Fluorescent Proteins As Markers in the Plant Secretory Pathway

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ABSTRACT The use of fluorescent proteins and live cell imaging has greatly increased our knowledge of cell biology in recent years. Not only can these technologies be used to study protein trafficking under different conditions, but they have also been of use in elucidating the relationships between different organelles in a noninvasive manner. The use of multiple different fluorochromes allows the observation of interactions between organelles and between proteins, making this one of the fastest-developing and exciting fields at this time. In this review, we discuss the multitude of fluorescent markers that have been generated to study the plant secretory pathway. Although these markers have been used to solve many mysteries in this field, some areas that require further discussion remain. Microsc. Res. Tech. 69:152–159, 2006. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Given the current widespread use of fluorescent proteins in visualizing and differentiating between intracellular compartments, it is difficult to believe that this technology has only developed over the past 10 years. Although fluorescent stains and immunofluorescence techniques have been used in imaging for several decades and continue to be widely used for some purposes, fluorescent proteins have certain advantages over these methods, particularly in observing the dynamics of protein transport and interactions. The use of combinations of fluorescent proteins with different excitation and emission wavelengths have led to the development of a variety of new methods to study protein movement and protein–protein interactions in living cells.

Fluorescent Proteins—A History

The first of the fluorescent proteins to be discovered was the green fluorescent protein (GFP), identified in the cnidarian Aequorea victoria by Shimomura et al. (1962). In the early 1990s, GFP was recognized as being of potential use to cell biologists and the revolution in microscopy began. In 1992, the gene encoding GFP was cloned (Prasher et al., 1992) and the ability of the protein to fluoresce when expressed in nonnative systems, as fusions, or when purified was demonstrated by several groups two years later (Chalfie et al., 1994; Inouye and Tsuji, 1994; Wang and Hazelrigg, 1994). These discoveries were an important breakthrough in themselves, allowing the observation of the localizations of proteins within cells or organisms. However, another significant breakthrough was made around the same time in the generation of new fluorescent proteins that emit light of different colors (Heim et al., 1994).

Fluorescence is caused when a fluorescent protein absorbs light that excites electrons within the protein fluorophore to a higher energy level. Different proteins absorb different wavelengths of light, giving them specific excitation and emission spectra. While excited, the electrons are less stable and eventually they revert to their original lower energy level, emitting energy in the form of light at the same time. In the case of GFP, the absorption takes place around 395 nm (blue light), while the wavelength of the emitted light is around 509 nm (Chalfie et al., 1994), giving the fluorescence a green color. Mutagenesis of certain residues in the GFP sequence gave rise to fluorescent derivatives with altered excitation and emission wavelengths (Heim et al., 1994, 1996; Rizzuto et al., 1996), meaning that these proteins could be excited independently of one another and their emissions observed separately with the use of appropriate filters. This breakthrough allowed more complex biological questions to be addressed using fluorescent tagging. The discovery of a red fluorescent protein (RFP/DsRed) in coral (Heikal et al., 2000) added to the variety of fluorescent proteins, although it was discovered that oligomerization of fluorescent proteins, although it required its fluorescence significantly (Baird et al., 2000). However, mutagenesis of DsRed has resulted in the generation of monomeric forms of RFP that are more strongly fluorescent and therefore more useful in imaging studies (Campbell et al., 2002; Shaner et al., 2004). Using different fluorescent proteins, it has become possible to observe the behavior of multiple proteins simultaneously, as well as using known markers to confirm the localizations of newly characterized proteins within the cell. The availability of multiple colors of fluorescent proteins led to the development of more complex technologies, such as fluorescence resonance energy transfer, which can be used to detect protein–protein interactions in vivo.

Over the past decade, many sequences have been fused to fluorescent proteins so as to investigate a wide variety of questions in many different fields. In this review, we concentrate on the multitude of markers...
developed to identify different organelles within the plant secretory pathway (Fig. 1). This pathway contains many of the same organelles as its counterparts in mammals and yeast, but significant differences between these systems have also been identified, meaning that each system must be examined independently to confirm that homologous proteins behave in similar manners.

**Nuclear Envelope/Endoplasmic Reticulum**

The first organelle in the secretory pathway is the endoplasmic reticulum (ER), which is continuous with the nuclear envelope (NE). This results in many ER markers labeling the NE and vice versa, making it difficult to differentiate between the two organelles. Indeed, even specific NE markers have been shown to redistribute into the ER during mitosis in plant cells (Brandizzi et al., 2004; Irons et al., 2003), indicating that the membranes of the plant NE may be absorbed into the ER during cell division. Observation of dividing tobacco BY-2 cells has permitted the identification of several markers that label the NE. For example, Ran GTPases are required for macromolecular transport in and out of the nucleus (reviewed by (Gorlich and Kutay, 1999)). These GTPases associate with different proteins during the GTPase cycle, including the GTPase-activating protein (GAP) that facilitates the hydrolysis of GTP to GDP. RanGAPs have been shown to be associated with the outer NE (Pay et al., 2002; Rose and Meier, 2001), with a heterogeneous distribution that suggests preferential association with the areas around the nuclear pores (Pay et al., 2002). Ran and RanGAP perform an additional function in regulating the formation of the mitotic spindle, and have been shown to localize at sites of microtubule nucleation, which include the NE in plant cells (Pay et al., 2002). However, the NE is not the only site of microtubule nucleation, meaning that RanGAP is not a specific NE marker protein. In an attempt to provide a specific NE marker in plants, Irons et al. (2003) have shown that GFP fused to the N-terminal 238 amino acids of the human lamin B receptor (LBR) localizes predominantly at the plant NE, a similar result to that observed in mammalian cells (Ellenberg and Lippincott-Schwartz, 1999). LBR-GFP labels the NE homogeneously, although it remains to be determined whether this fusion is associated with the inner NE, as is the case for the full-length protein in mammalian cells (Smith and Blobel, 1993). The components of the NE may vary widely among kingdoms, although little is known of the composition of the plant NE (reviewed by Meier, 2001). To study its structure and dynamics thoroughly it will be necessary to identify further markers that specifically label the inner or outer NE. Markers that are native

![Figure 1. Overview of the plant secretory pathway. Schematic representation of the organelles within the secretory pathway of plants. Note that the LV occupies the majority of the cell, while all other organelles are forced to the periphery. ERES are shown associated with Golgi bodies, as described by daSilva and others (2004). Multiple small PSVs are shown distributed throughout the cytoplasm, compared with a single large LV.](image-url)
to plants would be most useful, given the potential for variation in NE composition between species.

**Endoplasmic Reticulum**

In contrast with the situation for the NE, ER markers in plants are abundant, both membrane-bound and soluble. Indeed, many proteins when misfolded are retained in the ER. However, these proteins are not appropriate for use as ER markers when studying the secretory pathway, as the accumulation of misfolded proteins in the ER may well affect protein synthesis and export, as well as potentially causing changes in the morphology of this and subsequent organelles. Therefore, one must be careful to choose a marker protein that does not disrupt the pathway being studied. Some ER-localized proteins do not have a severe effect on the secretory pathway, but may still cause indirect alteration of certain steps. For example, fusions of GFP to the C-terminal ER retention signal H/KDEL provide soluble ER marker proteins (Boevink et al., 1996; Haseloff et al., 1997), as both HDEL and KDEL signals result in retention of soluble proteins within the ER in plant cells (Denecke et al., 1992). These fusions were among the first targeted GFP fusions to be used in plants. The ER localization of GFP-H/KDEL is thought to be achieved through an efficient retrieval system, whereby the H/KDEL signal is recognized by a receptor termed ER retention defective 2 (ERD2, Lee et al., 1993; Lewis et al., 1990; Semenza et al., 1990) in the cis-Golgi and transported back to the ER. It has been shown that saturation of this receptor is possible when HDEL-tagged proteins are over-expressed in plant cells (Crofts et al., 1999), resulting in the secretion of some H/KDEL-tagged proteins to the extracellular space. This could have deleterious effects on the protein folding/quality control machinery in the ER, since several of the major chaperone proteins, such as BiP and calreticulin, carry H/KDEL retrieval signals (Denecke et al., 1991, 1995). Secretion of these proteins from the cell would put extra stress on the ER, possibly leading to ambiguous results. It is therefore necessary to ensure that such soluble markers are not over-expressed before data can be considered valid.

In addition to the soluble markers discussed above, fluorescent fusions to ER resident transmembrane proteins have been created. Sec12 is a type II membrane protein that activates the small GTPase Sar1, thereby playing an important role in COPII-mediated export of proteins from the ER (reviewed by Hanton et al., 2005). A YFP (yellow fluorescent protein) fusion to Sec12 was created by daSilva et al. (2004), and was shown to be equally distributed throughout the ER membrane. This marker therefore appears to be ideal for live cell imaging; however, the untagged protein has been shown to inhibit ER export of soluble proteins in plants (Phillipson et al., 2001). This indicates that this marker too may disrupt the secretory pathway (daSilva et al., 2004) and may therefore result in the production of unsound data. The type I transmembrane protein calnexin is a membrane-bound homologue of calreticulin that is often used as an ER marker in cell fractionation processes, making it a useful target for fluorescent fusions (daSilva et al., 2005; Irons et al., 2003). No evidence for disruption of the secretory pathway through over-expression of this fusion has been presented, indicating that perhaps GFP-calnexin is the most useful fluorescent ER marker yet presented. It should be stressed, however, that over-expression of this marker may lead to the enlargement of the ER tubules and cisternae, resulting in the formation of sheet-like structures (Hanton and Brandizzi, unpublished results). This may be the result of saturation of the export machinery that determines accumulation of the markers into an enlarged ER membrane. As an alternative to these markers, which have specific retrieval mechanisms and activities in plant cells, it may be more useful to use TM17, a protein that is retained in the ER due to its shortened transmembrane domain (Brandizzi et al., 2002a). TM17 is a type I protein that was derived from the transmembrane domain of a mammalian lysosomal protein, and consists of a 17-amino acid transmembrane domain plus a short cytosolic domain of 4 amino acids. It would therefore not be expected to affect the function of the ER, and as it does not appear to be able to be exported from the ER (Brandizzi et al., 2002a), there should be no disruption to retrieval mechanisms from the Golgi apparatus.

**ER Export Sites**

The ER contains specialized regions that are thought to accumulate cargo destined for export in the anterograde direction through the secretory pathway. These are termed ER export sites (ERES) and were first identified through fluorescent labeling by daSilva et al. (2004). This study used Sar1, the small GTPase responsible for the regulation of COPII coat assembly as a marker protein. When coexpressed with a membrane-bound cargo protein destined for the Golgi apparatus, Sar1-YFP labels motile punctate structures that track with Golgi bodies, which are thought to be ERES. A GTP-restricted form of Sar1 is found to associate with these regions of the ER even in the absence of coexpressed cargo molecules, indicating a link between the GTPase cycle and accumulation of the GTPase at ERES. However, a recent publication using Sec13, a COPII coat protein, as a fluorescent marker (Yang et al., 2005) indicates that ERES do not continually track with the Golgi bodies, rather that they associate transiently with the Golgi but spend a considerable proportion of time moving independently. The discrepancies between the two studies may be explained by the use of two different expression systems (expression in tobacco leaves (daSilva et al., 2004), compared with tobacco BY-2 cells (Yang et al., 2005). Alternatively, the ERES markers used may behave differently, since Sar1 is a GTPase that mediates the recruitment of the COPII coat, while Sec13 forms part of the coat protein complex itself. Further study using these and other markers will be required to resolve the inconsistency revealed in these two studies.

**Golgi Apparatus**

Many proteins have been found to localize at the Golgi apparatus when fused to fluorescent proteins. However, many of these marker proteins also label other structures such as the ER or post-Golgi compartments. For example, ERD2-GFP (Boevink et al., 1998) and its YFP-fused counterpart (Brandizzi et al., 2002a) predominantly label the Golgi apparatus (Fig. 2). However, the function of ERD2 as the receptor that retrieves H/KDEL-tagged proteins to the ER that
it must cycle back to the ER from the Golgi, leading to a certain level of ER staining. ERD2 would be expected to localize specifically to the cis-Golgi in plants, concomitant with its role in retrieving proteins from the cis-Golgi to ER; however, electron microscopy data presented by Boevink et al. (1998) indicate that the ERD2-GFP fusion is equally distributed across the whole of the Golgi apparatus. Contrastingly, both a GFP fusion to the transmembrane domain of rat sialyl transferase (ST-GFP) and the native protein localize to the trans-face of the Golgi (Boevink et al., 1998; Wee et al., 1998). ST-GFP and the alternative fluorescent fusions ST-YFP and ST-CFP (cyan fluorescent protein) are all detected at the Golgi apparatus through fluorescence microscopy (Brandizzi et al., 2002b). Faint ER staining can also be observed (Brandizzi et al., 2002b), although this is much less pronounced than that of fluorescent fusions to ERD2. A GFP fusion to α-1,2-mannosidase I labeled Golgi stacks in BY-2 cells, and localized specifically to the cisternal rims (Nebenführ et al., 1999). Once again, however, the authors reported ER staining in some cells. Other proteins, such as GONST1-YFP (Golgi nucleotide sugar transporter 1, Baldwin et al., 2001; Handford et al., 2004), label structures that appear to be distal to the Golgi apparatus. In tobacco, GONST1-GFP has been observed at both the Golgi and other, smaller dots that do not colocalize with ERD2-YFP or ST-YFP (Handford et al., 2004), see also Figure 2. In contrast, GONST1-YFP expressed in onion cells labels only the Golgi apparatus (Baldwin et al., 2001). This reinforces the point that secretory pathways are not identical between species even when those species belong to the same kingdom. A newly characterized Golgi matrix protein, CASP (CCAAT-displacement protein alternatively spliced product, Renna et al., 2005), has been shown to label only the Golgi apparatus, making it a potentially valuable tool in further studies of the secretory pathway, particularly with regard to ER-Golgi transport. In addition to these proteins, which all possess one or more transmembrane domains, certain cytosolic proteins that label the Golgi apparatus have also been identified. These include the small GTPase ARF1 (ADP ribosylation factor 1, (Takeuchi et al., 2002) and several members of the Rab superfamily, such as the Rab11 homologues from pea, Pra2 and Pra3 (Inaba et al., 2002), or the Arabidopsis Rab5-related proteins Rha1 and Ara7 (Kotzer et al., 2004; Lee et al., 2004). However, similar to the situation for transmembrane proteins, these cytosolic markers can also label other, non-Golgi structures, nucleoplasm and cytoplasm.

**Prevacuolar Compartment**

The prevacuolar compartment (PVC) is a post-Golgi protein-sorting organelle, where proteins destined for transport to the lytic vacuole (LV) are delivered from the trans-Golgi network (TGN). Sorting of proteins to the LV involves the receptor protein BP80 (>80-kDa binding protein), first identified in *Pisum sativum* (Kirsch et al., 1994, 1996; Paris et al., 1997), but with several *Arabidopsis* homologues (Ahmed et al., 1997; Laval et al., 2003) and potentially more in other species. BP80 is thought to recognize its ligands in the TGN and transport them to the PVC, where the cargo can be deposited while the receptor recycles to the TGN. BP80 is one of several PVC marker proteins that have been identified (Brandizzi et al., 2002a; daSilva et al., 2005; Humair et al., 2001), see also Figure 2, and has been widely used to study vacuolar transport. It remains unclear how cargo molecules are transported from the PVC to the LV. There may be some form of vesicular transport between the two compartments, or alternatively the PVC itself may fuse periodically with the vacuole. The second hypothesis would require regeneration of the PVC, which could occur through the fusion of TGN-derived vesicles with one another. DaSilva et al. (2005) used a GFP-BP80 fusion to show that disruption of BP80 recycling between the TGN and PVC by the drug wortmannin results in the delivery of the BP80 fusion to the vacuole. This suggests that whichever transport mechanism is employed to carry cargo from the PVC to the vacuole is nonselective, as otherwise the receptor would accumulate in the PVC while only vacuolar proteins were delivered to the degradative compartment. It therefore seems likely...
that the entire PVC may fuse with the vacuole, in accordance with the second hypothesis outlined above.

In addition to BP80, several other proteins that label the PVC have been identified. PV72, a receptor protein from pumpkin that has considerable homology with BP80, is thought to be involved in sorting of cargo molecules to the protein storage vacuole (PSV, Shimada et al., 1997; 2002; Watanabe et al., 2002) rather than to the LV, as is the case for BP80. Expression of a GFP fusion of this receptor in tobacco BY-2 cells revealed a variety of staining patterns, apparently dependent on the age of the cells in which the fusion was expressed (Mitsushashi et al., 2000). After ~3 days, punctate structures were observed. The authors interpret these structures as Golgi bodies, based on the work of Nebenfuhr et al. (1999). However, in the absence of any coexpression data with known Golgi markers, it is impossible to say whether this is the case. It also appears that the punctate structures observed vary in size, suggesting that although some may be Golgi bodies, it is quite possible that others are PVC. In older cells, vacuolar staining was observed. This suggests that vacuolar sorting receptors may have a limited lifetime and that eventually they are transported to the vacuole for degradation. Alternatively, the result may be an artifact caused by expression of a protein normally found in developing pumpkin seeds in BY-2 cells.

Several plant homologues of Rab5 have also been reported to localize at the PVC. The localization of m-Rab_{mce} (Bolte et al., 2000) has been thoroughly studied in several different systems, giving similar but not identical results for each (Bolte et al., 2004a). A CFP fusion of m-Rab_{mce} was expressed in BY-2 cells, Arabidopsis protoplasts and tobacco leaves. In all of the studied systems both Golgi bodies and PVC were labeled by m-Rab_{mce}-CFP, but when coexpressed in BY-2 cells with known Golgi markers such as ST-YFP, it became obvious that only a part of the Golgi population labeled by ST-YFP was colabeled by m-Rab_{mce}-CFP. Contrastingly, in both Arabidopsis and tobacco leaves, most of the Golgi bodies were labeled with both markers. This indicates that there are differences between the secretory pathways of different plant expression systems, which must be considered when interpreting data. Two other Rab5 homologues have also been reported as labeling the PVC (Lee et al., 2004). Rha1 and Ara7 have a high degree of homology with one another, and have very similar labeling patterns. Both colocalized with AtPep12, a SNARE that has been shown to label the PVC (da Silva Conceição et al., 1997), and with AtBP80 (Ahmed et al., 1997; Sanderfoot et al., 1998). Rha1 has also been shown to be important in mediating vacuolar transport, supporting its localization at the PVC (Sohn et al., 2003); however, this work does not report on the distribution of the wild-type Rha1. Another study, this time in tobacco leaf cells, has confirmed the localization of Ara7 at the PVC (Kotzer et al., 2004). However, Kotzer et al. (2004) reported that Ara7 also colocalizes with ST-YFP, indicating that the Rab labels the Golgi as well as the PVC. It is not clear from the study by Lee et al. (2004) whether a similar result is achieved in their system, as no Golgi markers were used. Some debate regarding the localization of Ara7 in the plant secretory pathway has arisen, as the first study of this protein reported that Ara7 labels an endocytic compartment (Ueda et al., 2001). The authors studied Ara6 and Ara7, both of which have homology to Rab5 and appear to have similar intracellular distributions, which demonstrate colocalization with the styryl dye FM4-64. This dye labels the early endosomes in yeast (Vida and Emr, 1995), and is thought to label similar structures in plants. However, FM4-64 has been shown to label a variety of punctate structures in plant cells (Bolte et al., 2004b), including the Golgi apparatus and PVCs. In contrast to the work of Ueda et al. (2001), a more recent publication reports that a fluorescent probe that is endocytosed along with sterols in Arabidopsis, and might therefore be expected to label the endosomes, exhibits only a partial colocalization with Ara6, Ara7, and Rha1 (Grebe et al., 2003). A convincing marker for the endosome is required before this debate can be concluded. It may also be the case that the PVC acts as a late endosome. In mammalian cells, the late endosome receives cargo molecules from the early endosome, where sorting is thought to occur, and delivers it to the lysosome (reviewed by Luzio et al., 2000). If the plant PVC were to perform a similar function to that of the late endosome, this would explain the observation of FM4-64 accumulating in the PVC and then in the vacuolar membrane (reviewed by Bolte et al., 2004b).

**Vacuolar Compartments**

Plant cells have been shown to contain two distinct types of vacuoles (Paris et al., 1996), the LV and the PSV. These vacuoles can coexist in the same cell, but in general, LVs are found in vegetative tissues such as leaves and PSVs in storage tissues such as seeds. In addition to these types of vacuole, others have been reported by Otegui et al. (2005), although these are apparently senescence-induced and are therefore only present at certain developmental stages. Vacuolar sorting signals in plants have been classified into three basic types: sequence-specific, C-terminal, and structure-dependent (Matsuoka and Neuhaus, 1999), suggesting three sorting mechanisms and potentially also three destinations. The use of GFP has been important in determining the validity of this proposal, as it is possible to fuse specific sorting signals to GFP and observe its localization. However, the function of the LV by definition causes problems in observing fluorescent cargo molecules (Tamura et al., 2003). It has been suggested that incubating plants in the dark prior to observation may increase the fluorescence in the vacuole (Tamura et al., 2003), while the use of a modified form of GFP with increased levels of fluorescence (Cormack et al., 1996) has also been employed to observe targeting to the LV (Di Sansebastiano et al., 2001). The well-defined sorting signals from tobacco chitinase A (Neuhaus et al., 1991), barley aleurain (Holwerda et al., 1992), and sweet potato sporamin (Matsuoka and Nakamura, 1991) have all been fused to GFP. The fusion to the chitinase signal, a C-terminal sorting determinant, was delivered to small, nonacidic vacuolar structures in tobacco protoplasts (Di Sansebastiano et al., 1998), presumably the PSV. However, in some cells the fusion was also detected in a larger, acidic vacuole, most likely the LV. The cells in which this alternative delivery occurred were those in which large numbers of chloroplasts were present, suggesting that these cells are specialized for photosynthesis and therefore have a high protein turn-
over rate, requiring an active LV. In contrast to these results, a fusion of the sorting determinant of a-lerealain to GFP resulted in the large, acidic vacuole becoming fluorescent (Di Sansebastiano et al., 2001). The a-lerealain signal belongs to the sequence-specific class, supporting the theory that different classes of signals might deliver proteins to different types of vacuoles. Sporamin also bears a sequence-specific vacuolar sorting signal (Matsuoka and Nakamura, 1991, 1999), and a C-terminal GFP fusion was shown to accumulate in the large central vacuole of Arabidopsis protoplasts (Takeuchi et al., 2000). Investigation of other vacuolar sorting signals is required to confirm the specificity of sorting; expression in other tissues is also desirable, as not all leaf cells appear to contain the smaller, non acidic vacuoles (Di Sansebastiano et al., 1998).

In addition to the soluble vacuolar markers discussed above, several tonoplast markers have been identified. Tonoplast intrinsic proteins (TIPs) have been used as vacuole membrane markers in immuno-fluorescence studies (Jauh et al., 1998, 1999; Jiang et al., 2000; Paris et al., 1996), and in recent years, fluorescent fusions to these integral membrane proteins have also been created (Avila et al., 2003; Hicks et al., 2004; Saito et al., 2002). Different TIP isoforms label different vacuole types (Jauh et al., 1998, 1999; Paris et al., 1996); while γ-TIP is found on LVs (Jauh et al., 1999; Robinson et al., 1995), both α- and δ-TIP localize to the tonoplast of the PSV, although δ-TIP is thought to label a specialized subset of storage vacuoles (Jauh et al., 1998, 1999; Paris et al., 1996). The presence of both α- and γ-TIP on the same tonoplast has also been observed, likely indicating fusion of different vacuole types (Paris et al., 1996). DIP, another TIP isoform, has been shown to label putative precursors to the PSV (Jiang et al., 2000). These markers are therefore particularly useful in distinguishing between different vacuolar compartments. The use of GFP fusions of different TIPs has increased the knowledge of vacuolar function further. Independent studies using γ-TIP-GFP and δ-TIP-GFP have shown that both LVs and PSVs contain structures that are mobile inside the vacuole, the membranes of which are strongly labeled by the fusion proteins (Hicks et al., 2004; Saito et al., 2002). These structures may be autophagic in nature, as they contain cytoplasmic components such as mitochondria. Hwang et al. (2005) have identified other proteins that are localized at the tonoplast. Calcineurin B-like proteins (CBLs) are soluble proteins involved in calcium signaling (reviewed by Luan et al., 2002). The rice (Os) CBL1-4 carry an N-terminal myristoylation motif that is likely to be involved in their interaction with the membrane (Hwang et al., 2005). GFP fusions of these proteins show a variety of staining patterns when expressed in barley aleurone protoplasts; OsCBL1 does not associate specifically with any organelles, while OsCBL4 labels the plasma membrane. However, OsCBL2 and OsCBL3 both stain the PSV tonoplast, and RNAi studies of OsCBL2 demonstrate reduced vacuole biogenesis, indicating an important role in cell development (Hwang et al., 2005).

**Plasma Membrane**

The plasma membrane is the most distal location in the secretory pathway. Proteins can be secreted from the cell at this point, or can be specifically targeted to the plasma membrane as their final destination. Labeling of the plasma membrane can sometimes be confused with labeling of the cell wall or cytosol, as the large central vacuole in most cells squeezes the cytosol to the periphery of the cell and therefore causes it to resemble the plasma membrane. In such cases, it has been demonstrated that the three different locations can be distinguished by means of plasmolysis and osmotic rupture of the cell (Serna, 2005). Many proteins located in the plasma membrane are involved in the transport of cargo molecules in and out of the cell. For example, the tomato (Le) FBO1 is a membrane-spanning iron reductase that is important in the uptake of iron for the cell. When fused to GFP and expressed in onion epidermal cells, it localizes at the plasma membrane (Li et al., 2004). Similarly, the *Arabidopsis* polyl transporter AtPLT5 plays a vital role in transporting molecules such as glycerol and myo-inositol into the cell while exporting H+ ions into the extracellular space (Klepek et al., 2005). Although LeFRO1 and AtPLT5 are both integral membrane proteins, soluble proteins can also be targeted to the plasma membrane. The protein kinase LeCRK1 (Leclercq et al., 2005) possesses signals for myristoylation and palmitoylation, both of which appear to be necessary for its membrane association, as mutation of either signal causes the redistribution of the protein to the cytosol. LeCRK1 is thought to be involved in signaling pathways that lead to fruit ripening processes, rather than direct transport of molecules across the plasma membrane, which explains its presence on the cytosolic side of the plasma membrane.

**DISCUSSION**

It is clear that the number of fluorescent markers for the plant secretory pathway is constantly increasing and expanding our knowledge of the system. However, it is important to keep in mind a few ground rules when working with fluorescent fusions. The first of these is to ensure that the fusion of the fluorescent protein does not affect the function of the protein under investigation. When investigating the localization of a novel protein, it is desirable to confirm the data by coexpression of known fluorescent markers where possible. However, it must also be considered that such a coexpression may alter the transport of the protein being studied, and so appropriate controls are also required. It is also important that false data are not obtained by using fluorophores that emit light at similar wavelengths, as this can cause “bleed-through” of light from one channel to another.

Fluorescence microscopy has clarified many aspects of protein transport within the plant secretory pathway; however, several questions remain to be answered. An obvious example of this is the current controversy over ERES behavior in plant cells. To shed light on this matter, either the same fluorescence markers or the same expression system should be used to minimize variables that may alter the interpretation of data. Another topic that is under debate is the localization of Rab proteins to the endosomes or PVC. A specific endosomal marker has yet to be identified in plants, but PVC-localized proteins such as BP80 can be used to show whether the protein under scrutiny is targeted to the PVC or to other structures. Further studies using fluorescent
proteins will no doubt illuminate the plant secretory pathway and its organelles. Other techniques such as electron microscopy and biochemical assays can be combined with the rapidly developing field of fluorescence microscopy to provide accurate and unambiguous data that will increase our understanding of the system.

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