Methods for detection of protein interactions with plasmodesmata-localised reticulons

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Abstract

Plant reticulon family proteins (RTN) tubulate the ER by dimerization and oligomerisation, creating localized ER membrane tensions that result in membrane curvature. Two RTN ER-shaping proteins have been found in the plasmodesmata (PD) proteome which could potentially contribute to the formation of the desmotubule, an ER-derived structure that crosses primary PD and physically connects the ER of two cells. Here we describe two methods used to identify partners of two PD-resident reticulon proteins, RTN3 and RTN6 that are located in primary PD at cytokinesis in tobacco (*Nicotiana tabacum*): immunoprecipitations using GFP-Trap®_A beads to find novel interaction partners and FRET-FLIM to test for and quantify direct protein-protein interactions *in planta*.

Key words: plasmodesmata, reticulon proteins, endoplasmic reticulum, protein-protein interaction, co-immunoprecipitation, GFP-trap, FRET-FLIM.

1. Introduction

The cortical ER network of plants is involved in a plethora of functions and pathways including protein trafficking [1, 2] and pathogen responses [for review see 3, 4]. In plants, the protein family of reticulon proteins (RTN) contributes significantly to tubulation of the ER [5, 6, 7]. RTN proteins are integral ER membrane proteins found ubiquitously in eukaryotes and have been linked to a number of biological functions including neurodegenerative diseases [8, 9, 10] in animals. RTNs feature a reticulon homology domain that comprises two hydrophobic regions flanking a hydrophilic loop [11]. In Arabidopsis, 21 reticulons have been identified so far and over-expression of some of these induces a constriction phenotype to ER tubules and are capable of converting cisternae into tubules [5, 6, 7, 12]. Reticulons dimerise or oligomerise and so create ER membrane tension inducing membrane curvature [13]. This topology has been shown for the Arabidopsis reticulons 1, 2, 3, 4 and 13 [6, 13]. Arabidopsis coding for 21 reticulons in comparison to 4 mammalian ones, raises question about the potential of different functionality. A subgroup of RTN's, RTN17-21, contain terminal extensions that might allow for specialised functions within the RTN family. RTN19 and 20 contain some N-terminal extension homologues to 3β-hydroxysteroid dehydrogenase and have a role in sterol regulation in Arabidopsis roots via a novel mechanism [14]. Both RTN19 and 20 are not able to constrict the ER [14]. RTN1 and RTN2 have recently been proposed to act as receptors for autophagy-mediated ER turnover [15]. The ability of RTN proteins to constrict membranes is of interest for cell plate development and the formation of primary PD [16], the formation of which requires extensive remodeling of the cortical ER into tightly tubules to form the desmotubule [17, 18]. The desmotubule is only 15 nm in diameter

and hence one of the most constricted membrane structures found in nature [19]. How the ER is furled into desmotubules is poorly understood. RTN3 and 6 have been found in a plasmodesmata proteomics approach [20] and are so far unique in labelling of the desmotubule within plasmodesmata. Here they co-localise with viral movement proteins suggesting a role in cell-cell communication and plant immunity [16].

If two proteins are in close proximity (molecular distances of 1-10 nm) and therefore are most likely physically interacting, this can be measured using energy transfer processes described as Förster or fluorescence resonance energy transfer (FRET). This method was first described by Theodor Förster over 70 years ago [21] and is based on the non-radiative energy transfer from an excited fluorescent donor molecule to another non-excited fluorescent acceptor molecule in close proximity via dipole-dipole interactions. FRET will only take place if the donor emission spectrum has some overlap with the acceptor absorption spectrum with donor and acceptor dipoles displaying a mutual molecular orientation. The rate of the energy transfer k_T is calculated using equation 1:

$$k_T = \left(\frac{1}{\tau_D}\right) \left(\frac{R_0}{R}\right)^6 \qquad (1)$$

where τ_D is the donor excited-state lifetime in the absence of the acceptor, *R* is the distance between *D* (donor) and *A* (acceptor), and R_0 is the Förster radius. At the Förster radius, 50% of the donor molecules emit fluorescence while the rest undergoes energy transfer. During FRET, the decay rate is reduced due to quenching that depletes the excited state of the donor fluorophore and leads to shortening of the donor fluorescence lifetime. By measuring changes in the excited state lifetime of the donor at each pixel in the image, steady-state FRET is improved. This is described as FRET-fluorescence lifetime imaging

microscopy or FRET-FLIM.

We describe here two methods that have been used in protein-protein interaction studies for PD-localized reticulons. This work showed that RTN3 and 6 interact with a significant number of plasmodesmata proteins as well as proteins associated with ER-PM contact sites, lipid raft proteins, and proteins that interact with viral movement proteins [22].

The methods described are:

1) Immunoprecipitations using GFP-Trap®_A beads to find novel interaction partners for PD-localised reticulon proteins (Figure 1).

2) FRET-FLIM to test for and quantify real-time protein-protein interactions *in planta* (Figure 2).

2. Materials

2.1 Immunoprecipitation

1 *Arabidopsis thaliana* seedlings expressing the protein of interest fused to a fluorescent tag.

2 Murashige and Skoog (MS) medium: 4.4 g/l Murashige and Skoog basal salts, pH 5.7, 10 g/l agar.

3 Lysis buffer: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1 mM PMSF, protease inhibitor according to manufacturers' instructions.

4 Equilibration buffer: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA.

5 GFP-Trap®_A beads.

6 Petri dishes (large, with lid L x W x H: 245 mm x 245 mm x 25 mm).

7 50 ml Falcon tubes.

8 Glass bottles for buffers.

9 Cheese cloth.

10 Refrigerated table-top centrifuge.

2.2 FRET-FLIM using tobacco leaf Agrobacterium-mediated protein expression

1 *Nicotiana tabacum* plants about 5-6 weeks old, grown at 21°C, 14hr light, 10hr dark.

2 Plasmid constructs for Agrobacterium-mediated plant transformation fused to green fluorescent protein (here RTN proteins) and red fluorescent proteins (interaction test proteins), respectively.

3 YEB medium: 5 g/l beef extract, 1 g/l yeast extract, 5 g/l sucrose, 0.5 g/l $MgSO_4 \cdot 7H_2O$.

4 28°C shaker for falcons.

5 Infiltration buffer: 50 mM MES, 2 mM Na_3PO_4, 0.1 mM acetosyringone and 5 mg/ml glucose in 50 ml Falcon tube.

6 15-ml-Falcon tubes.

7 2-ml-Eppendorf tubes.

8 Table-top centrifuge.

9 Spectrophotometer.

10 1 ml syringes.

11 At least two-channel confocal and one-channel FLIM set up.

12 Becker and Hickl SPC 830 or SPC150 time correlated single photon counting card.

13 SPCImage analysis software version 5.1 or higher (Becker and Hickl).

3. Methods

3.1 Immunoprecipitation with GFP-Trap®_A beads

This part of the procedure requires between 4 and 5 h of time depending on sample size. This protocol uses GFP-Trap®_A beads (*see* **Note 1**) and the procedure is carried according to the company's protocol with slight modifications:

Arabidopsis thaliana seedlings expressing the protein of interest fused to a fluorescent tag in a stable manner are grown for two weeks on MS plates (*see* **Note 2**).

1. Grind 5 g of Arabidopsis whole seedling plant material in liquid nitrogen to a fine powder and then grind further in 5 ml lysis buffer until liquid (*see* **Note 3**). Distribute the extract into three 2-ml-Eppendorf tubes.
2. Incubate the extracts on ice for 30 min and then centrifuge at 10,000g for 10 min at 4°C.
3. Pour the supernatant (about 4 ml) into 50 ml Falcon tubes via 2 layers of cheese cloth.
4. For each plant sample, add 100 µl of GFP-Trap®_A agarose slurry to 500 µl equilibration buffer and centrifuge at 2,500 g for 2 min to pellet the beads. Discard the supernatant and repeat this wash twice.
5. Add the 100 µl washed agarose beads to the plant extract and shake the mixture on ice for 2 hours with the tubes being placed horizontally in the ice.
6. Centrifuge the tubes at 2,500 g for 2 min at 4°C and discard the supernatant carefully without disturbing the agarose pellet.
7. Wash the resulting agarose pellet twice with dilution buffer.
8. Use this pellet straight away for tryptic peptide digest and mass spectrometry analysis for containing proteins [22].

3.2 Real-time imaging of protein-protein interactions using FRET-FLIM

Potential interaction candidates resulting from the immunoprecipitation are verified *in planta* with the reticulon bait protein. The green fluorescent protein (GFP)-tagged RTN constructs are used whereas candidate proteins are fused to a red fluorescent protein (RFP). This procedure is done via FRET-FLIM using Agrobacterium mediated transient expression in tobacco leaf epidermal cells [22, 23].

1. Grow 5 ml overnight Agrobacteria cultures in 15-ml-Falcon tubes at 28°C with 200 rpm shaking in YEB with the appropriate antibiotics for constructs to be tested.
2. Transfer 1 ml per culture into separate labelled 2-ml-Eppendorf tubes.
3. Spin for 5 minutes at 4000 rpm and remove the supernatant.
4. Add 1ml infiltration buffer at RT and resuspend the pellet.
5. Spin for 5 minutes at 4000 rpm and remove the supernatant.
6. Resuspend the pellet in 1ml infiltration buffer.
7. Measure the OD_{600}.
8. Prepare your various construct mixes (the green RTN construct with each of your red test constructs) in individual tubes and in doing this dilute the resuspension with infiltration medium until the required OD is reached (*see* **Note 4**).
9. For the leaf infiltration take up the cells in a 1ml sterile syringe (no needle required).
10. Punch a small hole into the tobacco leaf avoiding using a P100 tip.
11. Place the syringe tip firmly against the underside of the leaf covering the hole and press the plunger down gently while exerting pressure against the other side of the leaf with your finger. The liquid will diffuse throughout the mesophyllar air space.
12. Repeat for all your constructs in at least two tobacco plants.
13. Mark the leaf upper epidermis with a permanent marker to keep track of the transformation sites.
14. Place the infiltrated plants back into growth conditions (21°C with 14 hours light, 10 hours dark) for at least 30 hours to allow transformation and gene expression to occur (*see* **Note 5**).

15. Cut out epidermal samples of infiltrated tobacco leaves are (~5 mm) with a scalpel, and perform both confocal/multiphoton FRET-FLIM data capture by microscope [24] (*see* **Note 6 and 7**).
16. Set laser light emission at a wavelength of 920 nm using a mode-locked titanium sapphire laser (Mira; Coherent Lasers), with 200fs pulses at 76 MHz, pumped by a solid-state continuous wave 532nm laser (Verdi V18; Coherent Laser).
17. To illuminate specimens on the microscope stage, focus the laser beam to a diffraction limited spot using a water-immersion objective (Nikon VC; numerical aperture of 1.2).
18. Collect fluorescence emission without pin-hole (non-descan), bypassing the scanning system, but instead passing through a BG39 (Comar) filter to block the near-infrared laser light.
19. Generate the raw time-correlated single photon (TCSPC) data by linking these via a time-correlated single-photon counting PC module SPC830 (Becker and Hickl). Analyse the pixel by pixel TCSPC data to generate a FLIM image or map (*see* **Note 8**).
20. Use a 633-nm interference filter to reduce chlorophyll autofluorescence that will otherwise strongly obscure the mRFP as well as eGFP emission.
21. Analyse data by obtaining excited-state lifetime values on a region of interest on the nucleus, and make calculations using SPCImage analysis software version 5.1.
22. Generate the range of lifetime values (for each pixel) within a region of interest and display as a distribution curve.
23. Fit decay data to a single exponential parameter as $f(t) = ae^{-t/\tau}$ (*see* **Note 9**). To allow for optimal fit, consider only values with a chi-square (χ^2) between 0.9 and 1.4 for statistical analysis (*see* **Note 10**).
24. Produce the range of lifetimes per sample by taking into account the median lifetime, minimum and maximum values for one-quarter of the median lifetime values from the curve.

25. Analyse at least three nuclei from at least three independent biological samples per protein-protein combination, and take the average of the ranges.
26. Calculate the degree or percentage efficiency of energy transfer (E), from one protein to the other, using equation 2:

$$E\% = \left[1 - \left(\frac{\tau_{DA}}{\tau_D}\right)\right] \times 100 \qquad (2)$$

Here τ_D and τ_{DA} are the measured excited state lifetime of the donor and acceptor, respectively. Example data is shown in Figure 2.

4. Notes

1. Gloves have to be worn for the whole procedure to reduce contamination with human proteins
2. This protocol can also be carried out with tobacco transiently expressing the protein of interest but will result in reduced number of interacting proteins and will of course only show interactors in tobacco.
3. Tissue can also be separated, e.g. if there is an interest in root proteins.
4. The optimum OD_{600} will vary according to the construct, hence different ODs have to be tested until sufficient expression levels are achieved. An OD_{600} of 0.1 is a good place to start with a new construct. After this lower ODs (e.g. 0.03) or higher ODs (e.g. 0.2-0.3) can be tested.
5. This growth period depends on the construct and protein longevity but 40-48 hours are mostly required before fluorescence can be observed.
6. For the experiments here, a two-photon microscope built around a Nikon TE2000-U inverted microscope is used with a modified Nikon EC2 confocal scanning system to enable near infra-red laser wavelength for FLIM [25].

7. Best measurement results for the ER have been shown for the nuclear envelope. Latrunculin B can be applied to the leaf discs to depolymerize the actin cytoskeleton and therefore inhibit movement of the ER. This is not necessary given sufficient expression levels of the donor protein which result in shorter time for data capture and therefore movement is less critical. FRET-FLIM measurements can also be taken on ER cisternae after application of Latrunculin B.
8. Prior to FLIM data collection, eGFP and mRFP expression levels in the plant samples within the region of interest are confirmed using a Nikon EC2 confocal microscope with excitation at 488 and 543 nm, respectively.
9. The intensity data provides the fluorescence decay function (*f*) at time, *t*. Single, double or triple exponential analysis of the decay yields the excited state lifetime (τ). The amplitude of the exponential components, *a*, defines the contribution to each lifetime component.
10. χ^2 describes the goodness of data fitted to the exponential function. So that a value of 1 represents analysis that perfectly describes the decay data points.

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Figure legends

Figure 1: Workflow for proteomic analysis. Arabidopsis plants expressing eGFP-RTN3 or eGFP-RTN6, respectively, in a stable manner are used for protein pulldowns with an anti-GFP-antibody (Chromotek). Interacting proteins are analysed using mass spectrometry. GFP-Calnexin (CXN) plants are used as negative controls and protein hits from CXN samples are deducted from the RTN samples. This is repeated at least twice. This resulted in 37 unique proteins interacting with the RTN proteins comprised of ER, PD, plasma membrane (PM) and cell plate proteins [22]. Size bar = 10 μm.

Figure 2: FRET-FLIM analysis of eGFP-RTN6. RTN6 without an interaction partner (A–E) or with mRFP-SMT1 (F–J). (A, F) Raw FRET-FLIM data; (B, G) pseudocoloured lifetime maps showing the lifetime values for each point within the region of interest; (C, H) distribution of lifetimes across the image. Blue shades represent longer eGFP fluorescence lifetimes than green ones. (D, I) Representative decay curves of a single point with an optimal single exponential fit, where $\chi 2$ values from 0.9 to 1.2 were considered an excellent fit to the data points (a binning factor of 2 was applied). The confocal images for the analysis in (E, J) show the eGFP construct in green and the mRFP construct in red. This example of FRET-FLIM analysis shows eGFP-RTN6 alone as a negative control and mRFP-SMT1 for protein–protein interaction. The fluorescence lifetime values for eGFP-RTN6 +mRFP-SMT1 are 2.25 ± 0.64 ns and therefore statistically lower than the lifetime values for the eGFP-RTN6 fusion alone (2.71 ± 0.57 ns).