# Green fluorescent protein reveals variability in vacuoles of three plant species

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## Abstract

Two vacuolar green fluorescent proteins (GFP) were stably inserted in *Nicotiana tabacum* and *Nicotiana benthamiana* genome, with unexpected difficulties, and compared with *A. thaliana* cv. Wassilewskaja transgenic plants expressing the same constructs. GFP fluorescence was strong in all tissues of *A. thaliana* but it was barely visible in *Nicotiana*. Confocal microscopy analysis revealed a variable distribution of the marker in those cells where GFP fluorescence was visible. The role of light dependent proteases was the variable pointing out more inter-species diversity. GFPs degradation was much higher in *Nicotiana* spp. than in *A. thaliana*. The version of GFP used appeared not to be a good vacuolar marker for *Nicotiana* differentiated tissues, although it can efficiently label vacuoles in protoplasts or calli. Nevertheless the sensitivity of the reporter protein can be used as an indicator of hidden characteristics of the plant vacuoles, revealing differences otherwise invisible. One of the markers in our system, GFP-Chi, evidenced a clear morphological difference in the vacuolar system of guard cells of the three species.

*Additional key words*: *Arabidopsis thaliana*, GFP, *Nicotiana benthamiana*, *Nicotiana tabacum*, protease.

## Introduction

Vacuoles are the largest organelles in plant cells. They are modified functionally and morphologically during development (Marty 1999) but little is known about their ontogenesis and physiology. Different types of vacuoles have been shown to co-exist in plant cells and tonoplast intrinsic proteins (TIPs) have been used as markers to identify them (Jauh *et al.* 1999, Jiang *et al.* 2000). Nevertheless their distinctions are not always clear. The biological functions of the different types of vacuole in many cell types are not fully understood. The study of soluble proteins sorted to different vacuoles has contributed to understanding of their functional characterisation (Vitale and Raikhel 1999, Jiang *et al.* 2000, Toyooka *et al.* 2000, Sanderfoot *et al.* 2001). The use of reporter proteins can allow comparative evaluations about vacuole physiology. At least in protoplasts, a valid reporter protein with vacuolar localisation is the green fluorescent protein (GFP). Targeting of GFPs to vacuoles of differentiated plant cells has been reported with a remarkable delay with respect to earlier papers dealing with protoplasts (Swanson *et al.* 1998, Di Sansebastiano *et al.* 1998, 2001). In fact just recent reports have shown vacuolar GFP expression in transgenic *Arabidopsis thaliana* (Fluckiger *et al.* 2003, Tamura *et al.* 2003) and *Nicotiana tabacum* (Di Sansebastiano *et al.* 2004) plants.

Vacuolar GFPs may now allow the monitoring of vacuolar development in different tissues during growth and differentiation (Fluckiger *et al.* 2003). Unfortunately, the possible degradation and/or protonation of GFP under the acidic conditions typical of vacuoles have been shown to be extremely different in the transgenic plants cited above.

Here is a comparative study of vacuolar GFPs accumulation in three plant species largely used as experimental tool in different laboratories: *A. thaliana, N. tabacum* and *N. benthamiana.*

*Received* 29 March 2005, *accepted* 7 January 2006.
*Abbreviations*: ER - endoplasmatic reticulum; GFP - green fluorescent protein; LV - lytic vacuole; PVC - pre-vacuolar compartment; SDS - sodiumdodecyl sulphate; TIP - tonoplast intrinsic protein; VSD - vacuolar sorting determinant.
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## Materials and methods

**Gene constructs and vector mobilisation into plant cells:** Vacuolar GFP variants expressed in *A. thaliana, N. tabacum* and *N. benthamiana*, were identical to those previously described by Fluckiger and co-workers (2003). In GFP-Chi the fluorescent marker was fused to a signal peptide, for translocation into the ER, at the N-terminus and to a C-terminal vacuolar sorting determinant (ctVSD) from tobacco chitinase A; in Aleu-GFP, the marker was fused to the first 150 amino acids from barley aleurain including signal peptide and sequence specific VSD (Di Sansebastiano *et al.* 2001). These modified genes were inserted into the binary vector pBin (Frisch *et al.* 1995, Haseloff *et al.* 1997, Fluckiger *et al. 2*003). *Agrobacterium tumefaciens* GV3101 strain was used in stable transformation because of its high virulence (Haseloff *et al.* 1997). *A. tumefaciens* was transformed by triparental mating using the *Escherichia coli* helper strain HB101 containing pRK2013. An identical binary vector pBIN was used in all three species. GFPs expression was driven by a single 35S promoter with no enhancing sequences (Fluckiger *et al.* 2003).

**Stable transformation and confocal microscopy:** *Arabidopsis thaliana* L. (cv. Wassilewskaja) transgenic plants were available in our laboratory (Fluckiger *et al.* 2003). *Nicotiana tabacum* L. (cv. SR1) and *Nicotiana benthamiana* L. plants, were transformed as described by Fisher and Guiltinan (1995). Part of the *N. tabacum* transformants was also described in a previous report (Di Sansebastiano *et al.* 2004).

Transgenic plantlets were grown from seeds of primary transformant on sterile solid Murashige and Skoog basal medium (MS, 3 % sucrose, 0.8 % agar) under continuous light (25 μmol $m^{-2}$ $s^{-1}$) at 24 °C. Observed samples consisted of plantlets at different stages of development from 7 to 14 d after germination or leaf fragments from adult plants 20 to 30 cm tall. These samples were mounted for microscopical observation in water under glass coverslips. Since *N. benthamiana* plants transformed with Aleu-GFP were always sterile, *in vitro* micropropagated shoots were used directly for microscopical observation.

For testing darkness effect, plantlets were incubated, on the same MS basal medium, for 48 h at 24 °C wrapped in aluminium foil and placed in a dark cabinet.

Plantlets and tissue samples were examined using a confocal laser-microscope *LSM Pascal Zeiss*. GFP was detected with the mirrors set to select 505 - 530 nm emission wavelength after excitation with 488 nm laser, while chlorophyll autofluorescence was detected with the filter set to select >650 nm emission wavelength after excitation with 633 nm laser.

**Protein extraction and immunoblotting:** Plant tissue samples (< 0.2 g) were reduced to powder in liquid nitrogen and incubated at 70 °C for 10 min in the extraction buffer (20 mM Tris-HCl pH 7,5; 500 mM NaCl; 2 % sodiumdodecyl sulphate, SDS). Insoluble residues were pelleted by centrifugation for 5 min at 12 000 *g*. Total soluble proteins were precipitated with 2 volumes of cold acetone.

Equivalent amounts of total proteins were separated in polyacrylamide gels with SDS (4 % stacking gel, 15 % separation gel) using the protocol described by Laemmli and Favre (1972). GFP detection was obtained using anti GFP (molecular probes *A6455*) primary antibodies and anti-rabbit secondary antibodies coupled to peroxidase (*Sigma A3687*) with ECL chemioluminescent protein detection kit from *Amersham* (*RPN2109*). Estimation of band intensity was obtained with the *Kodak 1D v3.6.2 Scientific Imaging System.*

## Results and discussion

**Stable transformation of plants:** Two markers were used to label vacuolar system of transgenic plants: a GFP with a C-terminal vacuolar sorting determinant (GFP-Chi) targeted to protein storage vacuoles (PSVs) and a GFP with a sequence-specific vacuolar sorting determinant (Aleu-GFP) targeted to the lytic vacuole (LV) and pre-vacuolar compartments (PVCs).

Regeneration of *Nicotiana* plants transformed with Aleu-GFP was problematic. In three independent experiments, only few transgenic lines were obtained from *N. tabacum* tissue transformed with Aleu-GFP, compared to transformants with GFP-Chi or control cytosolic GFP. Only 20 % of thirty plants survived in soil and produced seeds. Similar problems were experienced for *N. benthamiana* regenerants transformed with Aleu-GFP. None of the 12 transgenic plants produced seeds. Low regeneration efficiency from leaf discs suggests a negative effect of the transgene on cell differentiation. In fact, undifferentiated transformed callus cells were proliferating with no apparent problems (Fig. 1*A*,*B*). Compared to control transformations with cytosolic GFP, regeneration efficiency in *N. tabacum* was approximately 50 % for GFP-Chi and 10 % with Aleu-GFP; in *N. benthamiana* it was 10 % for GFP-Chi and 2 % with Aleu-GFP. Progeny segregation on kanamycin selection proved that all transgenic lines which produced seeds, were transformed by a single T-DNA insertion.

**Vacuolar GFPs fluorescent pattern in *A. thaliana*, *N. tabacum* and *N. benthamiana* plants:** Targeted to different vacuoles, the two GFP markers were expected to have different fluorescent pattern and this was previously described in *A. thaliana* (Fluckiger *et al.* 2003). Surprisingly it was hard to see GFP fluorescence in

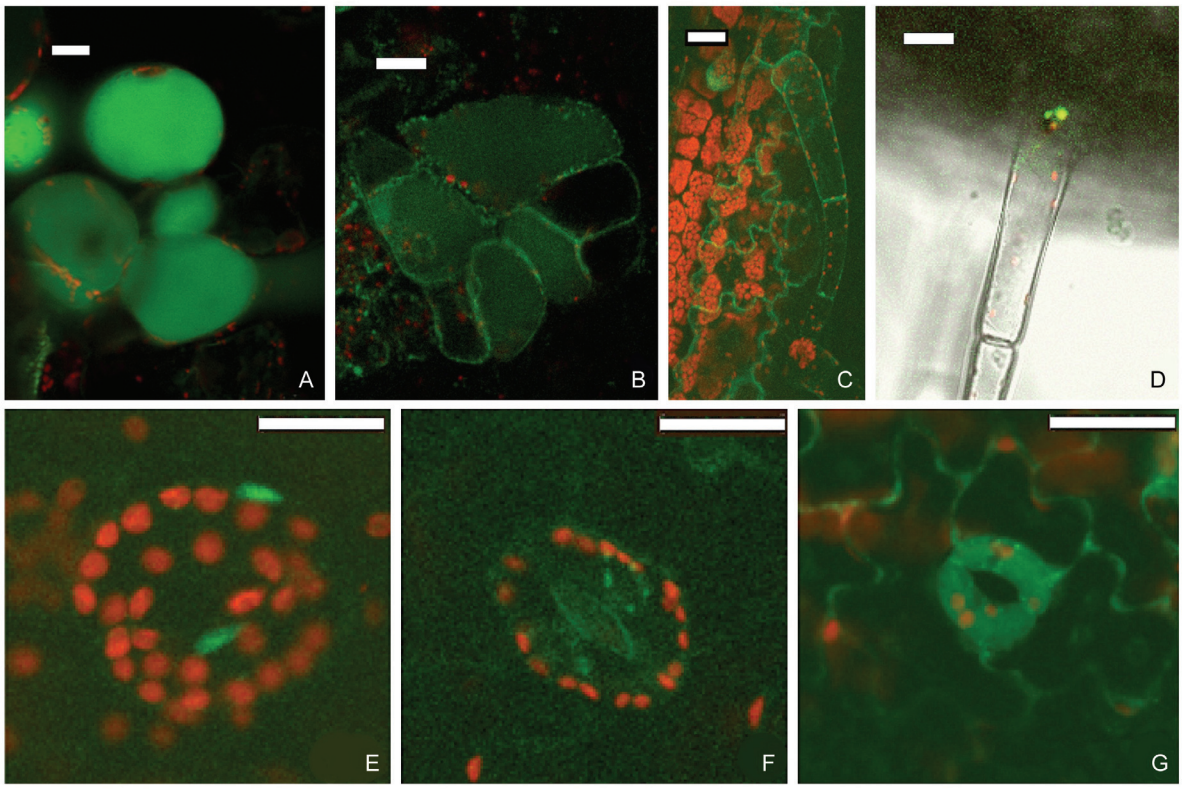


Fig. 1. Confocal images of cells from different plant species: *A* - *N. tabacum* callus cells expressing Aleu-GFP, 1.7 µm confocal section; *B* - *N. benthamiana* callus cells expressing Aleu-GFP, 1.7 µm confocal section; *C* - transgenic tissue, expressing GFP-Chi, from a regenerating shoot of *N. tabacum*, 12 µm confocal projection; *D* - trichome of a *N. tabacum* primary transformant expressing GFP-Chi, 10 µm confocal projection including transmitted light; *E*, *F*, *G* - guard cells expressing GFP-Chi, from transgenic leaves of *N. tabacum* (*E*), *N. benthamiana* (*F*), *A. thaliana* (*G*), 1.8 µm confocal sections. Green fluorescence is due to the expression of GFPs, chlorophyll autofluorescence is in red. *Scale bar* = 20 µm.

*Nicotiana* species.

Both primary transformant and progeny were observed for *N. tabacum*. GFP-Chi was only visible, before rooting, in ER of regenerating plants (Fig. 1*C*). In a shoot grown at the stage of three or four internodes, GFP-Chi appeared only in small compartments in cells at the base of trichomes (Fig. 1*D*) or in a single larger compartment of guard cells (Fig. 1*E*). No fluorescence was detected in flowering plants or in the second-generation plantlets. Aleu-GFP fluorescence was never detected in any *N. tabacum* transgenic plant.

Only primary transformant were used for *N. benthamiana* since no seeds were available for Aleu-GFP expressing plants of this specie. GFP-Chi fluorescence was detected in all transformants and in all cell types but it was faint and localised to the ER. This fluorescence distribution was confirmed in rooted plants and following generation. Also stomata guard cells shared the same pattern with a frequent punctuate distribution of fluorescence (Fig. 1*F*). Aleu-GFP fluorescence was never detected in any transgenic plant.

Observations for *A. thaliana* transgenic plants were done exclusively on the progeny of primary transformants (Fluckiger *et al.* 2003). In these plants GFP-Chi was initially kept separated from the large central vacuole, probably a lytic compartment while, after complete elongation and differentiation of cells, GFP-Chi reached the large central vacuole. On the contrary, Aleu-GFP was rapidly sorted to the large central vacuole. In fully differentiated stomata guard cells, both vacuolar GFPs were visible in the central vacuole. A brighter fluorescence was observed in the case of GFP-Chi (Fig. 1*G*) compared to Aleu-GFP fluorescence.

Fluorescence distribution within the cells transformed with GFP-Chi, the only vacuolar GFP visible in all three species, was dissimilar. The comparison was possible only in stomata guard cells because these cells were clearly identified and fluorescent (Fig. 1*E*-*G*).

**Effect of continuous darkness on vacuolar GFPs accumulation:** Plantlets of all three species, with the exception of Aleu-GFP expressing *N. benthamiana*, which consisted of *in vitro* propagated shoots, were grown in parallel for 48 h either under continuous light or darkness to be analysed by confocal microscopy and biochemical analysis. In fact Tamura and co-workers (2003) have demonstrated the activity of light-dependent proteases in *A. thaliana* cv. Columbia, reporting their effect on a chimerical GFP fused with the C-terminal peptide of pumpkin 2S albumin. If the proteases changed

the fluorescent patterns as described by Tamura *et al*. (2003), not only the results reported here but also those previously reported for *A. thaliana* (Fluckiger *et al*. 2003) might have to be reconsidered.

Confocal microscopy observations in *A. thaliana* revealed an increase in fluorescence under dark conditions (Fig. 2*A*,*B*) but fluorescent patterns were identical to those previously described by Fluckiger *et al*. (2003) in light conditions. Only in the hypocotyl parenchyma cells, as already described in the first description of these plants (Fluckiger *et al.* 2003), both GFP-Chi and Aleu-GFP were found not to be fluorescing in the central vacuole, however, 48 h of complete darkness were sufficient to make these compartments clearly fluorescent (Fig. 2*C*,*D*). Also in *Nicotiana*, no significant changes were observed in the dark and in this case Aleu-GFP remained not visibly fluorescent.

For the biochemical analysis of vacuolar GFPs, total

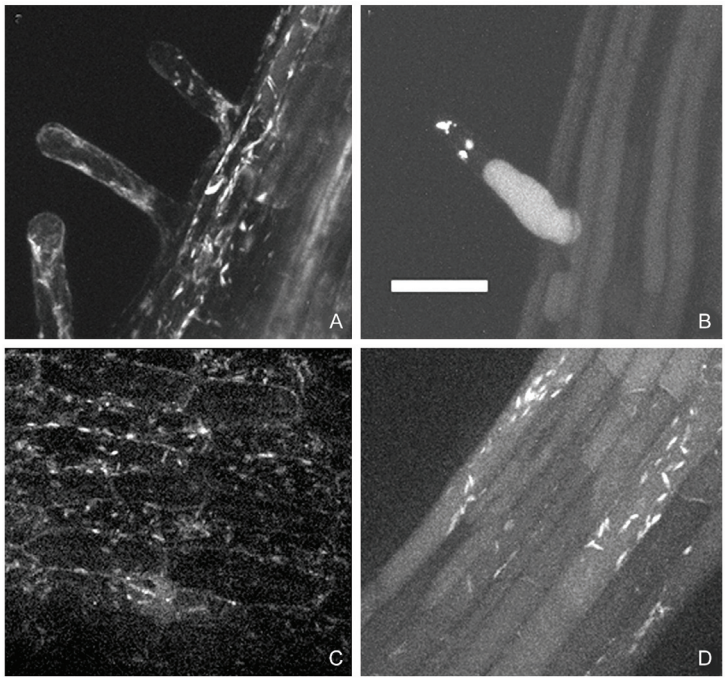


Fig. 2. Vacuolar GFPs in root hairs and hypocotyl cells of transgenic *Arabidopsis*. Projection of confocal laser scanning optical sections through the roots of *Arabidopsis thaliana* cv. Wassilewskaja plantlets, at an early stage of root hair formation, stably expressing two different vacuolar GFP-markers. During root hair expansion, GFP-Chi labelled only the ER and small vacuolar compartments both growing in continuous light (not shown) or after 48-h darkness (*A*), Aleu-GFP labelled directly a large central vacuole evidencing occasionally Golgi and pre-vacuoles both growing in continuous light (not shown) or after 48-h darkness (*B*). The hypocotyl parenchyma cells of either GFP-Chi (*C* and *D*) and Aleu-GFP (not shown) expressing seedling does not show fluorescence in the central vacuole when grown in continuous light conditions (*C*) but 48 h of complete darkness are sufficient to make these compartments clearly fluorescent (*D*). *Scale bar* = 50 µm.

soluble proteins were extracted from 7-d-old plantlets grown in either continuous light or dark conditions, and samples were prepared at similar concentration of proteins for Western-blot analysis. In most analysed cases, a strong increase in the amount of GFP was visualised through immunodetection when plantlets were grown in continuous darkness. This suggested that the GFP marker, whose expression was driven by the strong promoter 35S, was proteolytically degraded as described by Tamura *et al.* (2003) when *A. thaliana* (cv. Columbia) was grown in the presence of light. This observation was made for both *Arabidopsis* and *Nicotiana* plants (Fig. 3). In the case of *Nicotiana*, continuous darkness was the only condition in which a mature form of GFP-Chi showing the molecular mass of a matured GFP, typical of vacuolar processing, was observed (Fig. 3*B*,*C*). Variability can be observed among the different plant species. In *A. thaliana* cv. Wassilewskaja, darkness conditions determined an increase of Aleu-GFP of 53 % but did not induce a significant increase in GFP-Chi content (Fig. 3*A*). In *N. tabacum* grown in continuous light, Aleu-GFP was almost below detectable levels but its content increased by more than ten-folds in continuous dark conditions (Fig. 3*B*). In light conditions GFP-Chi was immunodetected exclusively as an immature form with higher molecular mass. Its content increased in continuous darkness by 33 % but two forms were then observed. In addition to the immature form, a mature form with the molecular mass of GFP without any additional peptide (about 28 kD) appeared (Fig. 3*B*). Similarly, in *N. benthamiana* the increase of Aleu-GFP amount in darkness was very high, up to 68 % (Fig. 3*C*),

also GFP-Chi content increased by 20 % and a second band corresponding to mature GFP was immunodetected (Fig. 3*C*).

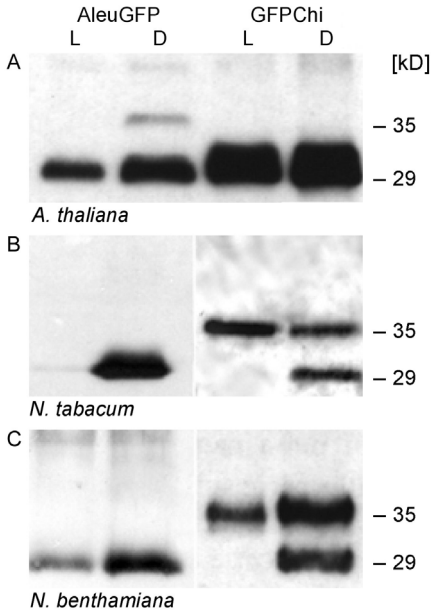


Fig. 3. Vacuolar GFPs extracted from transgenic plants grown in continuous light (L) or darkness (D) immunolabelled on Western blot. On the right indicative molecular mass are reported. *A* - *A. thaliana*, *B* - *N. tabacum* and *C* - *N. benthamiana*.

**General discussion:** In the present work we report the unexpected differences emerged during transformation of three plants species, broadly used for research, with two vacuolar GFP markers. *A. thaliana* and *N. tabacum* transgenic plants were previously described (Fluckiger *et al.* 2003, Di Sansebastiano *et al.* 2004). Nonetheless new transformation experiments were performed again for *N. tabacum* in parallel with the transformation, here described for the first time, of *N. benthamiana*.

The first observation reported in this study is that stable transformants of vacuolar GFPs were difficult to obtain in *Nicotiana* spp. This observation was surprising since we did not expect that a soluble vacuolar marker such as GFP might have any consequence on plant organogenesis. Nonetheless, considering the wide-ranging and unique functions of the vacuoles, over expression of vacuolar exogenous proteins may have affected the correct organisation of the vacuolar complex, indispensable for cellular activity. In the past, the importance of vacuoles organisation was evidenced when a knockout mutant with altered vacuoles morphology in *A. thaliana* (*vacuoless vcl1*) was shown to be not viable (Rojo *et al.* 2001). This represents the first demonstration that a functional vacuole is required for plant viability; in addition, the correct targeting of cellular material to vacuoles is necessary for cell viability as well as for cell growth and differentiation (Surpin and Raikhel 2004). The expression of exogenous proteins such as chimerical GFPs may saturate the vacuolar system and affect its activities, ranging from movement of ions and metabolites to hormonal signalling. Since vacuolar GFPs appear to fluoresce differently in *Arabidopsis* and *Nicotiana* spp., it is plausible to speculate that vacuoles saturated with GFPs have a negative effect on *Nicotiana* cell growth but not on *Arabidopsis*. This may explain the lack of fertile transformant for Aleu-GFP transformed *N. benthamiana*, and the low regeneration efficiency for *N. tabacum* transgenics. The use of vectors inserting the transgene under control of inducible promoters may bypass the regeneration problems but may not solve the problem of the lack of fluorescence in specific vacuolar compartments. In fact also transient expression of these constructs in leaves, after *Agrobacterium* infiltration, does not solve the problem of the lack of labelling in most of cell vacuoles. This technique was used in the past and showed already how the vacuolar GFPs used here, cannot label the large central vacuole of mesophyll and epidermal cells (Di Sansebastiano *et al.* 2004, Kotzer *et al.* 2004).

In the past GFP failed already to show fluorescence in transgenic plants (Haseloff and Amos 1995) and better vector were designed to overcome the problem (Köhler *et al*. 1997), but in the present work, the weak fluorescent signal is source of polymorphism and new information.

If we want to compare the organisation of the vacuolar system in these plant species, we have to restrict observations to specific cell types because not all cells were fluorescent. Stomata guard cells accumulating GFP-Chi appeared suitable for this purpose. In *A. thaliana* stomata guard cells GFP-Chi accumulated visibly in the large central vacuole but in *N. tabacum* it was visible only in a single small vacuole uniquely in regenerating shoots. In *N. benthamiana,* GFP-Chi fluorescence was not linked to plant development but was restricted to the ER or small punctuate structures. We know that GFP-Chi accumulated in large amounts within the cell even if it was not fluorescent, consequently we cannot say if fluorescent compartments observed are the exclusive accumulation sites of the protein. Anyhow it is very likely that the large central vacuole of the cells of different species does not share the same permissive conditions for GFP fluorescence. One of the parameters that can be taken into consideration is pH. Too acidic pH may alter protonation equilibrium of GFPs and make it not fluorescent but the pH indicator Neutral Red, that evidence pH below 5, showed in the past that the vacuolar GFPs described here are normally fluorescent in acidic vacuoles of undifferentiated callus cells (Di Sansebastiano *et al.* 2004).

Tamura *et al.* (2003) have recently described the expression in *A. thaliana* plants cv. Columbia of a GFP C-terminally tagged with a vacuolar sorting determinant from pumpkin 2S albumin (SP-GFP-2SC). In apparent contrast with Fluckiger *et al.* (2003) data, they showed that SP-GFP-2SC arrived to the large central vacuole in all cell types but its fluorescence was strictly dependent upon inhibition of protease activity by inhibitors or

growth under dark conditions.

Since fluorescence variability was already reported in light grown *A. thaliana* cv. Wassilewskaja (Fluckiger *et al.* 2003), the validity of the fluorescent patterns observed in those plants was again controlled considering the effect of light-dependent degradation. An increase of fluorescence was observed, but patterns were identical to those previously described (Fluckiger *et al.* 2003). We also tested vacuolar GFP-expressing *N. tabacum* and *N. benthamiana* plants under the experimental conditions indicated by Tamura *et al.* (2003) and, in spite of a large increase of GFP quantity revealed by Western blotting, no increase of fluorescence was observed. Again differences between the light-dependent proteolytic activity emerged between *Arabidopsis* and *Nicotiana* spp. The possibility of additional factors, at transcriptional or translational level, influencing GFPs accumulation, can not be ruled out because degradation of GFPs produce small fragments that are not efficiently immunolabelled by anti GFP serum. In fact GFP is a very compact molecule, it has a barrel structure formed by a β-pleated sheet and proteases probably do not cut the short N or C termini but the amino-acid bridges linking the β-pleated sheets, reducing the protein in much smaller fragments no more recognised by antibodies.

Even in the absence of fluorescence, vacuolar GFPs remain powerful tools to study the vacuolar-targeting machinery and to investigate organ-specific characteristics and differentiation of the endomembranes in plants (Hanson and Köhler 2001). In this study the markers revealed that the vacuolar compartments have different characteristics in different plants species from the point of view of morphology in differentiated cells and of light dependent proteolytic activity. *A. thaliana* cv. Wassilewskaja remains a valuable expression model system for the capacity of GFP to remain stable inside the vacuoles but the different behaviour of cv. Columbia (Tamura *et al.* 2003) and of *Nicotiana* spp. raises important questions about data interpretations. Many researchers are performing GFP expression in different plant species and, in some cases, they may have to deal with a number of parameters that have not usually been taken into account, such as GFP instability in chimerical constructs used in many laboratories as controls, and the unknown ratio between GFP protein quantity and visible fluorescence. However, very poor data are available in literature about localisation in transgenic *Nicotiana* spp. of stably expressed ER-retained (Hraška *et al.* 2005) or vacuolar GFP (Di Sansebastiano *et al.* 2005) fusion proteins, whereas several reports describe chimerical GFPs expression driven by strong tissue specific promoters (Ottenschläger *et al.* 1999, Wright *et al.* 2003) or in transient expression assays (Geelen *et al.* 2002, Brandizzi *et al.* 2003).

Vacuoles show different morphological, physiological and biochemical characteristics in different plant species and they can also be subjected to several modifications during cell growth and differentiation. Vacuole physiological modifications could explain the loss of fluorescence of GFP fused with the C-terminal peptide of pumpkin 2S albumin (Tamura *et al.* 2003), an event already described in tobacco BY2 cells relating to a precise stage of the suspension culture growth (Mitsuhashi *et al.* 2000). When calli were generated from the *Nicotiana* transgenic plants described here, the transition from differentiated to undifferentiated cells corresponded to the appearing of fluorescence in the central vacuole (Di Sansebastiano *et al.* 2004), we can then draw a parallel with BY2 in which, vacuolar GFP became fluorescent only after days of subculturing (Mitsuhashi *et al.* 2000).

Tamura *et al.* (2003) have shown an interesting way to shed light on differences between vacuoles in different plant species indicating the role of light-dependent proteases, which may also function as physiologic marker to characterise different vacuoles. Nevertheless we demonstrate here that morphological differences of the vacuolar system reported in *A. thaliana* cv. Wassilewskaja are not simply due to light-dependent degradation. In *Nicotiana* spp., more variability emerges and we deduce that vacuolar complex organisation is characteristic of cell types but also plant specie.

Huge differences in vacuolar GFPs stability and distribution can be expected among other plant species and also cultivars of the same specie. The above concerns may become an even more important point when related to the expression and stability of other eterologous proteins of interest for biotechnological applications.

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