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# Endocytosis in signalling and development

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After a long period of neglect, endocytosis in plants is finally coming of age. The constitutive recycling of plasma membrane proteins has been well established in the past few years, and recent studies report the ligand-induced endocytosis of receptors and other plasma membrane proteins. Signalling by ligand-bound receptors from endosomes has not, however, been demonstrated in plants. Although novel markers have been used to map endocytic pathways, the functional compartmentalisation of endosomes is still controversial. It is thus not clear where and how cargo proteins such as receptors are sorted towards either recycling to the plasma membrane or targeting to the vacuole for degradation.

## Addresses

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**Current Opinion in Plant Biology** 2006, **9**:589–594

This review comes from a themed issue on  
Cell biology  
Edited by Laurie G Smith and Ulrike Mayer

Available online 2nd October 2006

1369-5266/\$ – see front matter  
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DOI [10.1016/j.pbi.2006.09.011](https://doi.org/10.1016/j.pbi.2006.09.011)

## Introduction

Eukaryotic cell behaviour cannot be mechanistically understood without an appreciation of the precise, yet dynamic compartmentalisation of its molecular machinery. For example, plasma-membrane-localized receptors, which enable the environment to be scanned for diverse signals, change their subcellular localisation during their lifetime. They are made at the endoplasmic reticulum, secreted to the plasma membrane via the Golgi apparatus, and might undergo repeated cycles of endocytosis and recycling through endosomal compartments before being targeted to the lysosome/vacuole for degradation. This receptor trafficking is highly regulated and plays a crucial role in signalling. De-sensitisation by receptor downregulation is achieved by increased internalisation or decreased recycling [1,2]. Although less evident, endocytosis as a means of activating signalling appears to be a widespread phenomenon, at least in animals. In this review, we first summarize current knowledge of animal

receptor endocytosis and signalling. We then present a current view of the endosomal organization in plants as compared to that in animals and discuss recent data that link endocytosis to signal transduction and development in plants.

## Endocytosis and signalling in animals

The epidermal growth factor receptor (EGFR) is a popular model for studying general mechanisms of signal transduction and endocytic trafficking. EGFR is rapidly internalised and degraded upon ligand binding, which was initially regarded only as a mechanism for signal termination and possibly de-sensitisation of cells [3]. However, blocking endocytosis of EGFR with dominant-negative forms of dynamin severely reduced signal transduction to downstream mitogen-activated protein (MAP) kinases [4]. This requirement of endocytosis for signal transduction is now explained by the fact that p14, a necessary adaptor protein in signalling, is localized to endosomal membranes but not the plasma membrane [5]. Thus, ligand-activated receptor has to be moved to the location of downstream effector modules for signalling to occur; a process that might present cells with an additional level of control for their response to a given stimulus. Such a control mechanism has been suggested for transforming growth factor- $\beta$  (TGF- $\beta$ ) signalling. Here, the receptor needs to be endocytosed in order to meet an adaptor for the downstream Smad transcription factor, SARA (for ‘Smad anchor for receptor activation’), which localises exclusively to early endosomes [6–9]. Interestingly, TGF- $\beta$  receptor can be internalised from the plasma membrane by two different pathways, of which only the clathrin-dependent route delivers the activated receptors to early endosomes for SARA-dependent signalling [7]. Thus, the trafficking route for a receptor can determine how the signal is interpreted by the cell. In the case of growth factor morphogens, whose graded distribution determines cell fate in a position-dependent manner, endosomal sorting leads to changes in morphogen distribution and response [10–14].

The Notch receptor–Delta ligand system in *Drosophila* is used as a module for cell fate determination between two daughter cells, which requires differential endocytosis and unequal segregation of trafficking regulators. Asymmetric localisation and segregation of Numb, a protein that recruits endocytic adaptors to the Notch receptor, is thought to de-sensitize this cell to the Delta ligand by increasing the endocytosis of the receptor, therefore biasing signalling between the two cells [15]. In addition, the similarly localized ubiquitin ligase Neuralised causes ubiquitination of Delta, leading to its increased

endocytosis [16]. Furthermore, rab11-recycling endosomal compartments segregate unequally during division of the mother cell, increasing Delta recycling in one of the daughter cells [17]. Thus, it appears that unequal distribution of endosomal trafficking regulators is used at several seemingly redundant levels to ensure a difference in Notch–Delta signalling, which will eventually lead to cell-fate selection.

### General organization of the plant endosomal system

Many fundamental features of the endomembrane system are conserved across eukaryotes. For example, the conserved molecular machinery that is responsible for the sorting of proteins into the multivesicular body pathway has been recently elucidated in animals and yeast, and probably also exists in plants [18]. There are important differences between plants and non-plant organisms, however, which makes it necessary to distinguish between borrowed-by-homology and verified-by-experiment features of trafficking models. Plant cells easily match animal cells in the complexity of the endomembrane system [19,20] as evidenced by the *Arabidopsis* inventory of membrane trafficking components, which includes 54 soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors (SNAREs) and 57 Rab GTPases [21,22]. Homologies between animal and plant sub-classes of SNAREs and Rabs might be misleading, however, especially in the post-Golgi/endosomal system. For example, plants express close homologs of the rab5 family of early endosomal Rabs but lack clear homologs of the mammalian rab4 and rab9 families, which are involved in regulating early-to-recycling endosome and late endosome-to-trans-Golgi network (TGN) trafficking, respectively. *Arabidopsis* also has both many homologs of the rab11 GTPase, which is involved in endosome-to-plasma membrane recycling, and a myristoylated plant-specific rab that does not exist in animals. Systematic localisation of SNAREs and Rabs by transient overexpression in protoplasts [23,24], although a somewhat problematic technique, has led to the definition of different endosomal compartments: one was labelled by the canonical rab5 homologs *Arabidopsis* Rab-like 7 (ARA7; RabF2b) and Rab homolog of *Arabidopsis* 1 (RHA1; RabF2a) and by the SNARE vesicle-associated membrane protein 727 (VAMP727); another associated but distinct compartment was labelled by the plant-specific myristoylated ARA6 (RabF1) and by the SNARE syntaxin of plants 21 (SYP21). Both compartments were distinct from the SYP41-labeled compartment that was initially equated with the TGN and recently also identified as an ‘early endosomal’ compartment [25••]. The SYP41 compartment has been alternatively described as often dissociated from [23] or always associated with [25••] Golgi stacks. These descriptions resemble an earlier controversy about the association of the Golgi with the ultrastructurally defined ‘partially coated reticulum’,

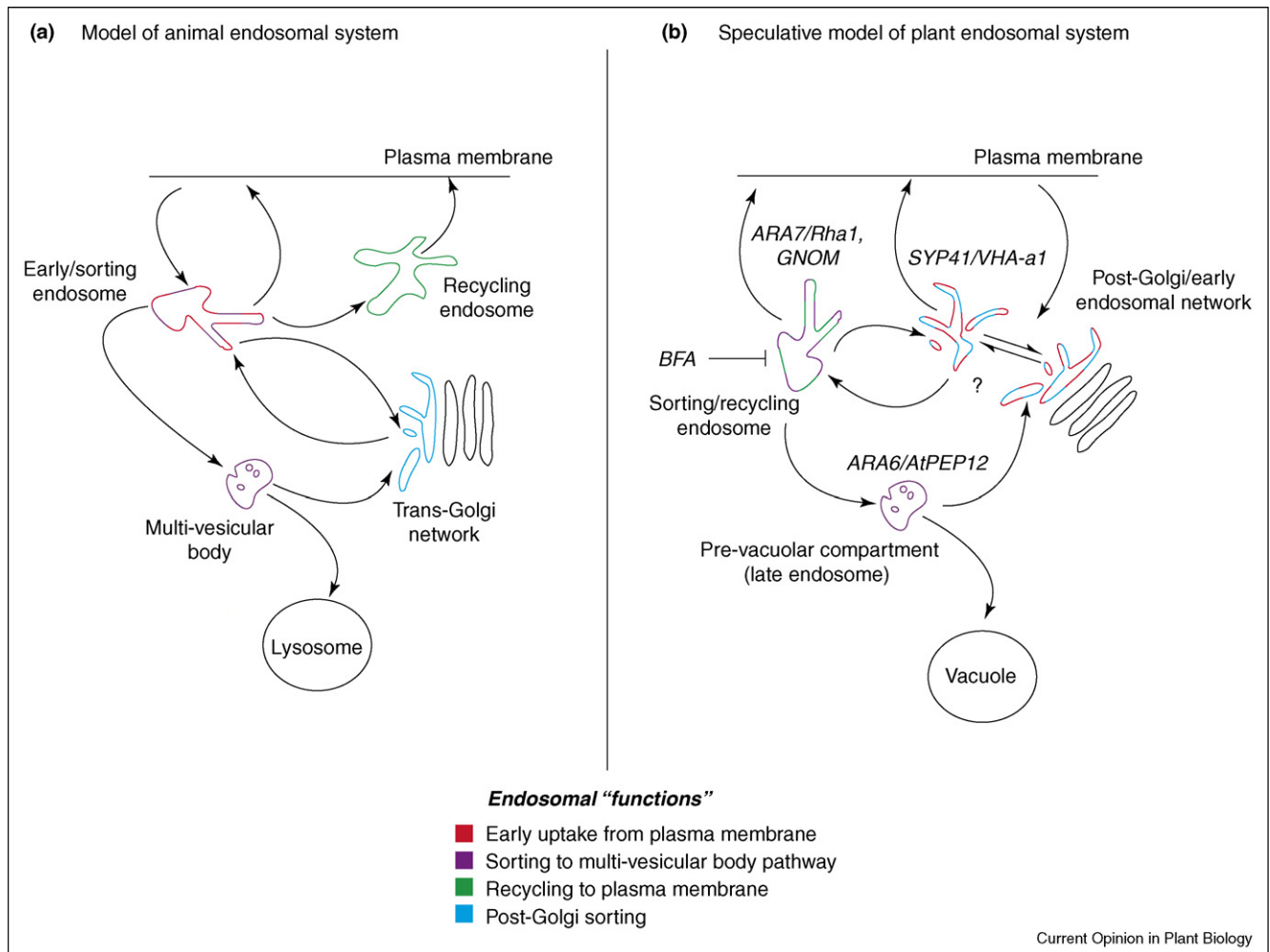
which is proposed to be an early endosomal compartment (see [26] for references).

Endomembrane compartments are functionally defined by the passage of distinct cargo molecules. For example, the fluorescent lipid dye FM4-64 has been used to trace the endocytic route from the plasma membrane to the vacuole, which also demonstrated that ARA6 and ARA7 compartments are ‘endosomal’ [27]. Its close homology to animal rab5-class proteins suggested that ARA7 marks early endosomes in plants. However, recent time-course studies of FM4-64 uptake indicate that vacuolar H<sup>+</sup>-ATPase subunit a1 (VHA-a1), which co-localises with SYP41 rather than ARA7, marks the ‘early endosomal’ compartment in plants [25••]. This suggests that the early endosomal and the late secretory functions might be performed by a single plant endomembrane compartment – a network often or always associated with the trans-side of the Golgi (TGN; Figure 1). By contrast, ARA7 highlights an endosomal compartment at which the adenosine ribosylation factor (ARF) guanine-nucleotide exchange factor GNOM acts to mediate the recycling of the auxin-efflux regulator PIN-FORMED1 (PIN1) to the plasma membrane [24,28]. Dominant-negative variants of ARA7 or RHA1 block the transport of vacuolar proteins from the Golgi to the vacuole [29,30], and ARA7 has been co-localized with pre-vacuolar, multi-vesicular body markers such as syntaxin SYP21 and the vacuolar-sorting receptor VSR [30,31]. However, other studies suggest that ARA7 and SYP21 label two different compartments [23,24]. These conflicting results are difficult to interpret within the animal model of endosome organization. We thus propose an alternative, speculative model in which plant early endosomes (probably corresponding to TGN) lack the degradation or recycling sorting function, which instead is associated with recycling endosomes (Figure 1).

### Endocytosis and auxin transport

In animals, the significance of endocytosis has been uncovered by investigating the trafficking of various model receptors in cell cultures and has only slowly been recognized in multi-cellular development. In plants, by contrast, the functional importance of endocytic trafficking was directly inferred from the analysis of developmental mutants. Mutants that are affected in the *GNOM* gene display severe tissue and organ polarity defects, resembling those caused by genetic or pharmacological interference with polar auxin transport [32]. Polarity of auxin transport is achieved by the coordinated localisation of efflux regulators of the PIN family to one end of cells [33], which requires continuous cycling between the plasma membrane and endosomal compartments [28]. PIN1 recycling and auxin transport are inhibited by brefeldin A (BFA), which interferes with vesicle budding by inhibiting GNOM and other ARF guanine-nucleotide exchange factors [34]. Engineered BFA-resistant variants

Figure 1



Functional organization of the endosomal system in plants as compared to that in animals. **(a)** Simplified model of the animal endosomal system. Note bi-functional early/sorting endosome (red/purple colour). **(b)** Speculative model of the plant endosomal system with altered functional organization, as indicated by specific markers. The early endosome appears to lack the sorting function and to have merged with the secretory TGN (post-Golgi network; red/blue colour). The sorting function would reside in the same compartment as that mediating recycling to the plasma membrane (green/purple colour). It is not known whether the sorting into the multivesicular body pathway generates or merely contributes to the prevacuolar compartment (purple) [54], from which a retrograde trafficking pathway seems to lead to the 'TGN' [55\*].

of GNOM render PIN1 localisation resistant to BFA, abolishing the BFA-sensitivity of auxin transport and plant growth [28]. Thus, GNOM mediates the recycling of PIN1 to the plasma membrane, which is necessary for the correct spatial distribution of auxin in the plant.

Auxin has long been proposed to promote its own transport in a positive feedback loop, which would induce the axial and polar organization of plant organs [32,35,36]. How might auxin canalise its own transport? A recent groundbreaking paper demonstrates that PIN1 endocytosis is inhibited by acute treatment with auxins, or in plants that have genetically increased auxin levels [37\*\*]. Moreover, this feedback occurs in a developmentally relevant context. During gravi-stimulation, for example,

endocytosis of the PIN2 auxin efflux carrier is diminished in the auxin-accumulating cells at the lower side of the root. In another study, increased endocytosis of PIN2 was demonstrated at the upper side of gravi-stimulated roots [38\*]. These findings suggest that the levels of auxin that are present in an unstimulated root significantly limit the endocytosis of PIN2, such that a drop in auxin levels leads to increased endocytosis at the upper side and auxin accumulation drastically reduces endocytosis at the lower side. In addition, auxin exposure eventually leads to increased degradation of PIN2, probably through ubiquitin-mediated sorting to the vacuole [38\*]. Together, these mechanisms might enable the flow of auxin to be reset once the root has re-aligned its growth to the gravity vector.

## Endocytosis and metal transporter trafficking and function

A vast array of metal transporters ensure the correct uptake and distribution of the appropriate metals throughout the plant [39]. These transporters have to be tightly controlled and adapted to the conditions encountered in the soil. For example, accumulation of the iron-uptake carrier IRT1 is strictly dependent on low iron conditions, even when expressed from the constitutive 35S promoter [40]. This indicates post-transcriptional control, which might involve the modulation of translational efficiency or substrate-induced degradation of the transporter by endocytic targeting to the vacuole. The latter mechanism has been demonstrated for AtBOR1, a boron uptake carrier [41•]. AtBOR1 accumulates at the plasma membrane in boron-starved cells, whereas toxic levels of extrinsic boron supply rapidly result in the internalisation and disappearance of the transporter. Interestingly, BFA treatment traps the boron transporter in intracellular compartments, suggesting that AtBOR1 is continuously re-cycled in the absence of boron. However, boron appears to inhibit this re-cycling and instead promotes the sorting of AtBOR1 into the multivesicular body pathway for degradation in the vacuole. It will be interesting to see how and where boron determines the turn-over of its transporter.

## Receptor trafficking in plants

Endocytosis research in plants has long been hampered by the lack of cognate receptor–ligand pairs that are amenable to cell-biological investigation. Plant receptors that have known ligands, such as the shoot meristem regulators CLAVATA1/CLAVATA3 [42] or the self-incompatibility mediators SRK (for *S*-locus receptor kinase)/SCR (for *S*-locus cysteine-rich) [43], were identified by genetic screens and are expressed in tissues that are challenging for cell biologists. By contrast, *Arabidopsis* CRINKLY4 (ACR4) is a widely expressed receptor that has a clear developmental phenotype. ACR4 localises to both endosomal and secretory compartments, in addition to the plasma membrane [44]. Inhibitor studies suggest that ACR4 has a significant turn-over, which does not require an active kinase domain. Although interesting, the absence of both cognate ligand and immediate downstream targets currently limits the biological relevance of these findings.

The brassinosteroid receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) is widely expressed, binds a known ligand and has a fairly well-understood signal transduction pathway. Thus, it has become a model for the activation and transduction mechanisms of the vast family of leucine-rich repeat (LRR) receptor kinases [45]. Förster resonance energy transfer (FRET) analysis of transfected cowpea protoplasts demonstrated that BRI1 and its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1; also known as

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3 [SERK3]) interact in endosomal structures but only in sub-domains of the plasma membrane [46]. Intriguingly, co-expression of both BRI1 and BAK1 led to their preferential accumulation in internal structures, suggesting that interaction of BAK1 with BRI1 regulates their endocytosis. It is unclear, however, whether cowpea protoplasts represent a functional system for brassinosteroid perception and BRI1–BAK1 interaction in endosomes. In addition, effects of BAK1 on BRI1 trafficking have not been confirmed in plants. Nonetheless, these findings raise the possibility that BRI1 signalling might occur from endosomes, as BAK1 is a necessary downstream component of BRI1 signalling [47,48].

Perhaps the best, yet indirect, evidence for endocytosis-mediated signalling of receptors comes from investigations of the plant immune response. Early attempts to visualize receptor-mediated endocytosis in plants were made with fluorescein-labelled elicitor preparations [49]. Although the uptake of this elicitor was time- and temperature-dependent, its receptor was not identified. Recently, a non-kinase receptor-like protein (RLP) for a fungal elicitor in tomato was shown to have a putative tyrosine-based endocytosis signal in the cytoplasmic tail that is necessary for elicitor perception and response [50]. Unfortunately, neither was it demonstrated that mutation of this signal indeed interferes with endocytosis nor can it be excluded that the mutation simply interferes with the interaction of downstream components at the plasma membrane. A genuine breakthrough came from the investigation of the *Arabidopsis* LRR receptor FLAGELLIN SENSITIVE2 (FLS2), which binds a flagellin peptide. FLS2 recognizes a ‘pathogen-associated molecular pattern’ (PAMP) and elicits the induction of basal defence responses [51]. Functional green fluorescent protein (GFP) fusions of FLS2 localize to the plasma membrane in leaf cells. Stimulation with a flagellin peptide promotes the accumulation of FLS2–GFP in endosomal structures, which is dependent on a biologically active ligand [52•]. Furthermore, it is an activated, phosphorylated form of the receptor that is preferentially endocytosed. FLS2 is the first plant receptor for which ligand-induced endocytosis has been demonstrated, and it will be intriguing to see how the endocytosis of FLS2 is linked to its signalling activity. Moreover, FLS2, together with AtBOR1, provides an urgently needed, biologically relevant model protein whose endocytosis can be easily induced and visualized. This will obviate the necessity to use BFA, which has been an important tool in investigating endocytosis but is also problematic because it alters the function and structure of the very compartment it is used to investigate.

## Conclusions

Endocytosis in plants has been established beyond doubt in the past few years. Several plasma membrane proteins

have been shown to undergo constitutive endocytosis and recycling, whereas others appear to be internalised by ligand-induced endocytosis and sorted to the multivesicular body pathway. Where and how the sorting between recycling and degradation takes place is still unclear. Additional markers and time-course studies of endocytosed proteins are needed to resolve this issue.

Another crucial issue concerns the significance of endocytosis in receptor signalling. To date, the evidence in favour of receptor endocytosis as a prerequisite for signalling in plants is circumstantial. Considering the differences in the endosomal organization and receptor repertoires of plants and animals, we cannot conclude with any certainty that endosomes also serve as signalling compartments in plants. Unlike many animal receptors, for example, the yeast model receptor Ste3p has been shown rather unequivocally not to require endocytosis for signalling [53]. It is not immediately obvious why endosomal signalling has evolved in the animal lineage. Nonetheless, it is tempting to speculate that it might overcome the limitations of diffusion-based signal transduction cascades in larger and more complex eukaryotic cells. Signalling from endosomes might have several advantages, such as immediate receptor downregulation after signalling, better shielding against inadvertent cross-activation of unrelated downstream elements, and efficient subcellular distribution of activated receptors by highly mobile endosomes. Plant cells should have similar problems related to cell size and complex signal transduction networks. Therefore, it is possible that endosomal signalling is also a reality in plants, possibly as part of a machinery that is conserved in animals and plants but more likely as an independent solution to the same mechanistic challenges.

## Acknowledgements

N.G. is recipient of a "Human Frontier Science Program" (HFSP) post-doctoral fellowship. Research in the laboratory of G.J. is funded by the Deutsche Forschungsgemeinschaft.

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