Imaging the intracellular targeting of vacuolar membrane proteins in living cells

1. Introduction

- This study is to observe trafficking of proteins in living cells of plants.
- Specifically Alpha-Tonoplast Intrinsic Protein (Alpha-TIP) for which very little is known about how it reaches the tonoplast membrane.
- For this, Red Florescent Protein (RFP-HDEL) was used to mark the Endoplasmic Reticulum (ER).
- Photo Activatable Green Fluorescent Protein (PA-GFP) was bound to Alpha-TIP and used to highlight its position in the cell.

2. Objectives

- Design a primer to clone RFP-HDEL from a DNA template and successfully produce a plentiful supply of the gene in a suitable vector.
- Co-infiltrate the RFP-HDEL construct alongside PA-GFP Alpha-TIP into *Nicotiana rustica* so that the plant could express these proteins.
- Activate the PA-GFP in the ER lumen under the confocal microscope and observe a time course of the movement of the protein over a number of hours.

3. Labwork & Preparation

- PCR and cloning techniques were used to introduce HDEL to the RFP sequence and produce *Agrobacterium* containing the gene for RFP-HDEL.
- This was infiltrated alongside PA-GFP Alpha-TIP into *Nicotiana rustica*.
- It was established that 5 days after infiltration was the optimum time to view the sample.
- A ratio of 1 RFP to 2 GFP was found to give the best contrast between the two.
- Pulsing the UV laser every 10 seconds for 5 minutes gives the best activation of PA-GFP.

4. Problems and Resolution

- The first 16 hour time course only gave 3 hours of images due to sample deterioration.
- Needed to try to activate PA-GFP on a transilluminator instead of removing the sample from the leaf and take less line averaging on the microscope to prevent photo bleaching.
- Could not activate in this way; the UV light from the transilluminator was not strong enough.
- Decided to use ST-RFP instead which localises to the golgi bodies.
- If Alpha-TIP goes via the golgi bodies, the green and red fluorescence would co-localise at some point.

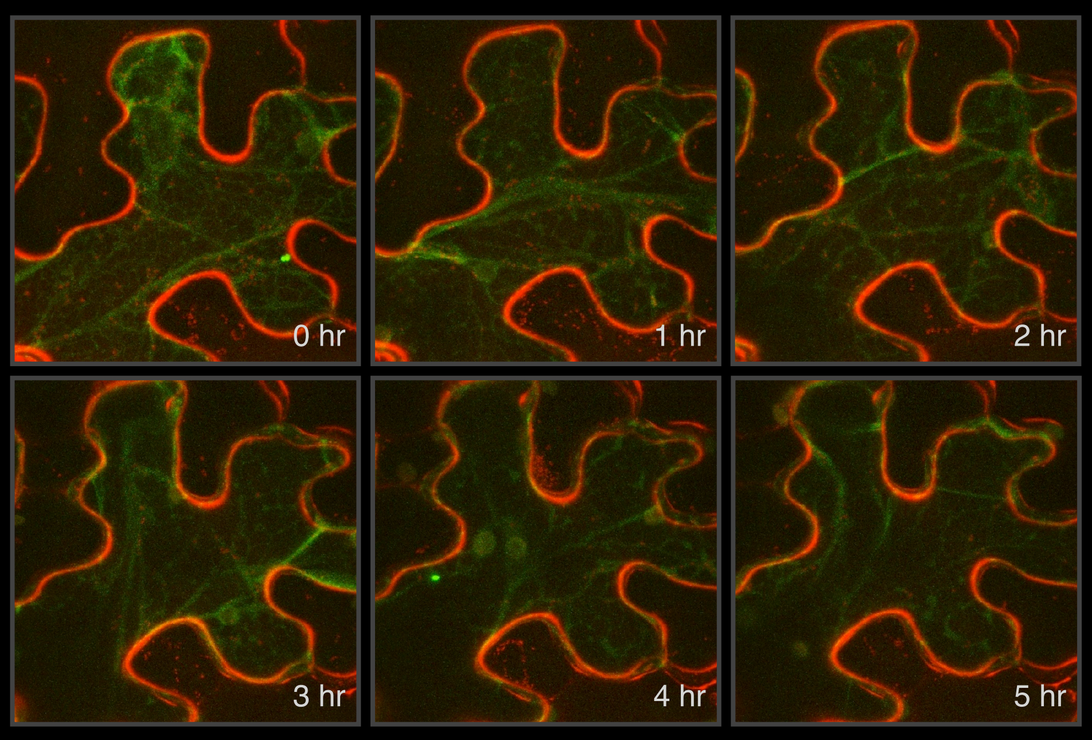

Figure 1 - A 5 hour time course, viewing the same cell. Red fluorescence identifies the golgi bodies whilst green fluorescence identifies the location of Alpha-TIP within the cell. No orange colour can be found throughout this time course, identifying that Alpha-TIP does not pass through the golgi body to reach the tonoplast membrane. Towards the end of this time course, it can be seen that Alpha-TIP is becoming dispersed, identifying that it no longer resides in just the ER lumen.

5. Results

- The second 16 hour time course gives 5 hours of images, after which the entire sample stopped fluorescing, presumably because the oil of the objective slipped off.
- During the 5 hours (shown in Figure 1) the fluorescence remained strong with a good resolution.
- Alpha-TIP appears to move from the ER lumen during the 5 hours, but at no point does it co-localise with the golgi bodies.
- An attempt to view a mutation of Alpha-TIP which has a C-terminal deletion failed since the plant refuses to express the protein.

6. Conclusions

- Some of the original aims were not fulfilled because of technical difficulties.
- However, a new marker for the ER lumen has been constructed (RFP-HDEL).
- It was discovered that dissected leaf samples cannot survive very long on a slide.
- PA-GFP cannot be activated by anything weaker than the UV laser at ~200x zoom.
- Using ST-RFP it is possible to find out that Alpha-TIP doesn't pass through the golgi.
- Within 5 hours, Alpha-TIP has begun to reach its destination in the tonoplast.

7. Future Work

- It is believed that the reason for the protein to avoid the golgi is a signalling sequence at the C-terminal end of the protein called a C-terminal Vacuolar Sorting Signal (ctVSS).
- Paul Hunter is trying to generate a viable protein for Alpha-TIP which is missing this sequence to find out if it then goes through the golgi.
- With more patience and time, the techniques used in this study to view the sample over a long period of time should reveal results, but better conditions for the sample will need to be designed to prevent deterioration.

8. References

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Poster and Project Work by:

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