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RESEARCH PAPER

Vacuolar system distribution in *Arabidopsis* tissues, visualized using GFP fusion proteins

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Abstract

Green fluorescent protein (GFP) allows the direct visualization of gene expression and the subcellular localization of fusion proteins in living cells. The localization of different GFP fusion proteins in the secretory system was studied in stably transformed *Arabidopsis* plants cv. Wassilewskaja. Secreted GFP (SGFP) and GFP retained in the ER (GFP-KDEL) confirmed patterns already known, but two vacuolar GFPs (GFP-Chi and Aleu-GFP) labelled the *Arabidopsis* vacuolar system for the first time, the organization of which appears to depend on cell differentiation. GFP stability in the vacuoles may depend on pH or degradation, but these vacuolar markers can, nevertheless, be used as a tool for physiological studies making these plants suitable for mutagenesis and gene-tagging experiments.

Key words: *Arabidopsis*, development, GFP, soluble protein sorting, vacuole biogenesis.

Introduction

The green fluorescent protein (GFP) allows the direct visualization of gene expression and also of the subcellular localization of fusion proteins in living cells, without the need for destructive techniques as required for β-glucuronidase, or the addition of cofactors as required for the luciferase assay (Chalfie *et al*., 1994; Haseloff *et al*., 1997; Misteli and Spector, 1997). The study of the secretory system is a good example of the new possibilities offered by the use of GFP *in vivo*. The distinct locations of different GFP fusion proteins in the secretory system of stably transformed plants confirmed the complexity of this membrane system (Boevink *et al*., 1998; Davies and Vierstra, 1998; Di Sansebastiano *et al*., 2001; Essl *et al*., 1999; Haseloff *et al*., 1997; Llopis *et al*., 1998).

The vacuolar system in plant cells has recently been shown to consist of different compartments characterized by specific tonoplast intrinsic proteins (TIPs) (Paris *et al*., 1996; Jauh *et al*., 1999; Jiang *et al*., 2000). The sorting of vacuolar soluble proteins has provided evidence of alternative pathways (Vitale and Raikhel, 1999; Matsuoka and Neuhaus, 1999). Soluble GFPs have been used to label at least two different compartments, the lytic vacuole (LV) (Di Sansebastiano *et al*., 2001) and the protein storage vacuole (PSV) (Di Sansebastiano *et al*., 1998, 2001).

The formation and evolution of single vacuoles in plant cells is a complex phenomenon. It is evident that different vacuoles can fuse and change the characteristics of their content as well as their tonoplasts (Jauh *et al*., 1998, 1999). Vacuolar GFPs have been successfully used in protoplasts and mini-protoplasts (Di Sansebastiano *et al*., 1998, 2001), but the production of protoplasts is likely to induce changes in function and composition of pre-existing vacuoles. Therefore, it is important to investigate the organization of the vacuolar system during differentiation in order to clarify its role in metabolic processes. In the past, ontogeny of vacuoles has been studied by microscopical observation (Moore and Smith, 1990), but this study was limited by the lack of knowledge on vacuole content sorting. After years of study on vacuolar protein sorting (Neuhaus *et al*., 1991; Holwerda *et al*., 1992;

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Schroeder *et al*., 1993; Matsuoka *et al*., 1995; Paris *et al*., 1996), reporter proteins have been developed and microscopy can be used with these new tools.

With the aim of clarifying the organization of the vacuolar system in different plant tissues, transgenic *Arabidopsis thaliana* plants expressing various GFP fusion proteins targeted to the secretory system were produced and analysed.

Materials and methods

Fusion gene construct

GFP variants expressed in *A. thaliana* were identical to GFPs expressed and described previously in tobacco protoplasts. SGFP, GFP-KDEL and GFP-Chi are encoded by the genes in plasmids pSGFP5, pSGFP5K and pSGFP5T (Di Sansebastiano *et al*., 1998), Aleu-GFP corresponds to Aleu-GFP6 (Di Sansebastiano *et al*., 2001). These modified genes were inserted into the binary vector pBin (Frisch *et al*., 1995; Haseloff *et al*., 1997) between *Bam*HI and *Sac*I restriction sites.

Constructs were prepared in *E. coli* (strain XL1) and transferred to *Agrobacterium tumefaciens* (strain GV3101) by triparental mating using an *E. coli* helper strain HB101 with the pRK2013 mobilization plasmid.

A. tumefaciens was grown on solid LB containing 50 mg l^{-1} kanamycin for at least 2 d to select for the presence of the binary vector.

Plant transformation and special treatments

A. thaliana cv. Wassilewskaja was germinated and grown on soil at 25 °C under continuous light. The main floral stem was cut to induce more stems and infiltration was performed after 5 weeks.

The protocol to obtain transgenic *A. thaliana* plants by the *in planta* transformation method using a pBIN derivate binary vector (Frisch *et al*., 1995; Haseloff *et al*., 1997), was described by Bechtold and Pelletier (1998).

To perform temperature tests, 7–14-d-old plantlets were incubated for 12 h in a thin layer of liquid MS medium at 15±2 °C .

To perform the protein synthesis inhibition test, 100 µg ml^{-1} cycloheximide (Sigma) was added to the liquid MS where the plantlets were incubated for 7 h at 25 °C in continuous light.

Protein extraction and western blotting

Before and after treatments, the whole plantlets (0.1–0.3 g) were ground to powder in liquid nitrogen and resuspended in extraction buffer (1× TBS; SDS 2%; 1× protease inhibitor cocktail, Complete, Roche 1697498), then incubated at 60 °C for 10 min. The soluble fraction after centrifugation (5 min, 14 000 *g*) was concentrated and about 1 µg was loaded on gel. SDS-PAGE electrophoresis and blotting followed standard conditions (Laemmli and Favre, 1972). Anti-GFP serum (A6455, Molecularprobes) was used for immunolabelling (dilution 1:15 000). Bands were revealed by chemiluminescent ECL reagents (Amersham Biosciences RPN2108); the intensity was estimated with Bio-Rad Molecular Analyst software.

Confocal microscopy

Transgenic *Arabidopsis* plantlets were grown on sterile solid MS and collected at different stages of development, from 7–14 d after germination, and mounted for microscopical observation in water under glass coverslips. The plantlets were examined using a confocal laser-microscope (Zeiss LSM 5 Pascal). The Argon laser excitation wavelength was 488 nm; GFP emission was detected with the filter set for FITC (505–530 nm).

Results

Production of transgenic Arabidopsis *plants*

A collection of plants expressing different GFP reporters was produced: a secreted GFP (SGFP) (Di Sansebastiano *et al*., 1998), a GFP retained in the ER because of a C-terminal KDEL peptide (GFP-KDEL) (Haseloff *et al*., 1997), a vacuolar GFP with a C-terminal vacuolar sorting determinant (VSD) (GFP-Chi) targeted to protein storage vacuoles (PSVs) (Di Sansebastiano *et al*., 1998), and a vacuolar GFP with a sequence-specific VSD (Aleu-GFP) targeted to the lytic vacuole (LV) and pre-vacuolar compartments (PVCs) (Di Sansebastiano *et al*., 2001). The Aleu-GFP sequence contained the F64L, S65T mutations (GFP6) to increase its quantum efficiency at 488 nm. Homozygous plants expressing GFP-KDEL, GFP-Chi and Aleu-GFP were selected to compare sorting to vacuoles (Flückiger, 1999).

The secreted SGFP was characterized by the accumulation of GFP in the intercellular space of all tissues and did not show strong GFP accumulation in young tissues (data not shown). These observations confirmed the necessity of targeting signals for retention in the secretory system.

Different *Arabidopsis* tissues from GFP-KDEL, GFP-Chi and Aleu-GFP expressing plants, were observed with a fluorescence and confocal microscope and compared.

GFPs accumulation in young roots

Young roots were observed, simply mounted in water, between 6 d and 14 d after seed germination. The clearest differences in fluorescent patterns of the three GFPs could be seen in cells behind the root tip, just starting elongation: GFP-KDEL labelled the ER and the nuclear envelope and no large compartments appeared to accumulate GFP (Fig. 1a); GFP-Chi also appeared to be associated with the ER, but was not homogeneously distributed there and also accumulated in large compartments that had previously been called neutral vacuoles (Di Sansebastiano *et al*., 1998, 2001) (Fig. 1b); Aleu-GFP labelled the central vacuole of the cell as well as small compartments not yet identified (Fig. 1c).

In roots from plantlets expressing GFP with the ER-retention signal (GFP-KDEL), most cells exclusively showed fluorescence in this compartment. Calyptrogen cells rapidly lost their fluorescence while differentiating into root cap cells. Meristematic cells of the root apex showed a diffuse fluorescence of the ER, but the nuclear envelope was usually evident, only in the elongating cells beside the apex did the ER appear well-defined and bright (Fig. 2a). Small compartments were visible in elongating cells (Fig. 2b), but these should be interpreted as part of the ER since a similar accumulation of GFP in the ER has already been observed associated to a reticular pattern (Hawes *et al*., 2001). This distribution persisted in the

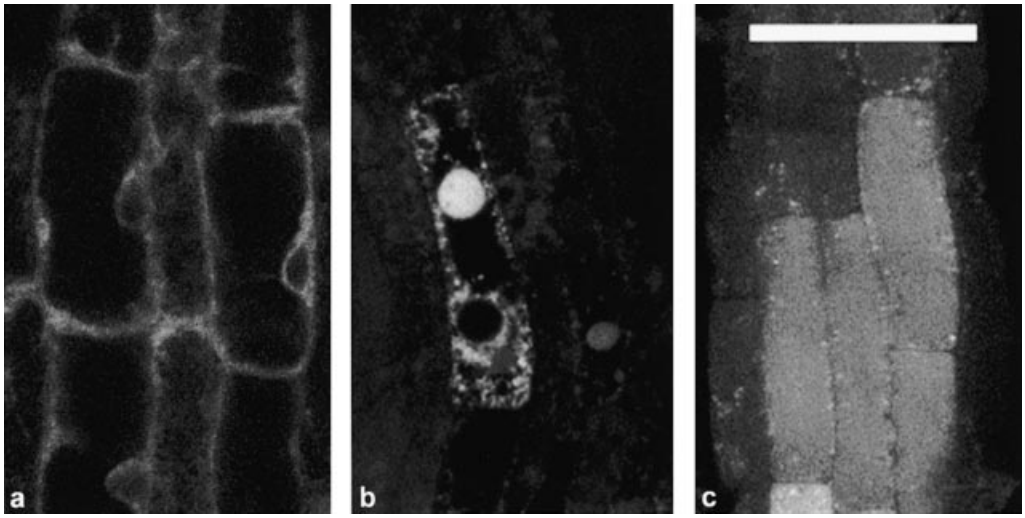

Fig. 1. GFP fluorescent patterns in elongating root cells. (a) ER-retained GFP-KDEL also labels the nuclear envelope; (b) the protein storage vacuole (PSV) marker GFP-Chi, apart from labelling the ER, also accumulates in small compartments and a small vacuole; (c) the lytic vacuole (LV) marker Aleu-GFP accumulates in the large central vacuole. It is also visible in a not yet identified punctate structure. Scale bar=50 μm.

elongated cells of the hairy region of the root (Fig. 2c), in the root hairs emerging from epidermal cells, and in the fully expanded hairs (Fig. 2d). Few compartments of different sizes were visible in all cells. Electron microscopy observations combined with immunogold labelling experiments, carried out on similar structures in other laboratories, showed that the larger compartments are ER-associated bodies, while the smaller ones are Golgi bodies (Hawes *et al*., 2001) but it is only possible to speculate upon their identity.

The accumulation pattern of the storage vacuole marker (GFP-Chi) in roots was more complex than the accumulation pattern of GFP-KDEL, because vacuoles were also labelled. As in plantlets expressing ER-retained GFP, the fluorescence in the calyptrogen decreased during the differentiation of the root cap cells. In the apex root cells GFP-Chi was found in the ER (Fig. 2e), but different vacuolar compartments and smaller sized compartments of an unknown nature were visible. Small vacuoles increased in size and number in the elongating cells beside the apex (Fig. 2f). Elongated cells of the hairy region belong to different cell types. In such cells, GFP-Chi was observed in ER and small vacuoles but occasionally also in the large central vacuole (Fig. 2g). Root hairs arise from position-specific cells, trichoblasts, after their expansive growth had ceased (Dolan, 2001). In trichoblasts GFP-Chi was always confined to ER. The earliest stages in the bulge formation resembled normal expansive growth and GFP-Chi remained located to ER (Fig. 2h). During rapid hair elongation, small vacuoles appeared and fused, finally labelling the central vacuole with GFP-Chi (Fig. 2i).

The differentiated root cap cells of plantlets expressing the lytic vacuole marker (Aleu-GFP) were fluorescent in a number of small compartments that are thought to be lytic vacuoles or pre-vacuolar compartments (PVCs). In the root apex cells, Aleu-GFP was concentrated in small compartments that could be either Golgi bodies, PVCs or small vacuoles (Fig. 2l) but cannot be identified definitively in the present study. ER was also visible but not very bright; it has been previously observed, in transient expression experiments (Di Sansebastiano *et al*., 2001), that this form of GFP is more rapidly exported from the ER to vacuoles than GFP-Chi. In the elongating cells of the smooth or hairy region, small compartments were less prominent while Aleu-GFP weakly labelled the central vacuole (Fig. 2m). In the elongated cells of the hairy region of the root the central vacuole was brightly fluorescent and no other compartments were visible anymore (Fig. 2n). The marker for lytic vacuoles was visible in the central vacuole in trichoblasts and in root hair from the beginning of bulge formation (Fig. 2o, p).

GFPs accumulation in aerial tissues

In cells from aerial tissues the distribution pattern was similar to the pattern described in roots. The observations were concentrated on the most accessible tissues: the leaf epidermis and hypocotyl parenchyma cells.

In the leaf epidermis (Fig. 3a), guard cells (Fig. 3d) and trichomes (Fig. 3g), of GFP-KDEL plantlets, the ER was clearly labelled. ER and small compartments were easily visible in hypocotyl parenchyma cells as well (Fig. 3l).

The observed patterns corresponded to the various aspects of transgenic *Arabidopsis* expressing similar constructs (Haseloff *et al*., 1997; Köhler, 1998; Ridge *et al*., 1999; Hawes *et al*., 2001).

In the leaf epidermal cells, storage vacuolar GFP-Chi was accumulated in ER but also labelled small vacuoles and the large central vacuole was very faintly fluorescent

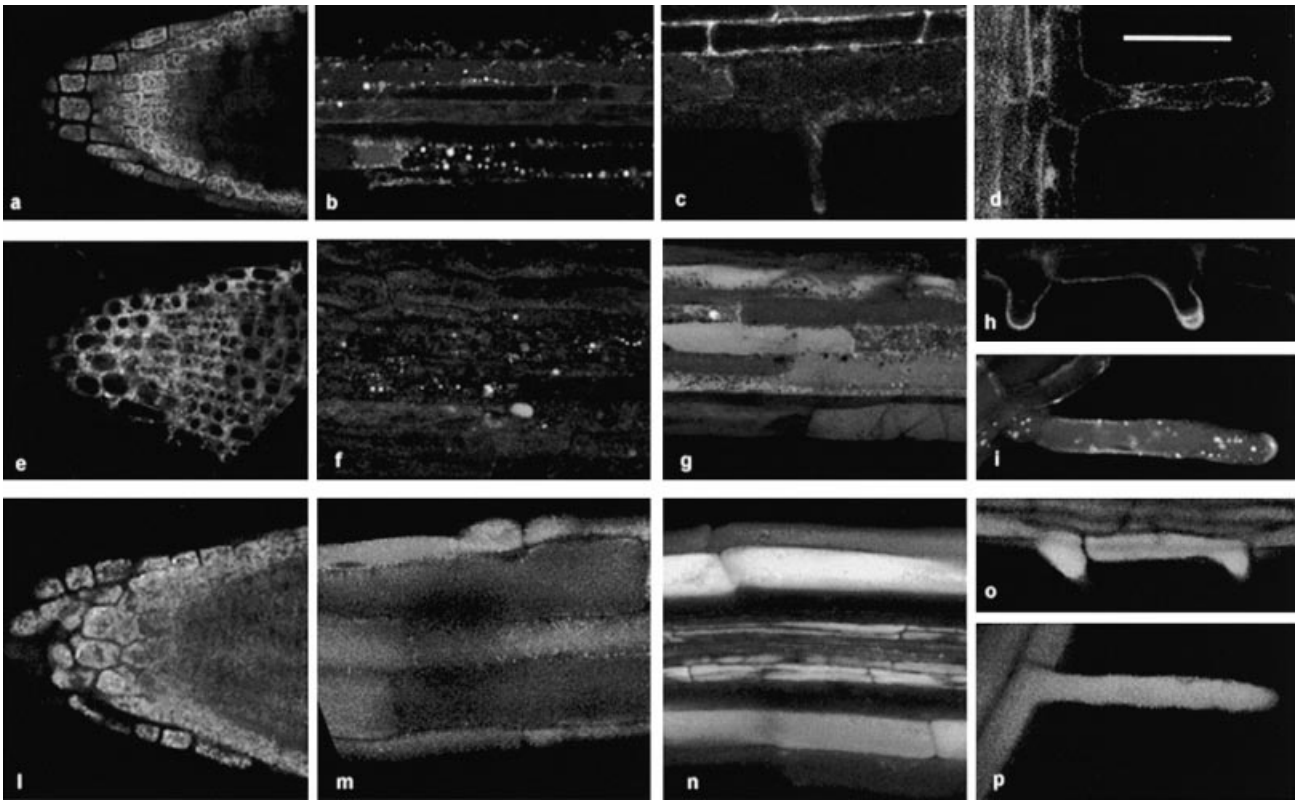

Fig. 2. GFP fluorescent patterns in roots of *Arabidopsis* plantlets. (a–d) ER-retained GFP-KDEL; (e–i) protein storage vacuole (PSV) marker GFP-Chi; (l–p) lytic vacuole (LV) marker Aleu-GFP. In the root apex cells GFP-KDEL labels the ER (a), GFP-Chi labels ER and small vacuoles (PSV) (e) and Aleu-GFP many small pre-vacuolar compartments (PVCs) (l). In the elongating region beside the apex, GFP-KDEL accumulates in small compartments and in the ER (b), GFP-Chi accumulates more in PSVs than in the ER (f), Aleu-GFP accumulates directly in the large central vacuole (LV) (m). In the hairy region of the root, GFP-KDEL is restricted to ER and no vacuoles are labelled in the main root body (c) or in root hairs (d). GFP-Chi is mainly in PSVs, but often labels LVs (g). GFP-Chi is limited to the ER of expanding root hairs (h) but sometimes labels their vacuole once fully expanded (i). Aleu-GFP labels the LV in root cells (n) and root hairs from their formation (o, p). Single confocal optical sections (1.5–1.8 μm). Scale bar=50 μm.

(Fig. 3b). Guard cells differentiate from protodermal cells called meristemoids, which continue to divide after surrounding cells have stopped dividing (Larkin *et al*., 1997). GFP-Chi labelled the ER of these meristemoids and remained limited to the ER even in young guard cells. It labelled the large central vacuole of guard cells only when the stomata were completely differentiated. In developing stomates, it was possible to observe that GFP-Chi was already in the large central vacuole in one of the two guard cells, while in the other it was still present in the ER and small compartments (Fig. 3e). Trichomes are the first cells that terminally differentiate on young leaf primordia (Larkin *et al*., 1996, 1997). In these cells GFP-Chi remained initially confined to the ER and small vacuoles. When trichomes were evenly spaced on the surface of the expanded leaf, GFP-Chi was also present in the large central vacuole, so that ER, small vacuoles and a fluorescent central vacuole were observed in the same cells (Fig. 3h). In the hypocotyl parenchyma cells, the central vacuole was never fluorescent and GFP-Chi was restricted to ER and small compartments with a pattern very similar to that of GFP-KDEL (Fig. 3m).

The leaf epidermal cells accumulating the lytic vacuolar Aleu-GFP showed a faint fluorescence apparently localized in the central vacuole (Fig. 3c). Aleu-GFP also was visible as a faint diffuse fluorescence in meristemoids but, in fully differentiated stomata, fluorescence decreased to the limit of detection. Differences were observed in the fluorescence of guard cells on the upper or lower face of the leaf blade. When located on the lower face, stomata rapidly lost their fluorescence (Fig. 3c) but, when located on the upper face, they maintained a brighter fluorescence and small compartments were also visible (Fig. 3f). Trichomes, like root hairs, showed a fluorescent central vacuole (Fig. 3i). They also appeared to differ in fluorescence distribution, depending on their location. The central vacuole was fluorescent in trichomes on the lower face of the leaf blade, but it was often negative in trichomes on the upper face, where fluorescence was often limited to smaller peripheral compartments.

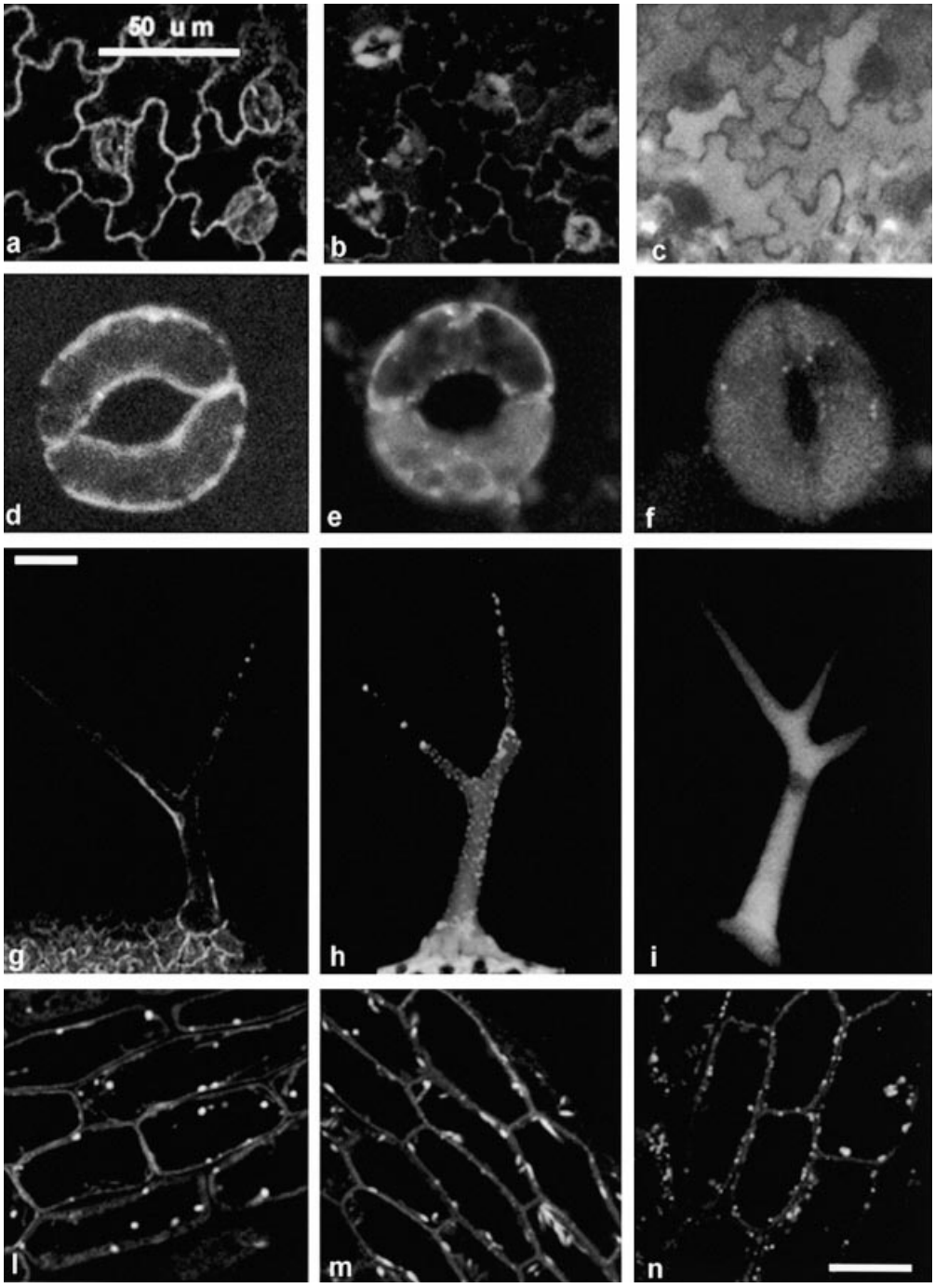

Fig. 3. GFP fluorescence patterns in leaf cells of *Arabidopsis* plantlets. (a–c) Epidermis; (d–f) stomata; (g–i) trichomes; and (l–n) parenchyma cells. In epidermal cells GFP-KDEL is restricted to ER, (a) GFP-Chi is similarly restricted to ER and associated structures, but it also weakly labels the central vacuole (b), Aleu-GFP labels the large central vacuole (c). Guard cells accumulate GFP-KDEL in the ER (d). GFP-Chi accumulates in the ER in young cells and moves into the central vacuole in mature guard cells. In a transition time one of the guard cell still has a fluorescent ER while the other has already a fluorescent LV (e). Aleu-GFP fluorescence is faint, but labels the LV from early development, PVCs were also visible. A stronger fluorescence is observed in stomata located on the upper face of the leaf (f). Trichomes accumulate GFP-KDEL in the ER (g), GFP-Chi is mainly in ER and PSV but also in the LV (h), Aleu-GFP accumulates essentially in the LV (i). In hypocotyl parenchyma cells no fluorescence is detectable in the LV. GFP-KDEL is restricted to ER and small compartments (l), similarly GFP-Chi is restricted to ER and associated structures (m), Aleu-GFP is visible in the ER and small PVCs (n). (a, b, c, d, e, f, l, m, n) are single optical sections (1.5–1.8 μm). (g, h, i) are 3D projections from six (4 μm) optical sections.

In hypocotyl parenchyma cells the lytic vacuolar Aleu-GFP did not label the large central vacuole, while small compartments, possibly PVCs, and ER were clearly visible (Fig. 3n). In this cell type both vacuolar markers and even the ER-retained marker gave very similar patterns.

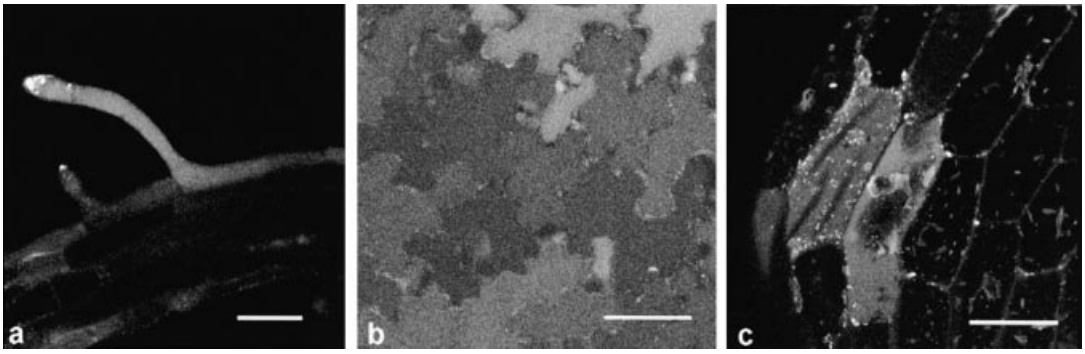

Fig. 4. GFP fluorescence patterns in tissues of *Arabidopsis* plantlets expressing Aleu-GFP and incubated for 12 h at 15 °C. (a) Root hairs show a fluorescent vacuole, but Aleu-GFP is also visible in the ER of growing cell tips. (b) Epidermal leaf cells exhibit an increased diffuse fluorescence. (c) Hypocotyl parenchyma cells are occasionally labelled by fluorescence in the central vacuole. Scale bar=50 μm.

GFP stability in transgenic plantlets

To test if the observed GFP distribution patterns were influenced by GFP stability in the different vacuoles, two tests were performed: (a) incubation of plantlets at low temperature; (b) incubation of plantlets in the presence of cycloheximide, a protein synthesis inhibitor.

The temperature test consisted of an incubation for up to 12 h at 15 °C before microscopical observations. The lytic vacuolar Aleu-GFP labelled the ER more than usual and, in polarized cells like the root hairs, it appeared concentrated at the growing tip of the cell (Fig. 4a). Epidermal leaf cells were much more fluorescent than in control tissues (Fig. 4b). Tracing a 'profile line' with the specific function offered by LSM 5 Pascal software, the maximal intensity of fluorescence in control stomata and epidermal cells was estimated as 150 and 50 units, respectively; after cold treatment they increased to 250 and 150 units. Some large vacuoles in hypocotyl parenchyma cells appeared fluorescent in a mosaic distribution (Fig. 4c). The other fluorescent markers were not affected by this cold treatment.

Protein synthesis inhibition was performed by incubating plantlets at normal temperature (25 °C) and in the presence of 100 μg ml^{-1} cycloheximide (Hörtensteiner *et al*., 1994). Immuno-blot analysis after 7 h treatment (not shown) revealed a stronger reduction of Aleu-GFP (–48%) than of GFP-Chi (–37%) or GFP-KDEL (–34%).

Discussion

To study the sorting of soluble proteins to vacuoles is not straightforward. Different vacuoles can appear optically similar and the use of reporter proteins could saturate the sorting machineries (Crofts *et al*., 1999; Vitale and Denecke, 1999). Finally, the endomembrane system is very sensitive to external conditions and sample manipulation can modify membrane distribution.

the organization of the vacuolar system in different tissues of *Arabidopsis* plantlets was analysed in this study, using soluble forms of GFP as reporters. These GFPs are fused to sorting determinants that label different subcompartments of the secretory system. In the transgenic plants expressing the secreted or the ER-retained GFPs, the pattern corresponded to previously published patterns (Haran *et al*., 2000; Haseloff *et al*., 1997; Köhler, 1998; Ridge *et al*., 1999). On the other hand, the fluorescent patterns due to the expression of the two vacuolar constructs GFP-Chi and Aleu-GFP are novel and offer a new insight into the organization of the endomembrane system during tissue differentiation. The vacuolar localization of these GFPs was first determined in tobacco mesophyll protoplasts (Di Sansebastiano *et al*., 2001). Aleu-GFP contains the mutations F64L, S65T that, increasing quantum yield, compensates pH quenching. GFP6-Chi with the same mutations was tested in tobacco protoplasts where it gave the same patterns as GFP5-Chi used in this work, but at higher intensities. For imaging purposes, Aleu-GFP included F64L, S65T mutations but GFP-Chi did not include the mutations. Both markers were clearly visible.

In transgenic *Arabidopsis* plants the two vacuolar GFPs are accumulated in some cells in distinct compartments, while in other cells they seem to accumulate in the same central vacuole. By contrast, GFP-KDEL, revealing an early station on the way to the vacuoles, is accumulated in all cells only in small and tubular compartments. The KDEL signal of this reporter is not very different from a C-terminal VSD and the saturation of its sorting mechanisms could cause overflow from the ER and sorting to vacuoles (Crofts *et al*., 1999), although accumulation of this GFP was never observed in vacuoles comparable to the two vacuolar GFPs. This indicated that over-expression driven by the 35S promoter was not sufficient to saturate the sorting machinery and to induce unspecific GFP accumulation in any vacuole. Indeed, in the protoplast system, which has higher expression levels, GFP accumulation in vacuoles was still not observed.

The root meristematic cells of the vacuolar GFP plants used in these experiments, were all characterized by small

fluorescent compartments with a very similar distribution, but they started to differ when the cells differentiated (Fig. 1).

In *Arabidopsis* plantlets, it was easy to follow the differentiation of epidermis and its specialized cells: trichomes, guard cells and root hairs. Trichome precursor cells and primary meristemoids differentiate from fields of equally competent protodermal cells and their patterning is regulated by several genes (Larkin *et al*., 1997). On the other hand, root hairs originate from trichoblasts precisely placed between two cortical cells in longitudinal stripes. Different genes regulate these differentiation processes and many plant species do not share the same spatial limitations (Dolan, 2001). Fluorescent patterns in very different cell types shared common characteristics. GFP distribution revealed an important feature of the vacuolar system in *Arabidopsis*: the marker for storage vacuoles (GFP-Chi) was kept separated from the large central vacuole, probably a lytic compartment, until the cell had fully expanded and differentiated. This was particularly evident in roots, where the formation of the root hairs marks precisely the end of expansion. In these cells, the lytic vacuole marker (Aleu-GFP) was rapidly sorted to the large central vacuole, the other marker being retained in the ER or accumulated in small compartments. After complete elongation and differentiation of these cells, GFP-Chi could reach the same large central vacuole where Aleu-GFP could be seen from the beginning of cell expansion. It appeared that vacuole biogenesis and organization could lead to a unique central vacuole only at the end of differentiation, as already observed during vacuole regeneration in miniprotoplasts (Di Sansebastiano *et al*., 2001). When mesophyll protoplasts were evacuolated, they regenerated separate lytic and storage vacuoles and only the lytic vacuole increased in volume to form a new central vacuole. At the end of expansion, small storage vacuoles seemed to fuse with the central vacuole in a part of the protoplasts.

Even if the common features now described were evident, the situation was complicated by the variability in fluorescence intensity within the same cell type. A differential accumulation of the lytic vacuole marker Aleu-GFP revealed a link between vacuole characteristics and cell type and position. The central vacuole of guard cells labelled by Aleu-GFP normally lost the fluorescence, but maintained a faint fluorescence when located on the upper face of the leaf blade. The fluorescence pattern also appeared to depend on cell location in trichomes. On the upper face the fluorescence disappeared from the central vacuole and persisted only in smaller compartments. This situation was opposite to that observed for guard cells. It may depend on the cell type considered and it may correlate to physiological conditions due to the environment (observations are *in vivo*).

The GFP fluorescence probably decreased or disappeared depending on pH or different proteolytic activities in the vacuoles of different cells. It is easy to imagine that cells located on opposite sides of the leaf blade have different proteolytic activities and vacuolar pH, in response to different environmental conditions. In hypocotyl parenchyma cells no fluorescent vacuoles were visible with either vacuolar GFPs. This may be due to increased degradation of GFP combined with a low pH.

The results of incubation at low temperature and treatment with cycloheximide supported this idea. Incubation at low temperature slowed down sorting of both markers. The treatment increased fluorescence in intermediate compartments like ER but it also slowed down degradative activity of vacuolar proteases. In fact, there was no major change in vacuolar accumulation of the storage vacuole marker, but visibility of the lytic vacuolar marker increased. This may be due, in part, to a reduction of pH quenching at low temperature. In plantlets incubated at normal temperature (25 °C) in the presence of cycloheximide to stop the synthesis of GFP, degradation of the lytic vacuolar marker was more evident. Western blotting experiments confirmed this higher degradation rate. Unfortunately degradation also occurs on GFP-Chi and GFP-KDEL and the difference does not appear very high. It is probably due to the mixed origin of the extracted material, which includes the full plantlet while only some tissues, for example, epidermis, may actually present a higher proteolytic activity in the central vacuole. In this study it is not possible to investigate this aspect, but a possible effect of differential degradation in addition to pH effects can be indicated.

Different aspects of the vacuolar system related to cell differentiation and environment appear to have been visualized. The vacuolar system, composed of vacuoles of different types (LV and PSV) and pre-vacuoles (PVCs), changed during cell development, while maintaining separate compartments. PSVs containing GFP-Chi remained separate from LV containing Aleu-GFP until the cell had fully differentiated and did not undergo further cell divisions. Cell expansion seemed to depend particularly on the presence of a LV separate from PSV.

The production of transgenic plants was necessary to study the vacuolar system in natural conditions and not only in artificial systems like protoplasts. Therefore, the *Arabidopsis* transgenic plants represent very good material for further investigations. these transgenic plants were produced to obtain a model in which alterations due to external stimuli or to mutations involved in the sorting of soluble proteins to the vacuoles could easily be identified. Considering, for example, the targeting to the vacuoles, alteration due to mutations may either prevent normal plant development by disturbing the regulated changes in cell volume or, on the contrary, they may cause no visible phenotype at all if the mis-targeted proteins are not

essential. The use of a visible reporter protein (GFP) stably expressed in transgenic plants makes possible the screening for otherwise undetectable mutations.

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