 3** (S1 fraction) on top of the sucrose cushion (*see*

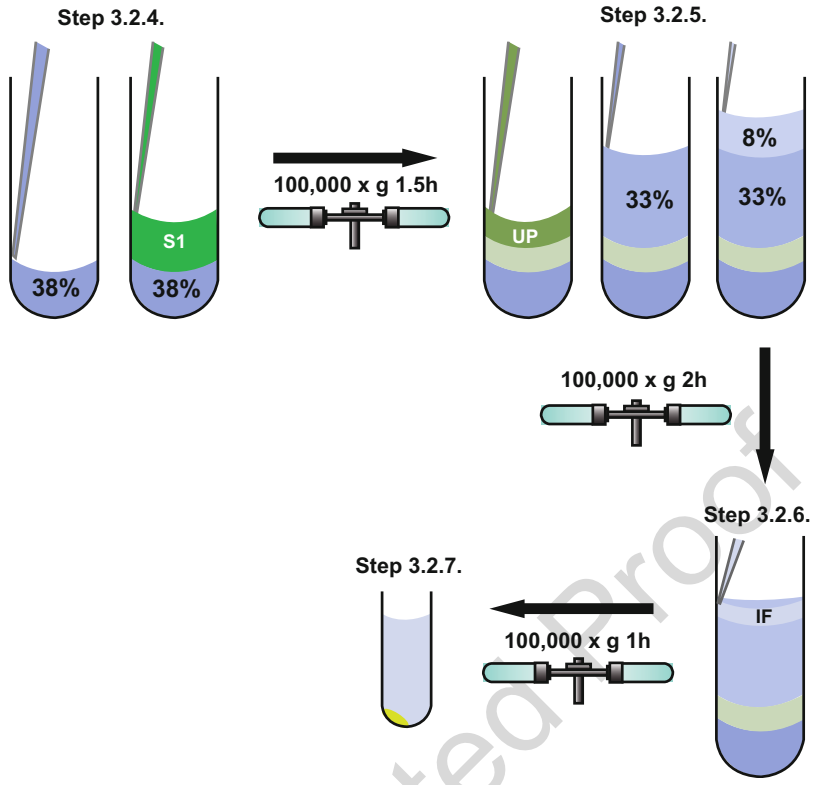

Fig. 2 Enrichment of Golgi/*trans*-Golgi Network compartments by sucrose gradient ultracentrifugation. Schematic illustration of the sequential sucrose gradient ultracentrifugation to isolate Golgi/TGN compartments. Briefly, the Supernatant (S1) fraction of plant homogenates is loaded onto a 38% sucrose cushion and centrifuged at 100,000 × g for 1.5 h (Subheading 3.2, **step 4**). The upper phase is removed and a discontinuous gradient is formed by adding the two 33 and 8% sucrose layers (Subheading 3.2, **step 5**). The interface between 8% and 33% sucrose fractions is collected (Subheading 3.2, **step 6**) and transferred onto a 30 mL centrifuge tube for centrifugation at 100,000 × *g*, 1 h (Subheading 3.2, **step 7**). The resulting pellet is kept for vesicle immunoisolation. UP, upper phase; IF, interface. Note: Sucrose gradients can be adjusted for the isolation of different vesicle populations

Note 11). Centrifuge at 100,000 × *g* at 4 °C for 1.5 h using a SW28 rotor or equivalent.

5. Place the tube on ice, and remove the plant extract liquid above the green interface band, without disturbing it. Using a 25 mL pipette, carefully add 15 mL of 33% sucrose on top of the collected green band, and then add 5 mL of 8% sucrose. Centrifuge at 100,000 × *g* at 4 °C in SW28 rotor or equivalent for 2 h (*see* **Note 12**).
6. Using a 5 mL pipette, slowly remove and discard 4–5 mL of the top gradient layer (8% sucrose). Using a Pasteur pipette, collect the interface band between the 8% and 33% sucrose layers into an ice-chilled 30 mL centrifuge tube and add 0.5× the volume of 50 mM HEPES (pH 7.5).

7. Centrifuge at 100,000 × *g* at 4 °C using a fixed angle 70Ti rotor or equivalent for 1 h. Decant the supernatant and keep the pellet at 4 °C overnight (*see* **Note 13**).

3.3 Immunoisolation of SYP61 Vesicles

SYP61 vesicles are isolated by immunopurification from the fraction obtained in the previous section.

1. Couple the GFP antibody to protein-A agarose beads. First, mix the protein-A agarose beads and place 25 μL into a 1.5 mL microfuge tube. Add 500 μL of ice-cold PBS, mix well by inverting the tube, and centrifuge at 10,000 × *g* for 30 s (*see* **Note 14**). Alternatively, use magnetic agarose beads directly coupled with GFP antibodies, for example, GFP-Trap®_MA ChromoTek. In this case, follow the protocol from Subheading 3.4, **step 1**.
2. Discard supernatant using a pipette and add 2 μL of the anti-GFP antibody to the pellet. Add cold PBS containing protease inhibitors to a final volume of 100 μL, mix well by inverting the tube and incubate on a rotator for 2 h. Centrifuge at 10,000 × *g* for 30 s and discard the supernatant.
3. Equilibrate the antibody coupled-agarose beads with 200 μL resuspension buffer on a rotator at 4 °C for 20 min. Centrifuge at 1000 × *g* for 30 s. Carefully discard the supernatant.
4. Meanwhile, resuspend the vesicle pellets from Subheading 3.2, **step 7** in 400 μL of resuspension buffer and incubate with 25 μL of protein-A agarose beads (*see* **Note 15**). Gently mix the suspension for 20 min using a rotator at 4 °C and then centrifuge at 1000 × *g* for 30 s. Collect the supernatant.
5. Add 300 μL of the supernatant collected in **step 4** to the antibody coupled agarose beads collected in **step 3** and mix for 1 h on a rotator at 4 °C. Centrifuge at 100 × *g* for 1 min (*see* **Note 16**). Discard supernatant.
6. Wash the pellet with 1 mL of wash buffer under gentle agitation at 4 °C for 2 min and centrifuge at 100 × *g* for 1 min. Repeat this step three times (*see* **Notes 17** and **18**). Keep the pellet.

3.4 Proceed to the Following Steps if You Are Using Beads Covalently Bound to the Antibody

1. Vortex GFP-Trap®_MA beads and pipette 25 μL bead slurry into 500 μL resuspension buffer. Magnetically separate beads until supernatant is clear. Carefully discard the supernatant and repeat the bead wash/equilibration step twice.
2. Add 300 μL of the supernatant collected in subheading 3.3, **step 4** to the equilibrated GFP-Trap®_MA beads collected in subheading 3.4, **step 1** and mix for 1 h on a rotator at 4 °C.

3. Magnetically separate beads until supernatant is clear. If desired, save 50 μL supernatant for immunoblot analysis. Discard remaining supernatant.
4. Wash the beads with 1 mL of wash buffer under gentle agitation at 4 °C for 2 min and magnetically separate until supernatant is clear. Discard supernatant. Repeat this step three times (*see* **Notes 17** and **18**). Keep the pellet.

3.5 Glycome Analysis

1. Dilute isolated vesicles to a final volume of 8 mL with double distilled water and sonicate on ice using a micro-tip with attachment, with output setting of 21.5 microns amplitude. Pulse-sonicate using 1s cycles followed by subsequent 1 s rest for a period of one min.
2. Repeat sonication three times to ensure complete disruption of vesicles.
3. Centrifuge for 15 min at 2200 × *g* at 4 °C. Collect the supernatant.
4. Coating of the ELISA plates: 15 μL per well of collected supernatant (after sonication and centrifugation) of each polysaccharide extract dilutions are added to the 384-well ELISA plates (with the number of coated wells equaling the number of mAbs to be tested plus controls) using a microplate sample processor. Evaporate to dryness overnight in a ventilated 37 °C incubator. Handling of the acid-resistant flat-bottom 384-well plate and incubation times are performed by the automated platform microplate mover.
5. Blocking: Nonspecific sites in the coated ELISA plates are blocked by adding 15 μL of blocking buffer per well, with the aid of a washer dispenser, followed by incubation for 1 h at room temperature.
6. Addition of primary antibodies (mAbs): The blocking buffer is aspirated and 15 μL of primary mAb are dispensed into each well using different washer dispensers (e.g., the MicroFlo™ Washer Dispenser for CCRC anti-mouse series and EL406™ for JIM and MAC for anti-rat series). Incubate plates with the primary antibodies for 1 h at room temperature.
7. Washing the plates: Aspirate the primary antibodies using MicroFlo™ and EL406™ washer dispensers. Wash each well with 20 μL wash buffer. Completely aspirate the buffer after 5 s. Repeat washes three times.
8. Secondary antibodies: After washing, add 15 μL of secondary antibody per well. Dispense anti-mouse or anti-rat secondary antibodies (mixed at a 1:5000 dilution in wash buffer) using the MicroFlo and EL406 washer dispensers into the respective mouse (e.g., CCRC series, using the MicroFlo™ washer

dispenser) and rat (e.g., JIM series, using the EL406™ washer dispenser) primary antibody-bound wells and incubate at room temperature for 1 h.

9. Washing secondary antibodies: Aspirate the secondary antibodies from each well after the incubation. Wash each plate well with 20 μL of wash buffer for 5 s and repeat four more times for a total of five washes using the washer dispensers.
10. Adding substrate and termination: Mix KPL TMB solution A with KPL TMB solution B in a 1:1 ratio (for 500 mL, mix 250 mL of Solution A with 250 mL of Solution B onto a separate container) without the stabilizer included in the kit. Dispense 10 μL of the prepared KPL TMB mix into each well. Allow each plate to incubate for precisely 30 min and stop the reaction with 10 μL of 0.5 N sulfuric acid per well using a microplate sample processor (*see* **Note 19**).
11. Quantitation: Immediately after termination, measure the net OD values of the color formation in the wells of the ELISA plates using a microplate spectrophotometer reader at 450 nm and subtract a background reading at 655 nm. Assemble the ELISA results into a heatmap using a modified version of the R-Console software [39] (Fig. 3) (*see* **Note 20**).

4 Notes

1. This protocol uses SYP61-CFP expressing plants for vesicle isolation. The CFP N-terminally fused to SYP61 interacts with the antibody during isolation, while the SYP61 C-terminus facilitates the attachment to the vesicle membranes. Special consideration should be given to the "bait" protein that will be used for isolation, in particular the accessibility to the antibody.
2. It is important to start with sufficient plant material. Only a selected fraction from the sucrose gradient will be used for vesicle isolation. Note that more than 12 g of plant tissues are required.
3. Growth in liquid media yields more root tissues. However, when the target protein is highly abundant in specific tissues or developmental stages, collect the appropriate tissues to obtain a higher yield.
4. The MS media used in this protocol are pH-adjusted. When other MS media are used, adjust the pH to 5.8–6.0.
5. Adjust growth conditions favoring the tissue expressing the bait protein.

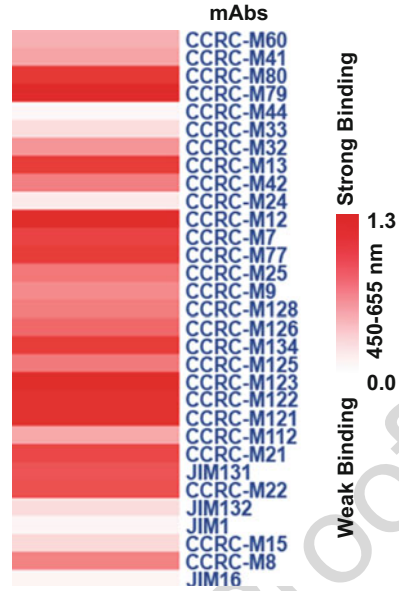

Fig. 3 Example of a vesicle glycome profiling showing diverse epitopes of pectic glycans in isolated SYP61 vesicles. The mean of three biological replicates is shown, after subtraction of negative controls. White to red scales indicates signal intensity in the ELISA assays, with white corresponding to no binding and intense red to strong binding

6. The sucrose gradient procedure is adapted from Drakakaki et al. (*see* Ref. 40). If necessary, the sucrose gradient can be adjusted with more layers or different sucrose densities to enrich for the specific target vesicles.
7. The composition of the resuspension buffer can be modified to enable the best binding of the bait protein.
8. Due to its ready availability and reactivity with CFP, the GFP antibody is used.
9. Rabbit IgG is used as a control, since the GFP antibody is raised in rabbit. Similarly, the control must be chosen according to the antibody host.
10. In this step, it is very important to keep the plant material cold, but not frozen. Ground tissue should be maintained as slurry.
11. A sharp interface between the sucrose cushion and supernatant should be visible. Carefully set up the sucrose layers and place the centrifugation tubes on the rotor without disturbing the sharp interface.
12. The extent of centrifugation might depend on the model of the centrifuge used. If the interfaces between the sucrose layers are not sharp, increase the centrifugation time.

13. Keeping the pellet overnight at 4 °C is fine. If time permits, the next steps can be performed immediately.
14. Instead of agarose beads, other types, such as magnetic beads, can be used.
15. This step is necessary to minimize nonspecific binding to the protein-A agarose beads.
16. After centrifugation, the supernatant can be stored and used to determine the vesicle purification efficiency by Western blot analysis.
17. Before the last washing step, keep a small fraction of ~10% of the suspension to test the isolation efficiency. Centrifuge at 100 × *g* for 1 min. Resuspend in 50 μL PBS and mix with appropriate SDS protein loading buffer to prepare the sample for SDS-PAGE and Western blot analysis.
18. In addition to the sample collected in [17], we recommend that samples from the original enriched vesicle fraction (Subheading 3.3, **step 4**), the flow through (Subheading 3.3, **step 5**) and the immunoisolated fraction (Subheading 3.3, **step 6**) are analyzed by Western blot. The presence of CFP-SYP61 in those samples can be evaluated using a monoclonal antibody against GFP. In addition, antibodies against subcellular markers for the endoplasmic reticulum (ER) marker, BiP [41] and the pre vacuolar compartment (PVC) marker, SYP21 [42], can be used to test the purity of the isolated SYP61 vesicles. The physical integrity of the isolated vesicles can be assessed by transmission electron microscopy [19].
19. The reproducibility and robustness of the data are superior when the ELISAs are performed with an automated platform, which minimizes human errors, particularly during the color development step.
20. Background noise from control data generated using no antigen (water) are subtracted from the mean values obtained, making the data more error resilient and statistically significant.

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