

Specific accumulation of GFP in a non-acidic vacuolar compartment via a C-terminal propeptide-mediated sorting pathway

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Summary

The green fluorescent protein (GFP) from *Aequorea victoria* can be detected in living plant cells after transient transformation of protoplasts. Expression of the GFP can be used to monitor protein trafficking in a mixed cell population and also to study the different function and importance of organelles in different cell types. We developed a vacuolar form of GFP that was obtained by replacing the C-terminal endoplasmic reticulum (ER)-retention motif of mGFP5-ER by the vacuolar targeting peptide of tobacco chitinase A. The vacuolar GFP was transported and accumulated in the vacuole as expected. However, we found two patterns of GFP accumulation after prolonged incubation (18–24 h) depending on the cell type. Most chloroplast-rich protoplasts had a fluorescent large central vacuole. In contrast, most chloroplast-poor protoplasts accumulated the GFP in one smaller vacuole but not in the large central vacuole, which was visible under a light microscope in the same cell. This differential accumulation reflected the existence of two different vacuolar compartments as described recently by immunolocalization of several vacuolar markers. We were able to characterize the vacuolar compartment to which GFP is specifically targeted as non-acidic, since it did not accumulate neutral red while acidic vacuoles did not accumulate GFP.

Introduction

The secretory pathway is one of the most complex transport routes in eukaryotic cells. Studies of vesicular trafficking *in vivo* can be addressed in real time by using the green fluorescent protein (GFP). The jellyfish (*Aequorea victoria*) GFP possesses a number of desirable traits as a universal reporter in living tissues and requires only blue or UV light for green fluorescence emission without any exogenous substrate (Heim *et al.*, 1994).

The successful use of GFP as a compartment marker in plants depends on its production in sufficient amounts, on its proper post-translational folding, on oxidative formation of its fluorophore, and finally on its correct targeting within the cell. A modified version of the GFP (mGFP4) has been produced recently where the AU content of the mRNA was

decreased in order to eliminate an aberrant splicing. Further mutations were made to improve the thermostability of GFP. The resulting GFP (mGFP5) was successfully targeted to the endoplasmic reticulum (ER) by adding a signal peptide and the ER-retention motif HDEL (Haseloff *et al.*, 1997; Siemering *et al.*, 1996).

Analysis of soluble vacuolar proteins in plants has revealed the existence of three classes of vacuolar sorting signals (VSS) (review by Neuhaus and Rogers, 1998): (i) a sequence-specific signal mostly found in N-terminal propeptides, as in sporamin or aleurain; (ii) internal surface determinants, as in phytohemagglutinin; and (iii) C-terminal propeptides, as in tobacco chitinase A or barley lectin. No conserved motif could be identified for the C-terminal propeptides, but sorting could be prevented by blocking the C-terminus with glycine residues or a glycosylation site (Dombrovski *et al.*, 1993; Neuhaus *et al.*, 1994).

Comparison of the sequence requirements for the three classes of VSS strongly suggests that the sorting system for C-terminal propeptides differs from the sorting system for the other VSS (Holwerda *et al.*, 1992; Nakamura *et al.*, 1993; Neuhaus *et al.*, 1991). The existence of at least two different sorting pathways for vacuolar proteins is also suggested by the inhibitory effect of wortmannin, a phosphatidylinositol 3-kinase inhibitor, on vacuolar targeting of a soluble protein with a C-terminal VSS but not of a protein with a sequence-specific VSS (Matsuoka *et al.*, 1995). Recently, the coexistence of functionally distinct vacuolar compartments has also been shown in non-differentiated plant cells using immunolabelling with vacuolar marker antibodies (Paris *et al.*, 1996). The vacuolar targeting was thus proposed to use (at least) two pathways; one would be mediated by a C-terminal VSS and would lead to a storage compartment, while the other would be mediated by a sequence-specific VSS and would lead to a lytic compartment.

To visualize the dynamics of vacuolar sorting along a C-terminal VSS-mediated pathway, we used the secreted GFP fused to the VSS from tobacco chitinase. This VSS has been shown to be sufficient to redirect a secreted form of chitinase to the vacuole (Neuhaus *et al.*, 1991).

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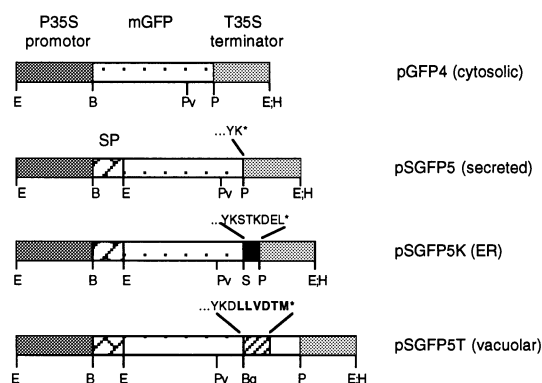


Figure 1. Schematic representation of GFP constructs. GFP4 and GFP5 are plant-adapted GFP sequences (Haseloff *et al.*, 1997). The C-terminal amino acid sequences of the fusion proteins are presented; (*) stop codon; bold, C-terminal VSS of tobacco chitinase; B, *Bam*HI; Bg, *Bg*II; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I.

Results

Constructs and controls

The plant-adapted mGFP4 (Haseloff *et al.*, 1997) was used as a control cytosolic form (Figure 1). For the secretory pathway, we used the thermostable, ER-retained mGFP5-ER (Siemering *et al.*, 1996), which also contains the signal sequence from an *Arabidopsis* chitinase and from which we removed the C-terminal HDEL motif to obtain the secreted SGFP5. We also replaced the C-terminus either by a KDEL motif, giving another ER-retained GFP (SGFP5K), or by the VSS from tobacco chitinase A, giving the SGFP5T (Figure 1).

We transiently transformed tobacco protoplasts with the control constructs GFP4, SGFP5 and SGFP5K, to check the ability of our system to properly sort soluble proteins. As expected, the secreted form was not accumulating inside the cell and was not detected in the medium, where it was probably degraded or diluted (data not shown). The cytosolic (not shown) and the ER forms (see below) of GFP accumulated in their respective compartment, leading to their typical patterns (Haseloff *et al.*, 1997).

The GFP forms that were retained in the cells were also a useful tool to optimize our transformation efficiency, which we estimated by the percentage of GFP-accumulating protoplasts. The transformation efficiency routinely reached 50% but could reach up to 80%.

Vacuolar GFP

When the construct SGFP5T with the vacuolar sorting sequence of tobacco chitinase was expressed transiently overnight, we observed green fluorescence in the majority of the cells (Figure 2a, left protoplast) while some other cells remained unlabelled (Figure 2a, right protoplast). The GFP fluorescence accumulated in an homogeneous way in

the whole cell, as shown in Figure 2(b) representing the same two cells under dual illumination (UV and white light). The green fluorescence was unfortunately partially masked by the red natural fluorescence emitted by the chloroplasts. In addition, the chloroplasts also absorbed some of the incident UV light and the emitted green light resulting in a lower intensity of the GFP signal of compartments located below them. For these reasons, if the red signal was eliminated by a filter this only resulted in dark shadows in place of the chloroplasts without improving the image quality (data not shown). We therefore decided to present images in real colours with both green (GFP) and red (chlorophyll) fluorescence, where the yellow represents a mixture of the two signals when chloroplasts happen to be below a green fluorescent compartment. Because GFP was not accumulating in small structures, confocal microscopy was not convenient.

In some cells the SGFP5T was found to accumulate within a smaller structure (Figure 2c), often adjacent to the chloroplasts on one side of the protoplast. Importantly, in this same cell a large central vacuole was also clearly visible by light microscopy but showed no green fluorescence (Figure 2d). We observed movement of the small green structure with respect to the chloroplasts, indicating that these cells were metabolically active.

Upon prolonged observation under the microscope some protoplasts were observed to burst and a fluorescent vacuole was seen to evaginate from the cell envelope (Figure 3a). If the vacuole happened to burst while we were observing the cell, the fluorescent content was released into the medium while the tonoplast remained unstained. This confirmed that the SGFP5T accumulated in the vacuole as a soluble protein.

To confirm further the localization of SGFP5T, we isolated vacuoles from protoplasts that had been incubated in the presence of a cell wall synthesis inhibitor, dichlorobenzonitrile. The protoplasts were lysed by a thermal shock and vacuoles were harvested by floatation (Gomez and Chrispeels, 1993). Among the isolated vacuoles we observed not only large vacuoles of ~ 50 μ m (Figure 3b, left) of cells as shown in Figure 2(a), but also small fluorescent vacuoles less than 10 μ m (Figure 3b, right) as observed in the other cell type (Figure 2c). In the pelleted cell debris examined after vacuole isolation, a faint labelling of the ER and the nuclear envelope was sometimes visible, due to the presence of residual GFP in the ER, but we never found large green fluorescent structures while we could stain nuclei by ethidium bromide. These results ruled out that the possibility that the small fluorescent structures could be nuclei and thus supported their vacuolar nature.

The two sizes of vacuoles correlate with two cell types

The starting material used for transient expression of SGFP5T was a heterogeneous population of protoplasts

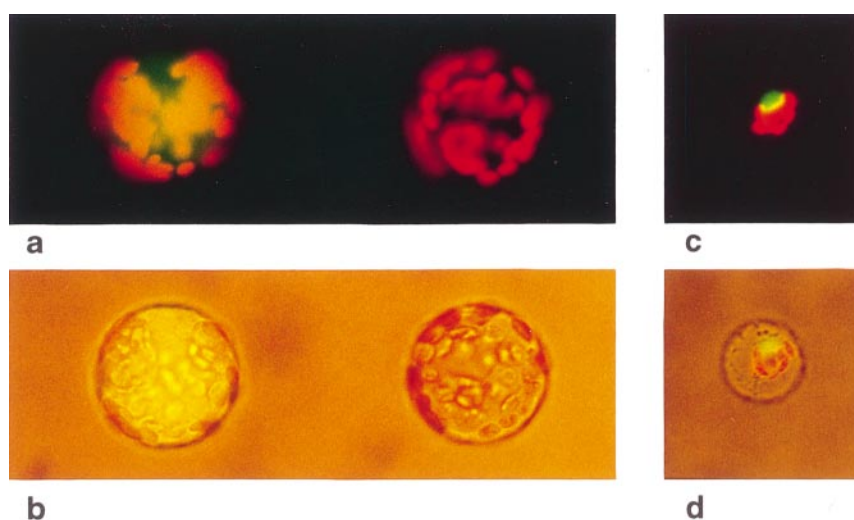


Figure 2. Protoplasts expressing the vacuolar SGFP5T.

(a) and (c) True colour fluorescence obtained with UV light; (b) and (d) were obtained with combined light, UV + visible, allowing simultaneous observation of the whole cell and of GFP. Chloroplasts emitted red fluorescence, while GFP fluoresced in green. Superposition of both signals yielded a yellow colour. (a) and (b) Chloroplast-rich cells, on the left a transformed cell, on the right an untransformed cell.

(c) and (d) Chloroplast-poor cell.

Magnification $\times 172$.

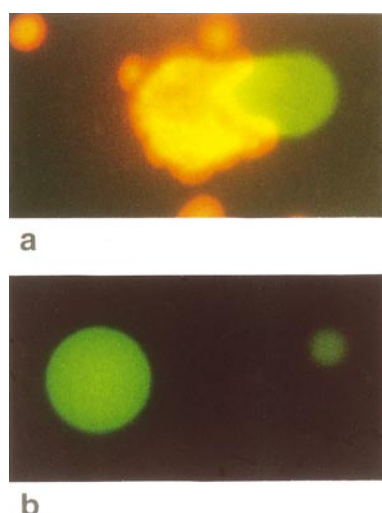


Figure 3. Vacuolar localization of SGFP5T.

(a) Bursting protoplast with an evaginating fluorescent vacuole.

(b) Isolated fluorescent large and small vacuoles.

Magnification $\times 172$.

derived from the various cell types present in the tobacco leaf blade. Two main cell populations could be distinguished: large cells of the palisade mesophyll had many chloroplasts, while other cells of various sizes had less than 10 chloroplasts and probably derived from the spongy mesophyll. We found a correlation between the pattern of vacuolar accumulation of GFP and the cell type 18–24 h after transformation. As shown in Table 1, 60% of the chloroplast-rich protoplasts had a big fluorescent vacuole (Figure 2a, left protoplast), while 68% of the chloroplast-poor protoplasts accumulated the GFP in a smaller vacuole

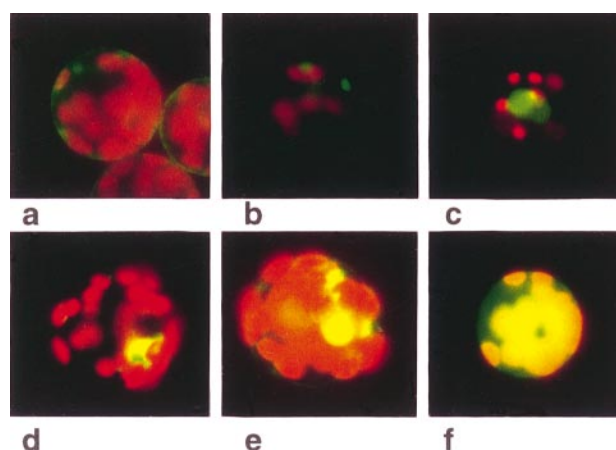


Figure 5. Time-course of the transiently expressed SGFP5T in chloroplast-poor cells (b and c), compared to chloroplast-rich cells (a, d–f) under UV light.

(a) 5–6 h after transformation, the ER contained most of the GFP.

(b) 12–15 h after transformation, in cells with few chloroplasts small compartments were filled with GFP.

(c) After 24 h or more, chloroplast-poor cells reached a final state with a small green vacuole.

(d) 12–15 h after transformation, in chloroplast-rich cells, GFP appeared limited to small vacuoles, as in the chloroplast-poor protoplasts.

(e) After 18 h larger vacuolar compartments became visible.

(f) After 24 h GFP occupied the large central vacuole.

Magnification $\times 172$.

that was far from occupying the entire internal space (Figure 2d). We also found an ER-type of staining (see below) sometimes associated with other much smaller structures in both cell types.

The presence of a large GFP-accumulating vacuole seemed clearly associated with cells from palisade meso-

Table 1. GFP patterns in two protoplast subpopulations expressing SGFP5T

Subpopulation	Localization		
	LV	SV	ER
Chloroplast-rich ($n = 494$)	60% (58–67)	1% (0–2)	39% (33–41)
Chloroplast-poor ($n = 153$)	3% (0–5)	68% (63–75)	29% (23–37)

After 24 h of expression, GFP was observed in a large vacuole (LV), in a small vacuole (SV) or in ER-like structures through the entire cell (ER). Protoplasts expressing GFP were classified into two subpopulations according to the number of their chloroplasts. Results are expressed as percentage within each population analysed, 100% corresponding to the total number of cells observed in either chloroplast-rich ($n = 494$) or chloroplast-poor ($n = 153$) protoplasts for three independent transformations. In parenthesis we report the observed minimal and maximal percentage in the independent experiments (min–max).

phyll, while smaller GFP-accumulating vacuoles were mainly found in spongy mesophyll cells.

Transient expression of vacuolar GFP highlights some steps of the protein transport

Six hours after transformation with the vacuolar SGFP5T, the fluorescence was observed in the ER and nuclear envelope (Figures 4c,d and 5a), as shown by the similarity with the pattern observed with the ER-retained SGFP5K for 6–24 h (Figure 4a,b). The same pattern was observed in chloroplast-rich and chloroplast-poor protoplasts. At the same early time point, the secreted SGFP5 produced the same pattern (data not shown) as the ER-specific dye DiOC₆(3) (Grabski *et al.*, 1993), confirming that it corresponds to an early ER accumulation pattern shared by every protein transiting through the secretory pathway. As shown in Table 2, this ER pattern was present after 6 h in a large majority of both cell types expressing the vacuolar SGFP5T.

Between 6 and 12 h this ER labelling became more intense and we started to detect larger structures. These vacuolar or prevacuolar compartments were visible in both chloroplast-poor (Figure 5b) and chloroplast-rich (Figure 5d) subpopulations. After 14 h, some chloroplast-rich cells already contained a much larger fluorescent vacuole (Figure 5e).

After 18 h, we quantified the GFP accumulation patterns for both cell types (Table 2) and found that 40% of the fluorescent palisade protoplasts had already reached the final stage of the large fluorescent vacuole described previously, while the ER staining was no longer visible in these same cells. Similarly, 40% of the chloroplast-poor protoplasts already accumulated the GFP in a typical single small vacuole after 18 h, while the large vacuole remained

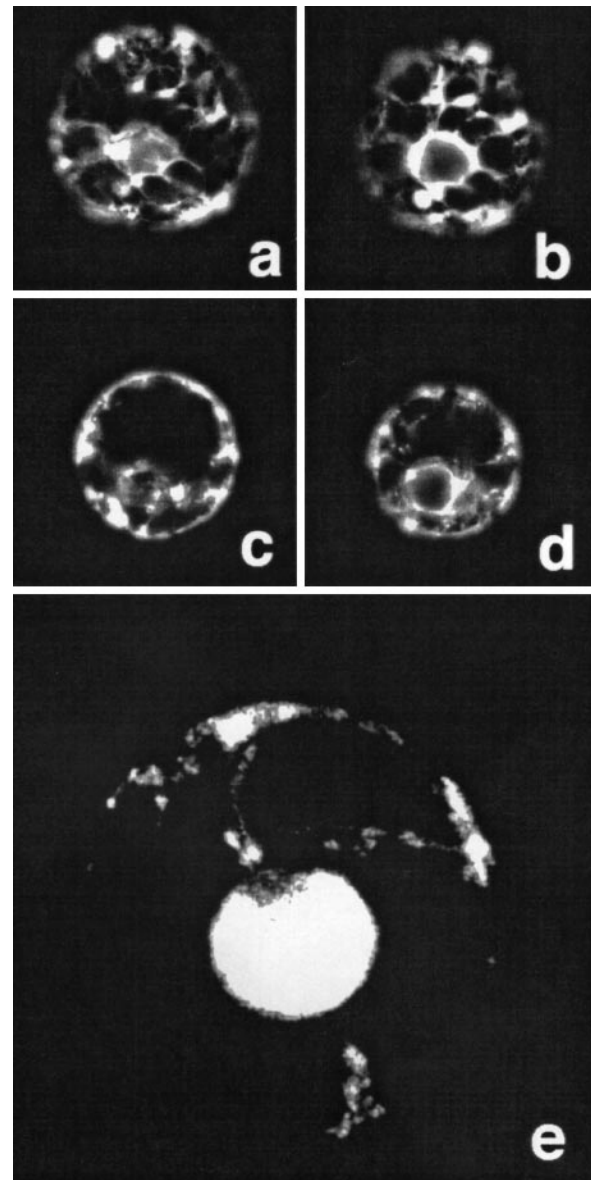


Figure 4. Confocal microscope images from single protoplasts emphasizing the role of the ER.

(a) and (b) Consecutive sections close to the surface of a protoplast expressing the ER-retained SGFP5K for 24 h.

(c) and (d) Consecutive sections close to the surface of a protoplast expressing the vacuolar SGFP5T for 6 h. Compare the nuclear envelope staining in (b) and (d).

(e) Stack of six consecutive sections of a protoplast accumulating the vacuolar SGFP5T in a small vacuole for 24 h. Residual ER-like fluorescence is visible next to the centrally located bright vacuole in strands crossing the unstained large vacuole and along the periphery.

Magnification $\times 1400$ (a–d) and $\times 2350$ (e).

unstained (Table 2). At this stage, the ER pattern was still highly represented in both cell types.

From 24 h on after transformation the labelling patterns no longer changed. In this final stage, 59% of the chloroplast-rich cells had a green large central vacuole (Figure 5f and Table 2) and 75% of chloroplast-poor cells contained

Table 2. Localization of vacuolar SGFP5T in tobacco protoplasts after various time of transient expression

Time	Subpopulation	Localization		
		ER	SV	LV
6 h	Cp-rich (<i>n</i> = 124)	98%	2%	0%
	Cp-poor (<i>n</i> = 60)	100%	0%	0%
18 h	Cp-rich (<i>n</i> = 141)	52%	8%	40%
	Cp-poor (<i>n</i> = 80)	60%	40%	0%
24 h	Cp-rich (<i>n</i> = 157)	38%	3%	59%
	Cp-poor (<i>n</i> = 88)	23%	75%	2%

Cells with typical GFP-accumulation patterns, ER-like (ER), small vacuole (SV) or large vacuole (LV), were counted in either chloroplast-rich (Cp-rich) or chloroplast-poor (Cp-poor) protoplasts at various times after transformation. Results from a single experiment are expressed as percentage, 100% corresponding to the number (*n*) of GFP-accumulating cells in each subpopulation.

a small green vacuole (Figure 5c and Table 2) which was typical for this cell type (Figure 2d). We followed the evolution of selected cells with a small vacuole for up to 2 h but never could observe any transition from small to large fluorescent vacuoles. This confirmed that a small vacuole represents the final destination for GFP in chloroplast-poor protoplasts. In both cell types, but more easily in cells with a small green vacuole, it was sometimes still possible to observe limited and faint green fluorescence in portions of the ER (Figure 4e). Even after a long incubation time, a proportion of both cell types still exhibited only ER staining (Table 2).

The transient expression system allowed the observation of some steps of protein transport. Since GFP synthesis continued during the incubation it was reasonable to detect some ER staining while GFP was accumulating in a large or small vacuole, depending on the cell type. At least for chloroplast-poor cells we could clearly identify two coexisting vacuolar compartments, a small vacuole that was the final destination for SGFP5T and a large vacuole that remained unstained.

The C-terminal VSS from tobacco chitinase A targets GFP to a pH-neutral compartment

Many plant vacuoles are acidified by ATP- and pyrophosphate-driven protein pumps located in the tonoplast. The low pH may be important for vacuoles with a primarily lytic function, while storage vacuoles need not to be acidic. The coexistence of different vacuolar compartments has recently been shown in single barley cells by immunostaining (Paris *et al.*, 1996). A presumably acidic compartment accumulated the thiol protease aleurain, while the other compartment accumulated the storage protein barley lectin.

To probe the pH of vacuoles in our protoplast population

we chose neutral red (NR), which has been used extensively for the vital staining of vacuoles (Ehara *et al.*, 1996). This dye can diffuse through membranes, but after protonation it is trapped within acidic compartments. The red coloration is therefore an indicator of the pH difference between cytoplasm and vacuole. Accumulation often leads to precipitation of the dye. We ensured that the presence of NR inside the cell was specific by rinsing the protoplasts before observation.

In control untransformed protoplasts, the large central vacuole of 98% of chloroplast-poor cells was red while a very small percentage of these cells remained unstained. In chloroplast-rich cells, a significant proportion (20%) of the large vacuoles remained NR-negative, while in the other 80% the colour varied from light pink to red (Figure 6c,d, right protoplast) and even precipitation was sometimes observed. In both subpopulations, small red vacuoles were only visible among neighbouring chloroplasts when NR precipitation occurred. Intracellular movement of organelles indicated that unstained cells were still metabolically active.

We then asked whether the GFP-accumulating compartments were also accumulating NR. We therefore stained tobacco protoplasts with NR after 24 h of SGFP5T expression. The proportions of GFP patterns were not affected by the NR staining procedure. The distribution of large NR-labelled vacuoles was identical in transformed cells as in control protoplasts. Under UV light we selected 51 cells with a GFP-accumulating large vacuole, such as in Figure 6(a), and after switching on to visible light we found that each of these large vacuoles was NR negative (Figure 6b). Similarly, we selected 15 cells with a small GFP-stained vacuole, such as in Figure 6(c, left), and found that, under visible light, 14 of them contained a large NR-stained vacuole (Figure 6d, left) while one of them remained devoid of any NR staining. In protoplasts with a large green vacuole, we could sometimes observe a precipitate of NR as a dark grain under visible light, indicating the presence of a small acidic compartment.

To confirm the differential vacuolar localization of SGFP5T and NR, we isolated vacuoles from NR-stained protoplasts expressing SGFP5T. Already during lysis we could observe separate green (Figure 7a,b, left) and red (Figure 7a,b, right) vacuoles emerging from lysing protoplasts. Some of the membranes from which the vacuole emerged fluorescent (Figure 7a) showed a more intense green fluorescence, a hint to the presence of some SGFP5T in compartments other than the large vacuole in this cell type. From the large vacuoles we obtained, the GFP-accumulating population (Figure 7c, right) was always devoid of any NR accumulation and therefore was impossible to photograph under white light (Figure 7d, right). Similarly NR-accumulating large vacuoles (Figure 7d, left)

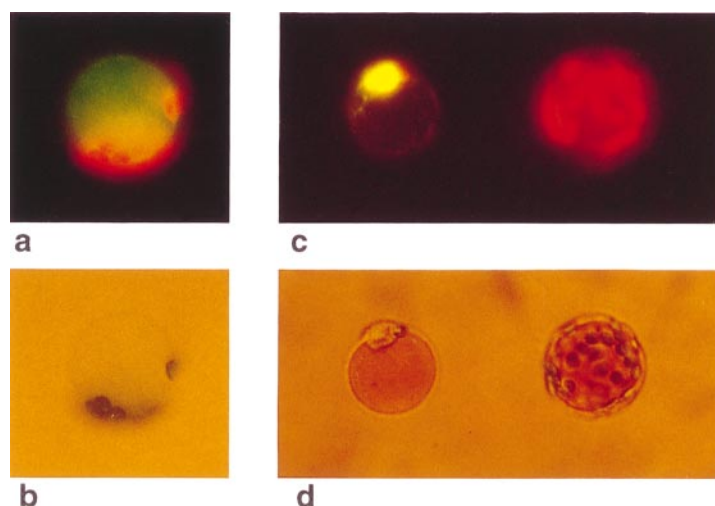


Figure 6. Distinct accumulation of SGFP5T and neutral red in protoplasts expressing SGFP5T.

(a) and (c) correspond to (b) and (d), respectively, under different illumination: UV light (a and c); visible light (b and d).

(a) and (b) A chloroplast-rich cell accumulated GFP in the large vacuole and NR was not visibly accumulated.

(c) and (d) A chloroplast-poor cell accumulated NR in the large vacuole while GFP was restricted to a smaller compartment (on the left of the picture).

A chloroplast-rich cell with no visible GFP accumulated NR in the large vacuole (on the right of the picture).

Magnification $\times 172$.

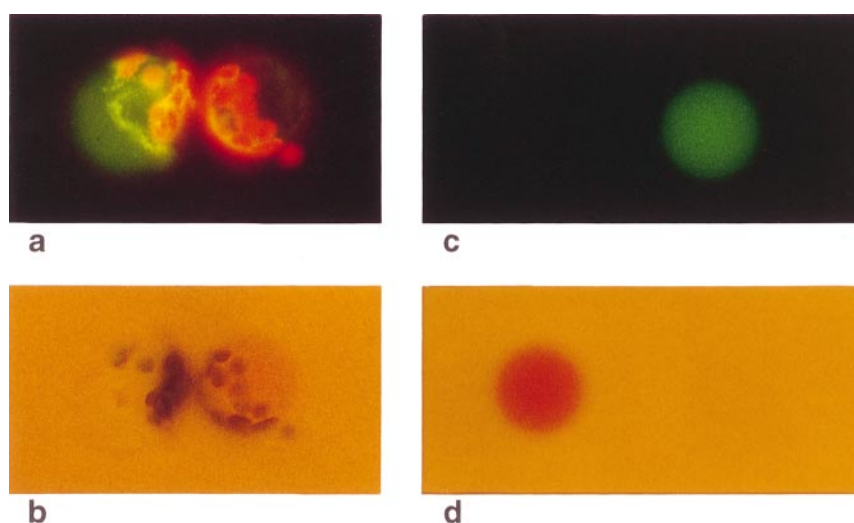


Figure 7. Specific accumulation of SGFP5T and neutral red in distinct vacuoles.

Cells were lysed by a thermic shock and vacuoles were purified. (a) and (c) Obtained with UV light, (b) and (d) with visible light.

(a) and (b) During the lysis, NR-accumulating and green fluorescent vacuoles could be observed to come out of the cells. In (a), green fluorescence could be also observed in the endomembranes of the lysed cell on the left, in addition to the green fluorescent vacuole. In (b) the red vacuole is visible in the right cell.

(c) and (d) The same field containing two purified vacuoles under UV light (c) and under visible light (d).

Magnification $\times 172$.

were invisible in UV (Figure 7c, left) since they did not contain any GFP.

These results show clearly that the GFP-accumulating vacuoles were non-acidic compartments in both cell types. At least for chloroplast-poor cells, a large acidic compartment was also present but it never contained any GFP.

To check if the acidity of some vacuoles might have caused the loss of part of the vacuolar GFP signal in certain cells, we tested whether preventing the acidification of the vacuole would affect the percentage of GFP-accumulating

vacuoles. The expression of SGFP5T was observed in protoplasts in the presence of NH_4Cl (10–50 mM) or monensin (5–10 μM). We found that while NR staining was strongly reduced, these compounds had no effect on GFP distribution. Bafilomycin A was also tested with inconclusive results. We cannot rule out, however, that a small percentage of the GFP did reach the acidic compartment and was degraded proteolytically, even at neutral pH. It is interesting to note that no fluorescence could be observed in the cell wall in leaves infected with a PVX vector encoding another

secreted GFP (Boevink *et al.*, 1996). This could mean that GFP is unstable in some cell compartments.

We used the lipid kinase inhibitor wortmannin, which has been shown to block specifically C-terminal VSS-mediated vacuolar sorting but not to affect the sequence-specific VSS-mediated vacuolar sorting (Nakamura *et al.*, 1993). We incubated protoplasts during the whole time of expression with various concentrations of wortmannin and found a maximal effect at 35 μ M. GFP no longer accumulated in either large or small vacuoles but was visible in the ER for a long time and then gradually disappeared, being either degraded or secreted by a default pathway. Because the inhibitor was present for the entire duration of expression, all GFP produced during 24 h was affected. The dose that affected the GFP distribution was in accordance with published results (Matsuoka *et al.*, 1995). Thus the targeting of GFP to a non-acidic vacuole in our tobacco protoplasts was wortmannin-sensitive, as is typical for a C-terminal VSS-dependent pathway.

Discussion

Based on previous studies of vacuolar targeting in plants, we have now developed a vacuolar marker visible in living cells. As expected, the vacuolar GFP was generally seen to accumulate in the central vacuole of protoplasts. Unexpectedly, however, a subpopulation of protoplasts, characterized by a low number of chloroplasts, presumably spongy mesophyll cells, accumulated the GFP in a single small vacuole distinct from the central vacuole.

Due to the transient expression of the marker protein, we could observe the movement of GFP synthesized in a burst of expression within the protoplast secretory pathway. Both vacuolar and secreted forms were seen to accumulate first in the ER. In the palisade-type protoplasts the vacuolar GFP could then be seen to accumulate in a single small vacuole which later disappeared when these cells typically accumulated the GFP in a large central vacuole. This suggests that palisade-type cells first accumulate GFP in a 'prevacuolar' compartment, the content of which later becomes part of the large central vacuole. In contrast, in the chloroplast-poor protoplasts the GFP accumulated in a small vacuole and remained there stably, although a large vacuole was also present in the same cells.

In both cell types and independently of the fact that the GFP-accumulating compartment was large or small, the pathway by which GFP was targeted to the vacuole was sensitive to wortmannin, showing that it is specific and typical for a C-terminal VSS-mediated vacuolar sorting (Matsuoka *et al.*, 1995). A current model (Okita and Rogers, 1996; Paris *et al.*, 1996) proposes that sequence-specific VSS target soluble proteins to a lytic compartment, while C-terminal VSS direct proteins to a storage vacuole. We predict that the sorting system based on sequence-specific

VSS would target the GFP to a different compartment and would not be sensitive to wortmannin. Preliminary results were obtained with GFP fused to a sequence-specific VSS. The GFP was expressed since we could observe an ER-like pattern at early expression times. At later times the fluorescence was weak and distributed in a pattern different from the pattern observed with the vacuolar SGFP5T described here, indicating differential sorting in our protoplast system.

We further characterized the vacuolar compartments of tobacco protoplasts in terms of acidity using NR, assuming that a lytic compartment should be more acidic than a storage vacuole. We found that, independently of their size, the GFP-accumulating vacuoles shared the common feature of being non-acidic, as they never accumulated NR. In the case of chloroplast-poor cells a small green fluorescent vacuole coexisted with a large vacuole that occupied most of the cell volume and accumulated NR. This led us to the conclusion that the large central vacuole varies in term of acidity depending mainly on the cell type, and that its pH is linked to its ability to accumulate GFP. The subpopulation of protoplasts where two vacuolar compartments coexist, one accumulating the GFP and the other accumulating NR, may be similar to some barley root tip cells that were found to accumulate barley lectin (a C-terminal VSS-containing protein) in a functionally distinct compartment from aleurain, a sequence-specific VSS-containing protease (Paris *et al.*, 1996). In these particular protoplasts the presence of two distinct vacuolar compartments, an acidic (lytic) and a non-acidic (storage) vacuole, allowed us to test if this type of cell is able to properly sort soluble proteins carrying VSS to their specific vacuolar destination. We have showed that this is the case for C-terminal VSS, as we obtained an exclusive and specific labelling of the storage compartment in living cells while the lytic compartment could be colabelled by NR. Whether these two distinct compartments coexist in all cell types isolated from tobacco leaves is difficult to conclude, since in chloroplast-rich cells a smaller NR-accumulating compartment can only occasionally be seen but may be often masked by the large number of chloroplasts. That not all plant cells contain two functionally distinct vacuoles (Paris *et al.*, 1996) explains why proteins targeted by the two different VSS types may accumulate in the same vacuole (Schroeder *et al.*, 1993).

Our results strongly support the existence of two vacuolar compartments that correspond to the destinations for the two different VSS-mediated pathways where C-terminal VSS send proteins to a non-acidic storage vacuole in a wortmannin-sensitive way. While the existence of at least two vacuole types was already known (Hohl *et al.*, 1996; Paris *et al.*, 1996), it is now possible to study specifically the biogenesis of the non-acidic vacuole in living cells. Ideally, two GFP isoforms with different colours will even

allow simultaneous staining of both vacuolar compartments in living plant cells.

Experimental procedures

Fusion gene constructs

The plasmids pBINmGFP4 and pBINmGFP5-ER, encoding a cytosolic and an ER-targeted GFP respectively, and adapted for expression in plants, were kindly provided by D. Haseloff (Cambridge, UK). For C-terminal fusions and transient expression in protoplasts, the coding sequences were cloned into the plasmid pGY1 (Neuhaus *et al.*, 1991) between 35S promoter and termination sequences. The coding sequence from pBINmGFP4 was isolated as a *Bam*HI–*Sac*I fragment, blunt ended with Klenow polymerase, cloned into the *Sma*I site of pGY1, producing plasmid pGFP4, which encodes a cytosolic GFP (E. Freydl, Zurich). The 3' *Bam*HI site was destroyed by partial *Bam*HI digestion, filling up and religation. A *Nhe*I site was introduced 5' of the start codon by PCR using the primer 1 (tctgc tagcg caatg agtaa aggag aagaa c; restriction site in bold.) and a 3' reverse vector primer 2 (tgtag agaga gactg gtgat ttc). An *Nhe*I site was introduced in the same reading frame into the coding sequence of tobacco chitinase A, at the end of the sequence encoding the signal sequence using a 5' forward vector primer 3 (tgacg caca tcca ctatc ctctg c) and primer 4 (gttct gcgct agcag aaagc agtag g). This allowed us to construct plasmid pSGFP4, encoding a GFP fused to the signal sequence of chitinase A. Furthermore a *Bgl*II site was introduced at the end of the coding sequence of the GFP, using 5' forward vector primer 3 and primer 5 (gtcga ctcta gagat ctttg tatag ttcat c). This replaced the stop codon by Glu and Ser codons. The *Bgl*II site was used to add the sequence encoding the vacuolar targeting peptide from tobacco chitinase A (from plasmid pSCM34 encoding the KD-7 mutant which contains a *Bgl*II site in the same reading frame; Neuhaus *et al.*, 1994) resulting in plasmid pSGFP4T. In another construction, a C-terminal STKDEL sequence containing an ER-retention signal was introduced by PCR using the forward primer 1 and primer 6 (cctgc agtca gagct cgtcc ttggt cgact tgtat agttc atc). This also introduced *Sal*I and *Sac*I sites within the coding sequence and a *Pst*I site after the stop codon in the resulting plasmid pSGFP4K.

The *Bam*HI–*Pvu*II fragments from pSGFP4, pSGFP4T and pSGFP4K were then replaced by the corresponding fragment from pBINmGFP5-ER containing the sequence encoding the signal peptide of *Arabidopsis* chitinase and most of the thermostable GFP. This produced the plasmids pSGFP5, pSGFP5T and pSGFP5K which were mostly used in this work (Figure 1). Plasmids were isolated by alkaline lysis in the presence of SDS (Sambrook *et al.*, 1989) and purified on an ethidium bromide–CsCl density gradient.

Protoplast transient expression

Nicotiana tabacum cv. SR1 protoplasts were isolated following the protocol of Nagy and Maliga (1976), cultured and rinsed using the indicated media and transformed by PEG-mediated direct gene transfer essentially as described elsewhere (Freydl *et al.*, 1995; Negrutiu *et al.*, 1987). Ten microgram of plasmid were used for the transformation of 600 000 protoplasts. After 2 h protoplasts were rinsed to remove the PEG, resuspended in 2 ml culture medium and incubated at 26°C in the dark. Protoplasts were observed by fluorescence microscopy in their culture medium at different times after transformation. Transformation efficiency

depended on the use of CsCl-purified plasmid and the use of a low protoplast density (up to 80% when reducing the number of protoplasts to 200 000 per transformation). It was also somewhat variable from one plasmid preparation to the next.

Vacuole isolation

Vacuoles were isolated essentially as described by Gomez and Chrispeels (1993). The protoplast suspension was supplemented with the cell wall synthesis inhibitor dichlorobenzonitrile at 2 $\mu\text{g ml}^{-1}$ (not 2 mg ml^{-1} as erroneously printed in the reference). After 24 h incubation, protoplasts were pelleted and resuspended in a lysis buffer (0.2 M mannitol, 10% Ficoll 400, 20 mM EDTA, 2 mM DTT, 5 mM HEPES, pH 8, 150 $\mu\text{g ml}^{-1}$ BSA) prewarmed at 42°C. The lysate was overlaid with an equal volume of a cold 5% Ficoll buffer (a 1:1 mixture of lysis buffer and vacuole buffer) and then with 0.25 volume of vacuole buffer (0.6 M mannitol, 10 mM HEPES, pH 7.5, 150 $\mu\text{g ml}^{-1}$ BSA). After centrifugation for 20 min at 1500 *g*, vacuoles were collected from the upper interphase and observed by fluorescence microscopy. The pellet was also checked for fluorescent particles and nuclei were stained with ethidium bromide at various concentrations.

Dyes and inhibitors

Neutral red (Fluka, Buchs, Switzerland) was dissolved directly in the culture medium at a concentration of 10 mg ml^{-1} . The final concentration for staining was 1 mg ml^{-1} . Cells were incubated with NR for 30 min at room temperature. One volume of osmoticum was added, cells were centrifuged for 5 min at 80 *g*, resuspended in 1 volume of fresh culture medium and evaluated within 30 min.

The following inhibitors were added to the culture medium used to resuspend the protoplasts after rinsing the PEG and were present for the entire incubation time.

NH₄Cl (Merck, Darmstadt, Germany) was filter sterilized as a 1 M stock solution and added to the protoplast suspensions at concentrations of 5, 10, 20 and 50 mM (Ehara *et al.*, 1996). Monensin (Sigma, Buchs, Switzerland) was dissolved in ethanol and used at the final concentrations of 5 and 10 μM (Ehara *et al.*, 1996). Bafilomycin A1 (Calbiochem, Lucerne, Switzerland) was dissolved in DMSO and used at concentrations of 2.5, 5 and 10 μM (Matsuoka *et al.*, 1997). Wortmannin (Sigma, Buchs, Switzerland) was dissolved in DMSO in a 10 mM stock solution. Final concentrations of 15, 25, 35 and 45 μM were used (Nakamura *et al.*, 1993).

Fluorescence microscopy

Protoplasts were observed with an Olympus BH2 microscope equipped with a mercury short arc lamp (OSRAM HBO). Pictures were taken on Kodak Elitell 400 colour film with an exposure time of 10 sec.

Confocal images were obtained with a confocal laser-microscope Leica DMR using the Leica TCS 4D operating system. GFP was detected with the filter set for FITC.

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References

- Boevink, P., Santa Cruz, S., Hawes, C., Harris, N. and Oparka, K.J.** (1996) Virus-mediated delivery of the green fluorescent protein to the endoplasmic reticulum of plant cells. *Plant J.* **10**, 935–941.
- Dombrovski, J.E., Schroeder, M.R., Bednarek, S.Y. and Raikhel, N.V.** (1993) Determination of the functional elements within the vacuolar targeting signal of barley lectin. *Plant Cell*, **5**, 587–596.
- Ehara, M., Noguchi, T. and Ueda, K.** (1996) Uptake of neutral red by the vacuoles of a green alga, *Micrasterias pinnatifida*. *Plant Cell Physiol.* **37**, 734–741.
- Freydl, E., Meins, F. Jr, Boller, T. and Neuhaus, J.-M.** (1995) Kinetics of prolyl hydroxylation, intracellular transport and C-terminal processing of the tobacco vacuolar chitinase. *Planta*, **147**, 250–256.
- Gomez, L. and Chrispeels, M.J.** (1993) Tonoplast and soluble vacuolar proteins are targeted by different mechanisms. *Plant Cell*, **5**, 1113–1124.
- Grabski, S., Feijter, A.W. and Schindler, M.** (1993) Endoplasmic reticulum forms a dynamic continuum for lipid diffusion between contiguous soybean root cells. *Plant Cell*, **5**, 25–38.
- Haseloff, J., Siemerling, R.K., Prasher, D.C. and Hodge, S.** (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. *Proc. Natl Acad. Sci. USA*, **94**, 2122–21270.
- Heim, R., Prasher, D.C. and Tsien, R.Y.** (1994) Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl Acad. Sci. USA*, **91**, 12501–12504.
- Hohl, I., Robinson, D.G., Chrispeels, M.J. and Hinz, G.** (1996) Transport of storage proteins to the vacuole is mediated by vesicles without a clathrin coat. *J. Cell Sci.* **109**, 2539–2550.
- Holwerda, B.C., Padgett, H.S. and Rogers, J.C.** (1992) Proaleurain vacuolar targeting is mediated by short contiguous peptide interactions. *Plant Cell*, **4**, 307–318.
- Matsuoka, K., Bassham, D.C., Raikhel, N.V. and Nakamura, K.** (1995) Different sensitivity to wortmannin of two vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. *J. Cell Biol.* **130**, 1307–1318.
- Matsuoka, K., Higuchi, T., Maeshima, M. and Nakamura, K.** (1997) A vacuolar-type H⁺-ATPase in a nonvacuolar organelle is required for the sorting of soluble vacuolar protein precursors in tobacco cells. *Plant Cell*, **9**, 533–546.
- Nagy, J.I. and Maliga, P.** (1976) Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana glauca*. *Z. Pflanzenphysiol.* **78**, 453–455.
- Nakamura, K., Matsuoka, K., Mukumoto, F. and Watanabe, N.** (1993) Processing and transport to the vacuole of a precursor to sweet potato sporamin in transformed tobacco cell line BY-2. *J. Exp. Bot.* **44**, 331–338.
- Negrutiu, I., Shillito, R.D., Potrykus, I., Biasini, G. and Sala, F.** (1987) Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. *Plant Mol. Biol.* **8**, 363–373.
- Neuhaus, J.-M. and Rogers, J.C.** (1998) Sorting of proteins to vacuoles in plant cells. *Plant Mol. Biol.* in press.
- Neuhaus, J.-M., Pietrzak, M. and Boller, T.** (1994) Mutation analysis of the C-terminal vacuolar targeting peptide of tobacco chitinase: low specificity of the sorting system, and gradual transition between intracellular retention and secretion into the extracellular space. *Plant J.* **5**, 45–54.
- Neuhaus, J.-M., Sticher, L., Meins, F. Jr and Boller, T.** (1991) A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. *Proc. Natl Acad. Sci. USA*, **88**, 10362–10366.
- Okita, T.W. and Rogers, J.C.** (1996) Compartmentation of proteins in the endomembrane system of plant cells. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 327–350.
- Paris, N., Stanley, C.M., Jones, R.L. and Rogers, J.C.** (1996) Plant cells contain two functionally distinct vacuolar compartments. *Cell*, **85**, 563–572.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.** (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schroeder, M.R., Borkhsenius, O.N., Matsuoka, K., Nakamura, K. and Raikhel, N.V.** (1993) Colocalization of barley lectin and sporamin in vacuoles of transgenic tobacco plants. *Plant Physiol.* **101**, 451–458.
- Siemerling, K.R., Goblik, R., Pines, J., Jackman, M. and Haseloff, J.** (1996) Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* **6**, 1653–1663.