New insights into the plant secretory pathway using virus delivered GFP

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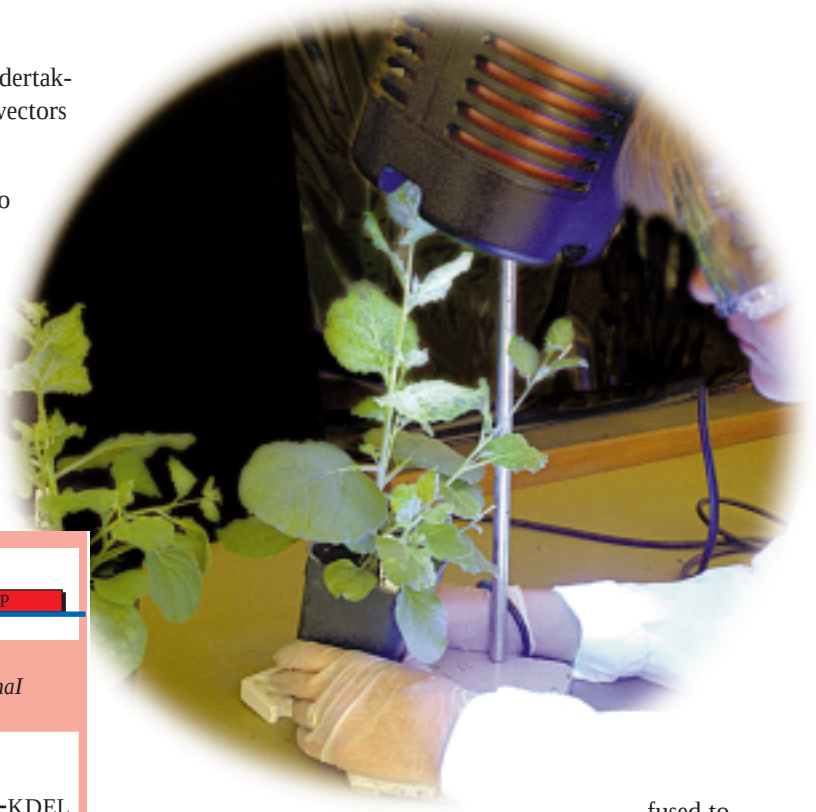
In addition to the study of basic virology undertaken at SCRI, we are developing virus-based vectors for fundamental cell biology research.

Potato virus X (PVX) has been engineered to express the green fluorescent protein (GFP) as a free protein and as a fusion to the viral capsid protein. These constructs are being used in a variety of ways to study the movement processes of PVX itself. Similar work is being carried out with cucumber mosaic virus, tobacco rattle virus and groundnut rosette virus. Recently, PVX also has been engineered to express GFP fused to proteins and peptides of interest in the secretory pathway. These modified GFPs act as powerful *in vivo* markers for protein localisation and illuminate the structure and dynamics of the plant secretory pathway.



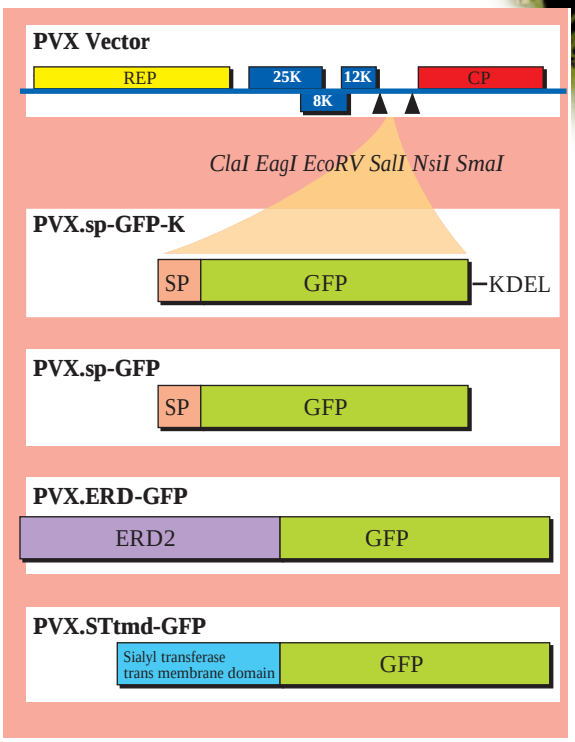


Figure 1 Diagrammatic representation of the GFP fusion constructs. The PVX genome has five genes: the replicase (REP), three overlapping genes encoding movement functions, and the coat protein (CP). The PVX vector contains duplicated coat protein subgenomic promoters, represented by the black triangles, and a polycloning site (expanded). Each GFP fusion was independently inserted between the *Eag*I and *Nsi*I sites.

Published work on the plant secretory system generally assumes that most processes are homologous, if not identical, to those in animals and yeast. However, our recent work suggests this may not be the case.

Targeting GFP to the ER In the first step of the secretory pathway, proteins enter the endoplasmic reticulum (ER). The translocation of nascent polypeptides from the cytoplasm to the ER is dependent on a signal peptide sequence being present at the amino terminus of the polypeptide. To determine whether GFP could be targeted to the ER when expressed from the PVX vector, a signal peptide sequence was fused to the amino-terminus of GFP[1]. Two different signal peptide sequences were tested, derived from the vacuolar storage proteins patatin and sporamin. To

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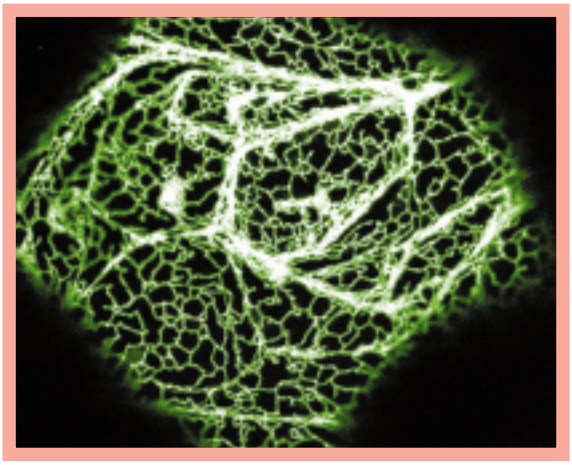

Figure 2 Fluorescent cortical ER in a PVX.sp-GFP-K infected *Nicotiana clevelandii* epidermal cell.

ensure high levels of GFP in the ER a four amino acid signal that specifies ER retention of proteins, KDEL, was fused to the carboxy-terminus of GFP (Fig. 1). Figure 2 shows an epidermal cell infected with the construct PVX.sp-GFP-K. The ER fluoresces brightly with the GFP and the polygonal network structure of the cortical ER is revealed. Since GFP allows imaging of live cells, we were able to observe that the cortical ER is a fairly stable structure, but on a smaller scale is constantly changing. The polygonal network constantly changes shape, new tubules extend from the network and fuse to another part or contract, and the small patches of sheet-like ER appear and disappear.

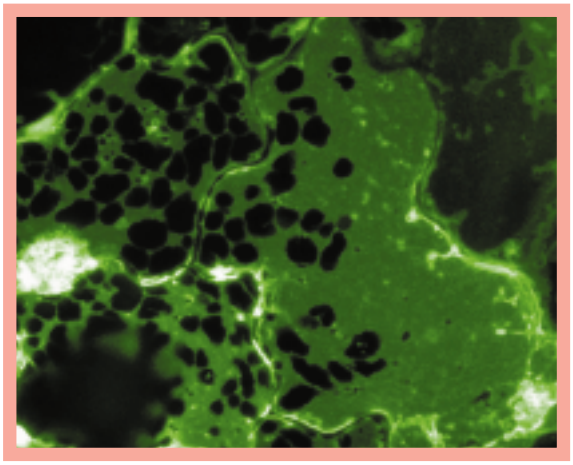

Figure 3 PVX.sp-GFP-K infected epidermal cells treated with 100µg/ml BFA for 4 hours. The cortical ER was transformed from a network of tubules into large sheet-like structures.

The *in vivo* effect of the fungal toxin, Brefeldin A (BFA), which inhibits secretion, was also investigated. High concentrations of BFA caused a dramatic and reversible change in the morphology of the cortical ER (Fig. 3). After several hours in BFA, the ER had formed an almost complete sheet.

Secreted GFP Proteins which enter the secretory pathway but which have no further targeting signals, are secreted. Secretion is therefore considered to be the default pathway.

To target GFP for secretion, a GFP was made with the sporamin signal peptide at the amino terminus and no other signals (Fig. 1). In cells infected with this construct, no GFP fluorescence was observed in the ER. A very faint fluorescence was seen which appeared to be in the cytoplasm. This fluorescence may be from GFP which did not enter the ER due to overloading of the ER translocation pathway by the high level of expression from PVX. This cytoplasmic fluorescence was possibly also present in the cells infected with the KDEL-containing construct but could not be detected due to the saturating fluorescence from the GFP-labelled ER.

Evidence that the GFP was being produced in cells and then secreted was obtained when secretion was inhibited by cold shock or with BFA. Fluorescence built up to detectable levels in the ER when secretion was inhibited. GFP was also detected in the apoplast by western blotting of fluid extracted from the extracellular space of PVX.sp-GFP infected leaves with anti-GFP antibody.

Targeting GFP to the Golgi apparatus The Golgi apparatus is a stack of membranous disks and associated budding vesicles and is considered to be the main processing and sorting organelle in the secretory pathway (Fig. 4). Proteins and other molecules enter the Golgi at the *cis* face in vesicles derived from the ER and

Figure 4 The *cis* face of the golgi stack, reconstructed from serial electron microscope sections.

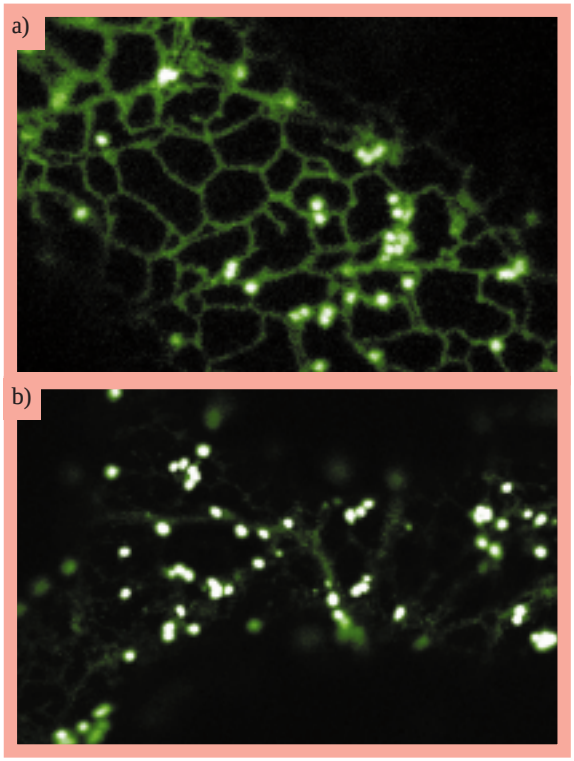

Figure 5 a) Fluorescent Golgi and ER in a PVX.ERD-GFP infected epidermal cell. b) Fluorescent Golgi in a PVX.STtmd-GFP infected epidermal cell.

leave from the *trans* face in specifically targeted vesicles. From electron microscope and immunofluorescence studies, it is known that plant Golgi are arranged very differently to animal Golgi. They are dispersed throughout the cytoplasm and are presumed to be subject to cytoplasmic streaming. It is thought that this arrangement may reflect a predominant function of the plant Golgi in the synthesis of cell wall components, as opposed to protein secretion for the animal Golgi[2].

In the first construct, the entire *Arabidopsis* ERD2 homologue sequence (provided by N. Raikhel, East Lansing) was fused to the amino terminus of the GFP (Fig. 1). In addition to targeting the GFP to the Golgi, it was hoped that this construct would help clarify the localisation and rôle of ERD2 in plants. From yeast and animal studies, ERD2 is known to be the receptor for the K/HDEL peptide signal. It resides predominantly in the Golgi apparatus where it binds to KDEL-containing proteins that have escaped the ER and recycles them back to the ER. For the second construct, the amino terminal 52 amino acids of the rat sialyltransferase (ST; provided by S. Munro, Cambridge), a protein localised to the *trans* Golgi and *trans* Golgi network of animal cells, was fused to the GFP (Fig. 1). The amino terminus of ST encodes the *trans* membrane domain (tmd) of ST and it has been shown that this region determines the localisation of the protein.

PVX.ERD-GFP infected cells displayed GFP fluorescence in the ER and the Golgi (Fig. 5a) while PVX.STtmd-GFP infected cells displayed GFP fluorescence almost exclusively in the Golgi (Fig. 5b). Evidence that the circular fluorescent bodies observed were in fact Golgi bodies was provided by immuno electron microscopy. Electron micrographs showed that the ERD-GFP was located throughout the Golgi, whereas the STtmd-GFP was located predominantly in the trans Golgi. This latter result demonstrated that there is sufficient homology between animal and plant systems for the animal Golgi targeting information to be functional. Observation of live cells with fluorescent Golgi revealed that the Golgi were in almost constant motion (videos of movement of GFP labelled Golgi can be seen at http://www.brookes.ac.uk/schools/bms/research/molcell/hawes/gfp/gfp.html). They appeared to be closely associated with the ER and could be seen moving around the outline of the polygonal network of the cortical ER. Their motion was multidirectional and at various speeds. They moved very rapidly along cytoplasmic strands, at a lower speed along grouped cables of ER and slowest around the cortical ER network. Pairs, or small groups, of Golgi appeared to move together for short periods of time and then move apart.

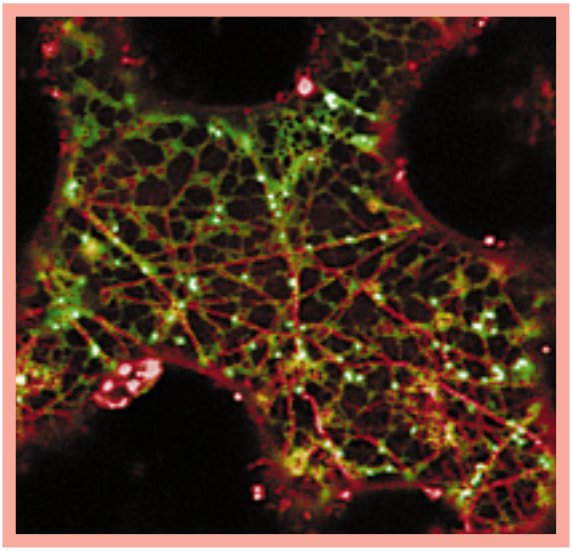

Figure 6 Double labelling of an epidermal cell to show labelled ER and Golgi (green) and actin filaments (red).

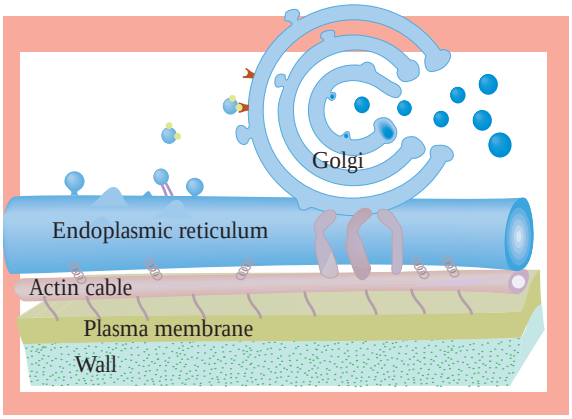

Figure 7 Model of Golgi association with the ER and actin.

Colocalisation of ER, Golgi and actin Previous studies have indicated a rôle for the actin cytoskeleton in plant Golgi function. When the cells with GFP-labelled Golgi were treated with an inhibitor of actin motor protein function, N-ethyl maleimide, the Golgi ceased to move. Treatment of the cells with cytochalasin, which depolymerises actin filaments, arrested Golgi movement and caused them to clump together at specific vertices on the polygonal ER network. The actin filaments in lightly fixed leaf cells were stained with rhodamine-conjugated phalloidin. Double imaging of the rhodamine and GFP in these cells revealed that the ER and the Golgi colocalised closely with the actin filaments (Fig. 6).

The close association of the ER, Golgi and actin has led us to propose a model in which the actin cytoskeleton underlying the ER network functions to transport the Golgi around the plant cell, using motor proteins that link the cytoskeleton to the Golgi stacks (Fig. 7). In this way, the Golgi remains closely associated with the ER and it is envisaged that in plants, transfer of proteins from the ER to Golgi occurs while the latter is in active transit.

References

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