Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set

Niko Geldner^{1,2,*}, Valérie Dénervaud-Tendon², Derek L. Hyman¹, Ulrike Mayer³, York-Dieter Stierhof³, and Joanne Chory¹

¹Howard Hughes Medical Institute and The Salk Institute, Plant Biology Laboratory, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA ²University of Lausanne, Department of Plant Molecular Biology, UNIL-Sorge, 1015 Lausanne, Switzerland ³ZMBP, University of Tübingen, Auf der Morgenstelle 3, 72076 Tübingen, Germany

SUMMARY

Plant membrane compartments and trafficking pathways are highly complex, and are often distinct from those of animals and fungi. Progress has been made in defining trafficking in plants using transient expression systems. However, many processes require a precise understanding of plant membrane trafficking in a developmental context, and in diverse, specialized cell types. These include defense responses to pathogens, regulation of transporter accumulation in plant nutrition or polar auxin transport in development. In all of these cases a central role is played by the endosomal membrane system, which, however, is the most divergent and ill-defined aspect of plant cell compartmentation. We have designed a new vector series, and have generated a large number of stably transformed plants expressing membrane protein fusions to spectrally distinct, fluorescent tags. We selected lines with distinct subcellular localization patterns, and stable, non-toxic expression. We demonstrate the power of this multicolor ‘Wave’ marker set for rapid, combinatorial analysis of plant cell membrane compartments, both in live-imaging and immunoelectron microscopy. Among other findings, our systematic co-localization analysis revealed that a class of plant Rab1-homologs has a much more extended localization than was previously assumed, and also localizes to trans-Golgi/endosomal compartments. Constructs that can be transformed into any genetic background or species, as well as seeds from transgenic *Arabidopsis* plants, will be freely available, and will promote rapid progress in diverse areas of plant cell biology.

Keywords

membrane compartments; vesicle trafficking; subcellular marker lines; live imaging

*For correspondence (fax +41 21 692 41 95; Niko.Geldner@unil.ch).

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INTRODUCTION

One of the major challenges for biologists in the post-genome era is to understand the precise functions of proteins within different cell types. A central determinant of protein function is the dynamic localization to distinct subcellular compartments, an aspect that is insufficiently resolved by biochemical fractionation. In plants, analysis of membrane compartments is difficult, because of their complexity, small size and dispersed arrangement, and a comprehensive set of proteins to mark these compartments is not yet available. Earlier attempts to generate such sets had limited success, being restricted to random cDNA fusions that did not allow for the parallel generation of multiple colors (Cutler et al., 2000). Other, more recent attempts have been limited in scope, and have focused on compartments that are already well defined (Nelson et al., 2007). Moreover, past and current vector systems often cause intolerable levels of overexpression in plants, a high incidence of gene silencing, and contain fluorescent proteins and tags that are incompatible or suboptimal for co-localization studies. Compartment mapping efforts in transiently transfected protoplasts have been useful; see Uemura et al. (2004), for example. Co-localization in these systems, however, is notoriously difficult, because wall-less protoplasts are an exceptionally artificial condition, and gene expression is both strong and variable, with no way to minimize the potential marker expression artifacts on membrane structures. In addition, protoplasts and other cellular systems cannot be used to analyze processes in a developmental context or a specific cell type.

RESULTS

We made a new series of plant transformation vectors that enable the rapid, parallel generation of differentially colored fluorescent protein (FP) fusion constructs (pNIGEL vectors; Figure 1a,b and Figure S1; Table 1). This vector set overcomes a number of the limitations of the vectors that are currently available, and provides the maximum versatility of the generated lines (Figure 1a). Fusion proteins can be generated using sequence-verified open-reading frames from the publicly available U-clone collection. This genome-scale collection enables the transfer of full-length ORFs by a one-step recombination procedure, using the univector plasmid fusion (UPS) system (Liu et al., 1998; Yamada et al., 2003). FPs with high spectral compatibility and increased sensitivity were selected (Rizzo et al., 2004; Shaner et al., 2004; Ai et al., 2007), and epitope tags were added for use in immuno-EM and biochemical fractionations (Figure 1a). The endogenous, intron-bearing promoter UBQ10 was used for ubiquitous expression at moderate levels (Norris et al., 1993), in order to minimize overexpression artifacts, and to avoid numerous problems associated with the widely used, viral 35S promoter, such as silencing, variable expression and expression bias. Lastly, vectors with different FPs also carried different resistance markers, enabling easy selection in genetic crosses, as well as in sequential transformations.

We used the YFP-bearing pNIGEL07 to generate fusion constructs of marker candidates from a selected set of 144 pUNI clones. Candidates were chosen based on predictions to localize to specific membranes and to tolerate fusions to their N-termini. About 100 recombined constructs were obtained after two rounds of recombination, and were then used for plant transformation. We selected lines that showed sufficiently strong and distinct

localization patterns, had no obvious developmental abnormalities and were fertile. A total of 63 marker lines, most of which are now available as homozygous seeds in the T₃ or T₄ generations, are now available. We named this marker collection ‘Wave lines’, as an allusion to the dynamic and constantly flowing nature of subcellular membrane ‘compartments’.

Table 2 gives an overview of the final Wave-line set. The signal strengths of the yellow and red variants were good, whereas the blue variants (carrying mCerulean) gave rather weak signals. Although these lines can be used, we sought an alternative in the blue/green range of the spectrum. We generated a set of fusion constructs with the recently described mTFP1, and are currently generating the respective transgenic plant lines (Ai et al., 2007). We found that the UBQ10 promoter drives expression in all cell types, organs and tissue layers investigated (Figure 1c). Importantly, UBQ10 showed very homogenous expression in the shoot meristem and primordia (Z. Nimchuk, personal communication), and was also detected at developmental stages in which the 35S promoter is useless (e.g. early embryos; Figure 1c). As expected, expression levels from UBQ10 were moderate, but were still readily detectable. Only a few cases of ‘patchy’, variable expression in root meristems were observed, which is indicative of transgene silencing, and is often observed in 35S marker lines. Also, for the large majority of lines, expression in the homozygous T₄ generation still did not show any sign of silencing. Figure 2(a) shows the signals of YFP variants of the Wave marker set in epidermal root meristem cells at subcellular resolution in homozygous T₃ lines.

Most of the selected Wave lines represent Rab (six of the eight major subgroups) or SNARE proteins, with a few exceptions (Table 2). Rab GTPases, as well as SNAREs, regulate membrane identity and fusion, and therefore represent good compartment ‘tags’. Some markers localized to morphologically distinct or otherwise characterized compartments, although many labeled unknown or poorly defined compartments (Figure 2a). To assess how well endosomal compartments were represented in our set, we employed the widely used endocytic tracer FM4-64. Endosomes are the most relevant compartments with respect to cellular communication and interaction with the environment; they are also the most divergent and least defined of plant membrane systems. We did an extensive, quantitative co-localization of the red FM4-64 dye with our yellow variants after short (5 min) and long (60 min) periods of dye uptake. The entire panel is shown in Figure S2, with selected examples presented here in Figure 3a,b. Many of the Wave lines showed significant, partial co-localization with FM4-64 after 5 min of uptake (Figures S2 and 3d), suggesting a good coverage of endosomal subcompartments. Intriguingly, our first simple survey with FM4-64 also yielded some surprising results. We found a significant co-localization of FM4-64 with representatives of the RabD class (RabD2a and RabD2b; Wave lines 29 and 33, respectively), which was increased upon treatment with brefeldin A (BFA; see below). The mammalian Rab1 family shows closest similarity to plant RabDs, and Rab1s are known as regulators in endoplasmic reticulum (ER)–Golgi transport (Allan et al., 2000). Analysis of RabD function by transient expression in tobacco strongly indicated that this function is conserved in plants (Batoko et al., 2000). The additional endosomal localization of RabDs in our survey now suggests a possibly much more extended role of Rab1 homologs in plant cells, also acting in endosomal trafficking.

Parallel visualization of compartment responses to chemical compounds

The Wave lines should be useful for rapidly assessing the effects of new chemical compounds on plant cell structure and function, and they will facilitate new types of chemical screens that rely on the parallel assessment of drug activities, by scoring multiple markers at the same time. To test the power of our lines for characterizing drug action, we did a parallel and quantitative analysis of the effects of the fungal toxin BFA. BFA is an inhibitor of endosomal trafficking in Arabidopsis (Geldner et al., 2001), and has become a major tool to manipulate and define plant endosomes (Geldner et al., 2003; Richter et al., 2007). Golgi stacks in Arabidopsis roots are largely insensitive to BFA, and tend to decorate the central BFA-induced aggregate in which endosomal compartments accumulate to varying degrees. However, differences between reports, marker lines, BFA concentrations and time scales make it difficult to more precisely define endosomal compartments, based on their responsiveness to the drug. The Wave lines allow a direct comparison of compartment sensitivities under precise conditions, performed in a rotating schedule of a few minutes for each observation. We compared the quantitative co-localization of each marker with FM4-64 between a 60-min BFA treatment and an untreated control (Figure 3b,c). The entire set of BFA treatments is shown in Figure S2. Our analysis demonstrates that low concentrations of BFA reliably distinguish between specific subclasses of endosomal compartments. The parallel acquisition facilitated the quantitative analysis of co-localization. The intensity correlation analysis of our data set, with and without BFA (Figure S2 and Figure 3d), revealed robust response patterns. For example, late endosomal markers (RabF class, Wave lines 2 and 7, respectively) showed response patterns that were distinct from Golgi markers (Wave lines 18, 22 and 127). Both show little co-localization with FM4-64, and do not shift into BFA-induced endosomal aggregations. Golgi markers (Wave line 18), however, separate more readily from the BFA-induced FM4-64 patch (Figure S2 and Figure 3c,d). Surprisingly, in our quantitative comparison, only Golgi markers, but not late endosome or other markers, showed some time-dependent increase in co-localization with FM4-64. In our view, this questions the simple model whereby FM4-64 only follows endocytic trafficking down to the vacuole. The trans-Golgi network (TGN) marker Wave line 13 (VTI12) is the only one to co-localize strongly with early FM4-64 uptake (Figure 3c,d), consistent with the current model of the plant TGN also being an early endosomal (EE) compartment (Dettmer et al., 2006). Importantly, our comparative analysis distinguishes one compartment type, marked by A1-class Rabs (Rab11-like, and Wave lines 34 and 129; data for 129 not shown) from all other compartments as having the most striking sensitivity to BFA. Under our conditions, only this type of marker shows complete aggregation into BFA compartments, although it only partially co-localizes with FM4-64 in the absence of BFA (Figure S2 and Figure 3c,d). This is in clear contrast to the TGN/EE marker Wave line 13, and many other markers. Similarly strong localization in the central BFA patch is only observed with recycling plasma membrane cargo, like the PIN1 auxin efflux carrier, for example. Therefore, we suggest that A1-class Rabs could be markers for a type of recycling endosome in plant cells. A recent paper implicated Rabs of the A2 and A3 subclass in cytokinesis, and reached similar conclusions about their localization. This paper pointed out that the A2/A3 compartments are earlier on the endocytic pathway than RabF2 compartments, which is entirely consistent with our comparative observations of RabA1 and RabF compartments (Chow et al., 2008).

Rapid combinatorial mapping of compartments using Wave lines

As a test of our Wave lines for multicolor imaging, and to further characterize the RabD- and RabA-class compartments, we crossed a set of five YFP-Wave (Y) lines with five mCherry-Wave (R) lines. Of the 25 possible combinations, 22 gave rise to F₁ progeny. The results are presented in Figure S3. Examples are given in Figure 3(e). From this first pass, we conclude that the RabD membranes overlap with, but extend beyond, Golgi stacks, and that they are distinct from RabA and RabG-labeled compartments (Figure 3e and Figure S3). Independent co-localization of Wave line 29Y (RAB-D2a) with ST-RFP and VHA-a1-RFP (labeling Golgi and TGN/early endosomes, respectively) confirm this notion (I. Moore, unpublished data). BFA treatment of the Wave line 33Y/Wave line 18R (RabD2b/Got1p Golgi marker) double marker line showed that the Rab1-homolog Wave line 33Y (RabD2b) accumulates into the core of the BFA patches, whereas only part of the label remains co-localized with the Golgi marker. This confirms the notion that, in contrast to animal cells, localization of Rab1 homologs in plants extends into the endosomal system. In addition, our systematic co-localization allows for some combinatorial definition of the RabD2b (Wave 33Y) compartment. Both RabD2a and RabA1e (Wave line 34Y) localize to a compartment in the proximity of the Golgi, and could possibly mark the same Golgi-associated endosomal compartment. However, the co-localization of Wave line 33Y with Wave line 34R (Figure 3e and Figure S3) clearly shows that both are again distinct from each other. This type of ‘triangulation’ should, and will, eventually be replaced by three-color analyses, adding blue Wave lines to the cross. However, our current analysis already supports the view of an extended Golgi-associated network that is subdivided into a number of functionally distinct domains.

Wave lines as markers for immunoelectron microscopy

Although our marker set will extend our ability to define and map subcellular structures in live-cell imaging, there are immanent limits of resolution, which will restrict the size and number of functional membrane subdomains that can be reliably defined. Because of this, it is important that the same marker lines used in live-cell imaging can be used for immunoelectron-microscopy analysis, which will allow the straightforward integration of the high-resolution structural and morphological information that can be acquired with immunoelectron-microscopy techniques. Figure 3(f) shows an immunoelectron-microscopy picture of Wave line 33Y (RabD2b) using GFP antibodies. The micrograph confirms the unexpected localization of RabD2b to non-Golgi compartments. Clearly, the strongest label can be observed at tubular compartments in proximity to the trans side of the Golgi, which is the expected localization for an endosomal compartment. Additional pictures are presented in Figure S4(a). As seen in Figure 3(g), immunoelectron-microscopy localization can also be performed on the mCherry-tagged set using the anti-RFP antibody. Staining in the Wave line 18R is largely confined to Golgi stacks; in contrast to Wave line 33Y. A very similar label was obtained when using myc or GFP antibodies on the YFP-tagged Wave line 18 (Figure S4c,d). This demonstrates that the co-localization of different markers using immunogold will be possible in red and yellow Wave-line crosses.

DISCUSSION

Our new set of marker lines puts a comprehensive set of subcellular markers at the disposal of plant biologists. The very broad and stable expression of this multicolor marker set strongly facilitates the dissection of subcellular structures, and responses, in a wide range of organs and cell types. It will thus help to move plant cell biology away from functionally inadequate cellular systems, and into the whole-plant system, where a multitude of important biological questions need to be analysed with respect to subcellular organization and trafficking. Examples are plant–pathogen and symbiotic interactions, responses to abiotic stresses or adaptive changes to environmental stimuli. In all of these cases, the cellular responses in a few, specific cell types are the key to understanding the process. We have demonstrated that the multiple fluorescent proteins and tags of the Wave lines enable straightforward compartment mapping and co-localization with genes of interest, as much in live imaging as in immunoelectron microscopy. This will facilitate the integration of data from different levels of resolution. The new vectors we developed can take advantage of public ORF collections, and provide a fast and cost-efficient way for generating translational fusions with improved fluorescent proteins in plant-expression vectors. This will catalyze the generation of entire sets of marked proteins and protein families, and further promote our understanding of the conserved and specific features of plant subcellular organization.

EXPERIMENTAL PROCEDURES

Plants and growth conditions

The transformation of plants was performed in the Columbia eco-type. For analysis, plants were grown upright on half-strength MS Agar plates in Percival chambers at 22°C, under long days (16 h light/8 h dark), and were used at 4–6 days after germination.

Construction of pNIGEL host vectors

pNIGEL vectors are based on the pGREENII vector series (Hellens et al., 2000; <http://www.pgreen.ac.uk>). The kanamycin resistance of the pGreenII vectors was replaced by ampicillin resistance of pBluescript. The fluorescent protein coding sequences used were EYFP and ECFP (Clontech, <http://www.clontech.com>), mCherry and mOrange (Shaner et al., 2004), Cerulean (Rizzo et al., 2004) and mTFP1 (Ai et al., 2007). These were cloned into lox recombination cassettes obtained from Steve Elledge's laboratory (<http://elledgelab.bwh.harvard.edu>) using standard cloning procedures. Sequences of all vectors have been submitted to GenBank (see Table 1 for accession numbers), and can also be downloaded from (http://www.unil.ch/dbmv/page49637_en.html). DNA stocks and transgenic lines will be made available through the ABRC (<http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm>).

CRE/lox recombination cloning

CRE/lox recombination cloning was performed according to the protocol described by Liu et al. (1998). A 500-ng portion of host vector DNA was used with an equal quantity of the open reading frame (ORF)-bearing pUNI vector, and then incubated with 1 U of Cre recombinase for 30 min in the supplier's buffer (New England Biolabs, <http://www.neb.com>).

www.neb.com). The reaction was transformed into DH5a cells after heat inactivation for 10 min at 70°C. Transformants were selected on carbenicillin/kanamycin plates. Generally, only a few colonies were recovered per reaction/transformation, but they were always found to contain the fusion product upon sequence confirmation.

Plant transformation

Plant transformation was performed essentially as described by Clough and Bent (1998), with the following modifications for increased throughput. pSOUP containing GV3101 *Agrobacteria* (see <http://www.pgreen.ac.uk> for information on pSOUP) were transformed with the recombined constructs, and then grown to high density in 50-ml cultures, allowing growth of up to 48 clones per shaker. Pellets were re-suspended into 200 ml of transformation medium, which provided sufficient volume for dipping. Recombined plasmids contain both ampicillin and kanamycin resistance. Kanamycin was used for selection in *Agrobacteria*.

Wave-line selection and confirmation

The initial screening for signals was performed on YFP-bearing Wave lines. Observation was carried out on non-selecting plates with segregating T₂ seedlings. Between three and six independent transformants per construct were scored, and those with the best expression levels were chosen for propagation. The lines were selected for those that showed distinct signals resembling membrane compartment structures, and which could be propagated until T₃, displaying good seed yield, consistent signals and no obvious developmental abnormalities. From those, red and blue spectral variants were generated. In these cases the lower number of transformants allowed us to score for up to 16 individual transformants per construct. This was especially necessary for the Cerulean-bearing Wave lines, which displayed much weaker signals on average than the mCherry and YFP-bearing lines. Homozygous lines were selected, and the presence of the correct insert was verified by PCR amplification/sequencing from plant tissues. The amplification from genomic DNA for YFP and Cerulean lines was performed with the primer pair: 5'-CTGGGGCACAAGCTGGAGTACA-3' (sense, in YFP/CER-ORF) and 5'-GACAGTGGGAGTGGCACCTTCC-3' (antisense, in the 3' untranslated region). The sequencing of the PCR product was carried out with the primer 5'-CTGCCCCGACAACCACTACCTGA-3' (sense, in YFP/CER-ORF). For mCherry lines the primers used were as follows: 5'-CAGAGGCTGAAGCTGAAGGACG-3' (sense) and 5'-GACAGTGGGAGTGGCACCTTCC-3' (antisense) for amplification, and 5'-CTGCCCCGACAACCACTACCTGA-3' for sequencing.

Imaging and quantitative co-localization analysis

All imaging was performed on an inverted SP/2 confocal microscope (Leica, <http://www.leica.com>). For quantitative co-localization, pictures of epidermal root meristem cells were taken with detector settings optimized for very low background, no pixel saturation and a complete absence of channel crosstalk. Dual-color images were acquired by sequential line switching, allowing the separation of channels by both excitation and emission. Quantification was performed using the intensity correlation analysis method published in Li et al. (2004), and as implemented in the IMAGEJ plugin of the MBF IMAGEJ bundle

(<http://www.macbiophotonics.ca/imagej/>). Intensity correlation analysis delivers a coefficient that ranges between 0.5 and zero for complete co-localization or non-colocalization, respectively. We found some advantages of intensity correlation analysis over other methods, in that it takes into account the relative intensities of co-localizing pixels, and can be performed without thresholding. We used biological reasoning to help define the significance of co-localization. We argued that positive intensity correlation of pixels (ICQ) values between early FM4-64 uptake and the vacuolar marker Wave line 9Y, which should not show any co-localization, can be taken as the 'background' co-localization caused by insufficient resolution and image noise. Only ICQ rates above that level were considered to be partially co-localized. For this analysis, regions of interest (ROIs) of entire cells, excluding the plasma membrane, were analyzed. Each line and condition is an average of between five and 10 ROIs. Error bars indicate the standard deviation of the mean.

Immunoelectron microscopy

Root tips were fixed with 4% formaldehyde in microtubule-stabilizing buffer (MTSB; 30–60 min), followed by treatment with 8% formaldehyde in MTSB for another 45–90 min. Fixed samples were embedded in 10% gelatin, infiltrated with a mixture of polyvinylpyrrolidone and sucrose (Tokuyasu, 1989), and were then frozen in liquid nitrogen. Thawed ultrathin cryosections were labeled with rabbit anti-RFP antibodies (1:500; MBL Medical & Biological Laboratories Co., Ltd, <http://www.mbl.co.jp>), rabbit-anti-GFP antibodies (1:500; Torrey Pines Scientific, <http://torreypinsscintific.com>) or monoclonal mouse anti-myc antibodies (approximately 5 µg ml⁻¹; 9E10) diluted in blocking buffer. Bound primary antibodies were detected with goat anti-rabbit or anti-mouse IgG coupled to Nano-gold (1:80 in blocking buffer; Nanoprobes, <http://www.nanop-robex.com>). After silver enhancement (HQ silver, 8.5 min; Nanoprobes), labeled sections were stained with uranyl acetate and embedded in methyl cellulose.

Inhibitor and fluorescent tracer treatment

FM4-64 (Invitrogen, <http://www.invitrogen.com>) was applied at a concentration of 3 µM; BFA (Sigma-Aldrich, <http://www.sigmaaldrich.com>) was applied at a concentration of 25 µM. Stock solutions were prepared in DMSO at 3 mM and 100 mM, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ai HW, Shaner NC, Cheng Z, Tsien RY, Campbell RE. Exploration of new chromophore structures leads to the identification of improved blue fluorescent proteins. *Biochemistry*. 2007; 46:5904–5910. [PubMed: 17444659]
- Allan BB, Moyer BD, Balch WE. Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. *Science*. 2000; 289:444–448. [PubMed: 10903204]
- Batoko H, Zheng HQ, Hawes C, Moore I. A rab1 GTPase is required for transport between the endoplasmic reticulum and golgi apparatus and for normal golgi movement in plants. *Plant Cell*. 2000; 12:2201–2218. [PubMed: 11090219]
- Boursiac Y, Chen S, Luu DT, Sorieul M, van den Dries N, Maurel C. Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. *Plant Physiol*. 2005; 139:790–805. [PubMed: 16183846]
- Carter C, Pan S, Zouhar J, Avila EL, Girke T, Raikhel NV. The vegetative vacuole proteome of Arabidopsis thaliana reveals predicted and unexpected proteins. *Plant Cell*. 2004; 16:3285–3303. [PubMed: 15539469]
- Chatre L, Brandizzi F, Hocquellet A, Hawes C, Moreau P. Sec22 and Memb11 are v-SNAREs of the anterograde endoplasmic reticulum-Golgi pathway in tobacco leaf epidermal cells. *Plant Physiol*. 2005; 139:1244–1254. [PubMed: 16244155]
- Choi WG, Roberts DM. Arabidopsis NIP2;1, a major intrinsic protein transporter of lactic acid induced by anoxic stress. *J Biol Chem*. 2007; 282:24209–24218. [PubMed: 17584741]
- Chow CM, Neto H, Foucart C, Moore I. Rab-A2 and Rab-A3 GTPases define a trans-golgi endosomal membrane domain in Arabidopsis that contributes substantially to the cell plate. *Plant Cell*. 2008; 20:101–123. [PubMed: 18239134]
- Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J*. 1998; 16:735–743. [PubMed: 10069079]
- Conchon S, Cao X, Barlowe C, Pelham HR. Got1p and Sft2p: membrane proteins involved in traffic to the Golgi complex. *EMBO J*. 1999; 18:3934–3946. [PubMed: 10406798]
- Cutler SR, Ehrhardt DW, Griffiths JS, Somerville CR. Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. *Proc Natl Acad Sci USA*. 2000; 97:3718–3723. [PubMed: 10737809]
- Dettmer J, Hong-Hermesdorf A, Stierhof YD, Schumacher K. Vacuolar H⁺-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. *Plant Cell*. 2006; 18:715–730. [PubMed: 16461582]
- Geldner N, Friml J, Stierhof YD, Jurgens G, Palme K. Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*. 2001; 413:425–428. [PubMed: 11574889]
- Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jurgens G. The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell*. 2003; 112:219–230. [PubMed: 12553910]
- Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Mol Biol*. 2000; 42:819–832. [PubMed: 10890530]
- Li Q, Lau A, Morris TJ, Guo L, Fordyce CB, Stanley EF. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. *J Neurosci*. 2004; 24:4070–4081. [PubMed: 15102922]
- Liu Q, Li MZ, Leibham D, Cortez D, Elledge SJ. The univector plasmid-fusion system, a method for rapid construction of recombinant DNA without restriction enzymes. *Curr Biol*. 1998; 8:1300–1309. [PubMed: 9843682]
- Nelson BK, Cai X, Nebenfuhr A. A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. *Plant J*. 2007; 51:1126–1136. [PubMed: 17666025]
- Nielsen E, Cheung AY, Ueda T. The regulatory RAB and ARF GTPases for vesicular trafficking. *Plant Physiol*. 2008; 147:1516–1526. [PubMed: 18678743]

- Norris SR, Meyer SE, Callis J. The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. *Plant Mol Biol.* 1993; 21:895–906. [PubMed: 8385509]
- Rancour DM, Dickey CE, Park S, Bednarek SY. Characterization of AtCDC48. Evidence for multiple membrane fusion mechanisms at the plane of cell division in plants. *Plant Physiol.* 2002; 130:1241–1253. [PubMed: 12427991]
- Richter S, Geldner N, Schrader J, Wolters H, Stierhof YD, Rios G, Koncz C, Robinson DG, Jurgens G. Functional diversification of closely related ARF-GEFs in protein secretion and recycling. *Nature.* 2007; 448:488–492. [PubMed: 17653190]
- Rizzo MA, Springer GH, Granada B, Piston DW. An improved cyan fluorescent protein variant useful for FRET. *Nat Biotechnol.* 2004; 22:445–449. [PubMed: 14990965]
- Rutherford S, Moore I. The *Arabidopsis* Rab GTPase family: another enigma variation. *Curr Opin Plant Biol.* 2002; 5:518–528. [PubMed: 12393015]
- Sanderfoot AA, Assaad FF, Raikhel NV. The *Arabidopsis* genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. *Plant Physiol.* 2000; 124:1558–1569. [PubMed: 11115874]
- Sanderfoot AA, Kovaleva V, Bassham DC, Raikhel NV. Interactions between syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the *Arabidopsis* cell. *Mol Biol Cell.* 2001; 12:3733–3743. [PubMed: 11739776]
- Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol.* 2004; 22:1567–1572. [PubMed: 15558047]
- Tokuyasu KT. Use of poly(vinylpyrrolidone) and poly (vinyl alcohol) for cryoultramicrotomy. *Histochem J.* 1989; 21:163–171. [PubMed: 2722561]
- Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K, Sato MH. Systematic analysis of SNARE molecules in *Arabidopsis*: dissection of the post-Golgi network in plant cells. *Cell Struct Funct.* 2004; 29:49–65. [PubMed: 15342965]
- Yamada K, Lim J, Dale JM, et al. Empirical analysis of transcriptional activity in the *Arabidopsis* genome. *Science.* 2003; 302:842–846. [PubMed: 14593172]
- Zheng H, Bednarek SY, Sanderfoot AA, Alonso J, Ecker JR, Raikhel NV. NPSN11 is a cell plate-associated SNARE protein that interacts with the syntaxin KNOLLE. *Plant Physiol.* 2002; 129:530–539. [PubMed: 12068098]
- Zheng H, Camacho L, Wee E, Batoko H, Legen J, Leaver CJ, Malho R, Hussey PJ, Moore I. A Rab-E GTPase mutant acts downstream of the Rab-D subclass in biosynthetic membrane traffic to the plasma membrane in tobacco leaf epidermis. *Plant Cell.* 2005; 17:2020–2036. [PubMed: 15972698]

Figure 1.

New plant expression vectors for the high-throughput generation of fluorescent fusion constructs.

(a) The pNIGEL vector series drives expression in Arabidopsis from the endogenous, intron-bearing UBQ10 promoter. Based on the pGREENII plant transformation vectors, pNIGELs carry an Amp, instead of Kan, resistance for compatibility with the pUNI open reading frame (ORF) library. The loxP site following the different fluorescence-tag sequences allows for translational fusion to the N-terminus of pUNI ORFs.

(b) Schematic diagram of the recombination/selection procedure, as described in Liu et al. (1998). CRE recombinase mediates a single recombination event that leads to fusion of the

pUNI and HOST vectors. Recombined products are selected on Kan/Amp double-selection plates. In addition, the origin of pUNI is incompatible with standard *Escherichia coli* strains. (c) The UBQ10 promoter drives detectable expression in many cell types of interest. The YFP signals from a plasma membrane-localized marker protein are presented here. The expression is homogenous, and shows no sign of patchiness or variability in expression levels, as is often observed in 35S-driven marker lines. Scale bars: early embryo, 10 μm ; all other panels, 50 μm .

Figure 2.

The multicolor Wave markers allow the rapid, parallel visualization of subcellular structures *in planta*.

(a) The YFP-signals of the 20 selected Wave lines all show reproducible staining of membrane compartments, and could all be propagated as homozygotes into the T_4 generation. Root meristem cells at the onset of elongation are shown here. Most of the central membrane compartments, such as Golgi, vacuole or plasma membrane, are covered by multiple markers. In addition, a number of markers highlight compartments of pleiomorphic, dotted appearance, many of which apparently define subpopulations of endosomes. Scale bar: 10 μ m.

(b) Examples of signals of the same compartment marker tagged with Cerulean (blue), YFP (yellow) or mCherry (red). Note that all three fluorophores allow clear spectral separation, based on their excitation/emission characteristics, thereby circumventing the use of spectral-unmixing algorithms. Scale bars: 10 μ m.

Figure 3.

Functional mapping of membrane compartments by quantitative and combinatorial co-localization.

- (a) Co-localization of YFP-labeled compartments (green) with the FM4-64 lipidic, endocytic tracer (red) after 5–10 min of uptake. Some compartments can be clearly defined as co-localizing or non-colocalizing, whereas many show partial co-localization.
- (b) As in (a) but after 60–90 min of FM4-64 uptake.

- (c) Co-localization after co-treatment of FM4-64 with 25 μ M brefeldin A (BFA) for 60 min. Partially FM4-64 co-localizing compartments can be distinguished by their different sensitivity towards the drug, compared with FM4-64. Some partially co-localizing compartments are largely resistant to BFA, whereas others completely aggregate in the BFA compartment, and strongly co-localize with FM4-64.
- (d) Quantification of co-localization based on the images shown in (a–c), measured as intensity correlation of pixels (ICQ), with a value of zero indicating no co-localization, and a value of 0.5 indicating complete overlap. Co-localization measured after 5 min (yellow) and 60 min (orange) of FM4-64 uptake. Note that some compartments show no increase in overlap, or even apparent separation from FM4-64, in response to BFA, whereas others become completely confounded. Also, note the lack of a time-dependent increase of co-localization with FM4-64.
- (e) Co-localization YFP-tagged Wave lines (Y) with mCherry-tagged lines (R), either with or without BFA, treated as in (d). Scale bar for panels in (a–c) (in the upper left panel in a): 10 μ m. Scale bar for (d) (in the leftmost panel): 5 μ m.
- (f) Immunogold labeling of Wave line 33Y (RabD2b) using antibodies against the additional 3 $\times$ myc epitope in the Wave Y lines. Note that most of the label appears in the trans-Golgi network (TGN) region and beyond, whereas only some of the labeling can be seen throughout the stack.
- (g) Immunogold labeling of Wave line 18R (AtGot1p) using antibodies against RFP. Scale bar: 500 nm.

Table 1

Overview of pNIGEL vectors

Vector name	Fluorophore	Additional tag	Plant resistance	Accession number
pNIGEL 07	EYFP	3 × myc	Basta	FJ493465
pNIGEL 09	ECFP	3 × HA	Basta	FJ524330
pNIGEL 11	–	3 × myc	Basta	FJ524331
pNIGEL 13	–	3 × HA	Basta	FJ524332
pNIGEL 15	mCherry	–	Basta	FJ524333
pNIGEL 16	mOrange	–	Basta	FJ524334
pNIGEL 17	mCherry	–	Hygromycin	FJ493466
pNIGEL 18	mCerulean	3 × HA	Kanamycin	FJ493467
pNIGEL 19	mTFP1	3 × HA	Kanamycin	FJ530950

Table 2

Overview of Wave marker lines

Wave number	Name	Name ref.	Ar number	U clone	FM4-64 co-localization	BFA sensitivity	Assignment localization	Loc. ref.	Remarks
1	Tag only	N.A.	N.A.	pUNI51 vector	N.A.	N.A.	Cytosol/nucleus	N.A.	–
2	RabF2b (ARA7)	Rutherford and Moore (2002)	A4g19640	U09899	Partial	Low	Late endosome/pre-vacuolar compartment	Nielsen et al. (2008)	Extensive experimental evidence for localization to multivesicular bodies and function in trafficking to vacuole
3	Rab C1	Rutherford and Moore (2002)	A1g43890	U09677	Partial	Intermediate	Post-Golgi/endosomal	–	No experimental data found
5	RabG3f	Rutherford and Moore (2002)	A3g18820	U10069	Partial	Very low	Late endosome/vacuole	Carter et al. (2004), Nielsen et al. (2008)	Homologs identified in vacuolar proteome
6	NIP1;1	Boursiac et al. (2005)	A4g19030	U11812	Non-significant	Very low	Endoplasmic Reticulum/plasma membrane	Choi and Roberts (2007)	Marker also observed to localize to plasma membrane, possibly upon prolonged immersion
7	RabF2a (Rha1)	Rutherford and Moore (2002)	A5g45130	U12647	Partial	Low	Late endosome/pre-vacuolar compartment	Nielsen et al. (2008)	Extensive experimental evidence for localization to multivesicular bodies and function in trafficking to vacuole
9	VAMP711	Sanderfoot et al. (2000)	A4g32150	U14984	Non-significant	Very low	Vacuole	Carter et al. (2004), Uemura et al. (2004)	Consistent with localization in protoplasts and identification in vacuolar proteome
11	Rab G3c	Rutherford and Moore (2002)	A3g16100	U23198	Non-significant	Very low	Late endosome/vacuole	Carter et al. (2004),	Homologs identified in

Wave number	Name	Name ref.	At number	U clone	FM4-64 co-localization	BFA sensitivity	Assignment localization	Loc. ref.	Remarks
13	VTI12	Sanderfoot et al. (2000)	At1g26670	U60796	Strong	Intermediate	Trans-Golgi network/early endosome	Nielsen et al. (2008)	vacuolar proteome
18	Got1p homolog	TAIR annot.	At3g03180	U63080	Non-significant	Very low	Golgi	Sanderfoot et al. (2001), Uemura et al. (2004)	pUNI clone truncated, fusion product contains only aa 126–222 Localization consistent with that reported for full-length protein
22	SYP32	11	At3g24350	U20852	Non-significant	Very low	Golgi	Conchon et al. (1999)	Localization to Golgi, as described in <i>Saccharomyces cerevisiae</i> ; no experimental data found in plants
24	Rab A5d	Rutherford and Moore (2002)	At2g31680	U22946	Partial	High	Endosomal/recycling endosome	Rancourt et al. (2002), Uemura et al. (2004)	Consistent with localization in protoplasts, possibly different from SYP31
25	Rab D1	Rutherford and Moore (2002)	At3g11730	U50900	Partial	Intermediate	Post-Golgi/endosomal	Chow et al. (2008)	See text
27	Rab E1d	Rutherford and Moore (2002)	At5g03520	U09494	Partial	Intermediate	Post-Golgi/endosomal	–	Similar to Wave lines 29 and 33
29	Rab D2a	Rutherford and Moore (2002)	At1g02130	U13716	Partial	Intermediate	Golgi/endosomal	Zheng et al. (2005)	Different from reported Golgi localization by transient expression in tobacco cells
								Nielsen et al. (2008)	Broad localization, clearly extending beyond Golgi into endosomal compartments, see text

Wave number	Name	Name ref.	A# number	U clone	FM4-64 co-localization	BFA sensitivity	Assignment localization	Loc. ref.	Remarks
33	Rab D2b	Rutherford and Moore (2002)	A5g47200	U10162	Partial	Intermediate	Golgi/endosomal	Nielsen et al. (2008)	Broad localization, clearly extending beyond Golgi into endosomal compartments, see text
34	Rab A1e	Rutherford and Moore (2002)	A4g18430	U63334	Partial	High	Endosomal/recycling endosome	Chow et al. (2008)	See text, also strong cell-plate localization; data not shown
127	MEMB12	Sanderfoot et al. (2000)	A5g50440	U50585	Non-significant	Very low	Golgi	Uemura et al. (2004), Chatre et al. (2005)	Consistent with reported localization of MEMB11 to Golgi in tobacco cells
129	Rab A1g	Rutherford and Moore (2002)	A3g15060	U51331	Partial	High	Endosomal/recycling endosome	Chow et al. (2008)	See text, also strong cell-plate localization; data not shown
131	NPSN12	Sanderfoot et al. (2000)	A1g48240	U60291	N.A.	N.A.	Plasma membrane	Zheng et al. (2002)	Different from reported NPSN11 localization to the cell plate
138	PIP1;4	Boursiac et al. (2005)	A4g00430	U16966	N.A.	N.A.	Plasma membrane	Boursiac et al. (2005)	Consistent with reported PM localization of PIP1;1