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The C-terminal tetrapeptide of phaseolin is sufficient to target green fluorescent protein to the vacuole*

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Summary

Phaseolin is a vacuolar storage glycoprotein synthesized as a precursor with a short C-terminal propeptide. We have recently shown that deletion of the last four C-terminal amino acids (AFVY, which are part of, or constitute the propeptide) abolishes vacuolar targeting, causing phaseolin to be secreted. Here we provide biochemical and microscopical evidence that the AFVY tetrapeptide, when fused to a secreted version of green fluorescent protein (GFP), inhibits GFP secretion and leads to its accumulation in vacuoles, where it is processed. This demonstrates that the tetrapeptide contains sufficient information for vacuolar sorting.

Key words: plant secretory pathway – vacuolar sorting – phaseolin – green fluorescent protein

Abbreviations: BFA brefeldin A. – ER endoplasmic reticulum. – GFP green fluorescent protein

Introduction

Secretory proteins that are destined to plant vacuoles must carry discrete determinants for vacuolar sorting. These determinants are heterogeneous in nature and specificity (Vitale and Raikhel 1999). This reflects, at least in part, the fact that cells may contain functionally different vacuolar compartments (Paris et al. 1996). Vacuolar sorting signals are found to be necessary by deletion or mutation in the original protein, and sufficient if, once fused to a reporter protein, they are capable of targeting it to vacuoles (Neuhaus et al. 1991). Three classes of sequences involved in plant vacuolar sorting have been established: sequence-specific sorting signals, physical structure sorting determinants and C-terminal signals (reviewed in Matsuoka and Neuhaus 1999). This classification is purely descriptive, as the univocal attribution of one class to a specific vacuolar targeting pathway remains problematic. All the signals clearly identified to date are part of, or constitute, propeptides (Matsuoka and Neuhaus 1999).

C-terminal signals have been identified in propeptides of several vacuolar proteins (Vitale and Raikhel 1999, Koide et al. 1999, Miller et al. 1999). They are very variable in length and do not possess a conserved sequence motif; their common feature is the presence of a hydrophobic amino acid

* This paper is more focussed than the oral presentation at the meeting since a more general review has been published recently (Vitale and Raikhel 1999).

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patch within the propeptide (Vitale and Raikhel 1999, Matsuoka and Neuhaus 1999).

We have studied the intracellular trafficking of phaseolin, the major vacuolar storage glycoprotein of common bean seeds. In developing bean cotyledons, phaseolin is transported to the protein storage vacuoles (PSV): upon arrival at the PSV, a short propeptide is proteolytically removed from phaseolin (Bollini et al. 1982, D'Amico et al. 1992). This trimming event occurs at the C-terminus and involves only four or five amino acids (Bollini et al. 1982, Bollini and Vitale, unpublished). We speculated that this short propeptide might contain vacuolar sorting information. We therefore generated a phaseolin mutant (Δ418) in which the last four C-terminal amino acids, AFVY, were deleted (Frigerio et al. 1998). When Δ418 was expressed in transgenic tobacco plants, it was no longer targeted to vacuoles but quantitatively secreted into the apoplast. We thus concluded that AFVY was necessary for vacuolar sorting, although we could not conclude whether it was a signal, sufficient for sorting, or rather represented just a portion of a larger vacuolar sorting determinant (Frigerio et al. 1998). The sequence AFVY resembles the tetrapeptide IAGF, which is the C-terminal propeptide necessary for the vacuolar sorting of the 2S albumin storage protein of Brazil nut (Matsuoka and Neuhaus 1999, Saalbach et al. 1996), and the two tetrapeptides are the shortest C-terminal vacuolar sorting determinants identified to date. A longer C-terminal portion of 2S albumin, composed by 16 amino acids (including IAGF) is capable of redirecting secreted yeast invertase to the plant vacuole, whereas the IAGF tetrapeptide is not sufficient, suggesting that the 2S propeptide is not a complete signal (Saalbach et al. 1996).

In this work we show that the tetrapeptide AFVY alone is sufficient to efficiently redirect the reporter green fluorescent protein (GFP) to the vacuole in tobacco and *Arabidopsis* cells.

Materials and Methods

DNA

mGFP5 was obtained by PCR amplification from the pBIN-GFP-ER construct (Siemering et al. 1996; a gift from J. Haseloff). The coding sequence was modified to contain the additional AFVY residues at the 5′ end by PCR to yield GFP-AFVY. Both sequences were cloned downstream to the CaMV 35S promoter in expression vector pDHA (Tabe and Higgins 1998). For stable transformation, pDHA containing GFP-AFVY was linearised with HindIII and inserted into binary vector pGA470.

Transient and stable transformation

Protoplasts were prepared from axenic leaves (4 to 7 cm long) of *N. tabacum* cv. Petit Havana SR1., or from cultured suspension cells of *Arabidopsis thaliana* (Axelos et al. 1992). Protoplasts were subjected to polyethylene glycol-mediated transfection as described by Pedrazzini et al. (1997) and incubated overnight at 25 °C in the dark before pulse labelling, or 24 h at 25 °C in the dark before microscopical observation.

Transgenic plants expressing GFP-AFVY were generated by *Agrobacterium*-mediated transformation as described previously (Pedrazzini et al. 1997).

Cell labelling and protein analysis

Pulse-chase labelling of protoplasts using Pro-Mix (a mixture of ^{35}S-methionine and ^{35}S-cysteine; Amersham) was performed as described (Pedrazzini et al. 1994). Where indicated, cells were pre-incubated with 10 µg/mL brefeldin A for 45 min before labelling. Homogenisation of protoplasts and incubation media was performed by adding to the frozen samples 2 volumes of ice-cold homogenisation buffer (150 mmol/L Tris-Cl, 150 mmol/L NaCl, 1.5 mmol/L EDTA and 1.5 % Triton X-100, pH 7.5) supplemented with Complete (Boehringer) protease inhibitor cocktail. Immunoprecipitation of expressed polypeptides was performed as described previously (Pedrazzini et al. 1994), using rabbit polyclonal antisera raised against GFP (Molecular Probes). Immunoselected proteins were analyzed by 15 % reducing SDS-PAGE and fluorography.

For sucrose gradient fractionation, small (4–6 cm-long) leaves were homogenated with ice-cold buffer A (2 mmol/L $MgCl_2$, 10 mmol/L KCl, 100 mmol/L Tris-Cl, pH 7.8) supplemented with 12 % (w/w) sucrose, using 1 mL of buffer for 200 mg of leaf. The homogenate was centrifuged at 1000 × g, 4 °C for 10 min. The supernatant (1 mL) was loaded on 4 mL of a 16–55 % (w/w) sucrose gradient made in buffer A. The gradient was centrifuged for 90 min at 154,000 × g, 4 °C in a SW 55 rotor (Beckman). Thirty-five µL of each fraction were analyzed by SDS-PAGE and protein blot as described (Pedrazzini et al. 1997), using antisera against GFP (Clontech) and BiP.

Microscopy

Arabidopsis protoplasts transfected with GFP constructs were incubated in the dark for 24 h at 25 °C before observation. Cells were visualised with a GFP filter set on a Leica DMR confocal laser scanning microscope using the Leica TCS 4D operating system and equipped with a 40X oil immersion objective (Di Sansebastiano et al. 1998).

Results

We engineered GFP5 fused to the *Arabidopsis thaliana* basic chitinase signal peptide (mGFP5, Siemering et al. 1996) to contain the C-terminal tetrapeptide AFVY (GFP-AFVY). Both mGFP5 and GFP-AFVY were placed under control of the constitutive CaMV 35S promoter in the expression vector pDHA. We initially characterised biochemically the fate of both recombinant proteins: we transfected tobacco protoplasts and subjected them to pulse labelling with ^{35}S methionine and ^{35}S cysteine, followed by chase for 0 or 24 hours. GFP was then immunoselected from cell homogenates and incubation media with rabbit polyclonal antiserum against native GFP (Fig. 1). As expected (Boevink et al. 1999) mGFP5 is secreted in the incubation medium during the chase (lanes 1–4). Secreted mGFP5 presents a slightly higher electrophoretic mobility, suggesting that it is subject to extracellular processing.

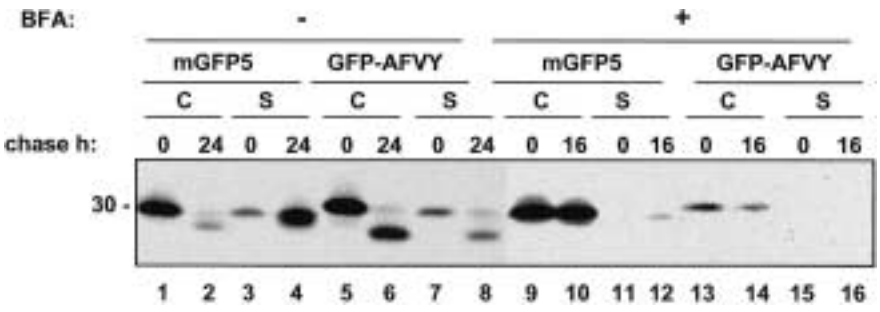

Figure 1. GFP-AFVY is not secreted and undergoes intracellular processing. Tobacco leaf protoplasts were transfected with mGFP5 or GFP-AFVY and subjected to pulse labelling for 1 h in the presence (+) or in the absence (–) of 10 μg/mL brefeldin A (BFA) and chased for 24 h. Cells (C) and incubation media (S) were homogenised and subjected to immunoprecipitation with anti-GFP antiserum, followed by SDS-PAGE and fluorography. The number at left indicates molecular mass marker in kilodaltons.

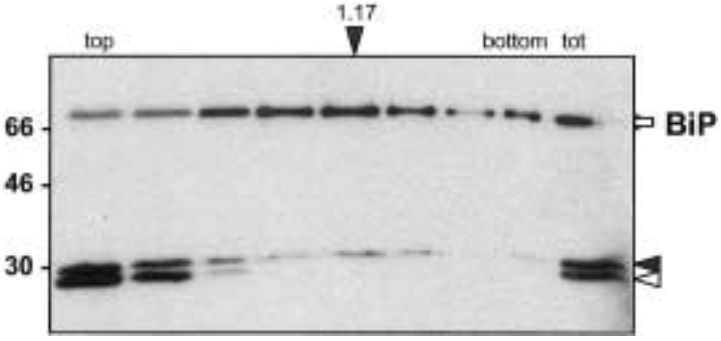

Figure 2. Subcellular fractionation of transgenic tobacco leaves. Leaves from transgenic tobacco plants expressing GFP-AFVY were homogenised in 12 % (w/w) sucrose buffer and subjected to centrifugation on a 16–55 % (w/w) isopycnic sucrose gradient. Gradient fractions were collected and resolved by SDS-PAGE followed by protein blot with anti-BiP or anti-GFP antisera. The number at the top indicates fraction density in mg/mL. Numbers at left indicate molecular mass markers in kilodaltons. The arrowheads on the right indicate intact (black arrowhead) and processed (white arrowhead) GFP-AFVY.

This event has been observed previously for other secreted proteins, and is probably due to the fact that protoplasts actively secrete proteases in the medium (Frigerio et al. 1998). The small amount of protein that remains intracellular after the chase has a greater reduction in molecular weight compared to secreted mGFP5, indicating that it is not on the way to secretion. Upon subcellular fractionation (not shown), intracellular, processed mGFP5 is recovered in a soluble fraction, which contains cytosolic as well as soluble vacuolar proteins and is absent from a total microsomal fraction, suggesting that a minor proportion of mGFP5 is delivered to the vacuole. We conclude that mGFP5 is rather efficiently secreted by the protoplasts. The behaviour of GFP-AFVY is different: the protein remains intracellular and during the chase is subjected to a marked decrease in molecular weight (Fig. 1, lanes 5–8). Retention seems complete: although we cannot exclude that a small proportion of GFP-AFVY is secreted and processed, the protein recovered extracellularly is probably contamination from cells, because it does not increase in amount in the incubation medium during the chase. Therefore, the presence of the C-terminal tetrapeptide AFVY is sufficient to prevent secretion of GFP. Where does processing of GFP-AFVY occur? We performed pulse-chase labelling in the presence of brefeldin A, a fungal metabolite that inhibits vesicular trafficking along the secretory pathway (Gomez and Chrispeels 1993, Pedrazzini et al. 1997, Staehelin and Driouich 1997). We observed that, in the presence of brefeldin A, both secretion of mGFP5 and processing of GFP-AFVY are prevented (Fig. 1, lanes 9–16). This indicates that traffic is needed for the processing of GFP-AFVY. Thus, the vacuole is a likely candidate as the compartment of destination for GFP-AFVY.

We generated transgenic plants expressing GFP-AFVY: we then homogenised leaves from transgenic plants in the presence of sucrose and subjected the homogenates to isopycnic sucrose gradient centrifugation. We resolved the gradient fractions on SDS-PAGE and protein blot analysis with a mixture of anti-GFP and anti-binding protein (BiP) antibodies (Fig. 2). BiP is a major chaperone of the endoplasmic reticulum (ER) and therefore it is a marker for this compartment. The anti-GFP antiserum detects two polypeptides, the sizes of which correspond to intact and processed GFP-AFVY as identified by pulse-chase (Fig. 2). Unprocessed GFP-AFVY is detected in part in the same fractions as BiP. Conversely, the processed polypeptide is recovered exclusively in the top fractions of the gradient. The top fractions contain cytosolic, secreted and vacuolar proteins, because vacuoles break and release their content during homogenization (Pedrazzini et al. 1997). Given that GFP-AFVY is not secreted by protoplasts (Fig. 1), the presence of intact GFP-AFVY in the ER fractions and of both intact and processed GFP-AFVY in the soluble fractions of the gradient strongly suggests that GFP-AFVY is transported from the ER to the vacuole, where it is processed.

The conclusive demonstration that AFVY redirects GFP to the vacuole was provided by direct microscopical observation. We observed GFP in transfected protoplasts from *Arabidopsis thaliana* suspension cultured cells. These cells have

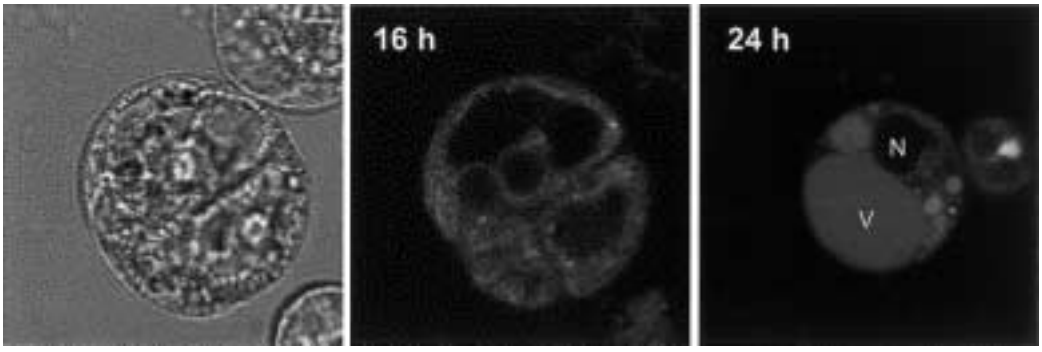

Figure 3. GFP-AFVY accumulates in the vacuole of *Arabidopsis* protoplasts. Protoplasts from *Arabidopsis* suspension cultured cells were transfected with GFP-AFVY and incubated for 16 or 24 h. Cells were observed with a confocal microscope using a FITC filter set. The brightfield image on the left panel refers to the transfected cell observed after 16 h (central panel). N: nucleus; V: vacuole.

the main advantage of being devoid of chloroplasts; furthermore, they seem to present only one type of large, central vacuole, as opposed to tobacco mesophyll protoplasts, which are a heterogeneous population of cells, in which at least two cell types have been shown to contain morphologically different vacuolar structures (Di Sansebastiano et al. 1998, 2001). We transfected protoplasts from *Arabidopsis* cells grown in suspension with GFP-AFVY (Axelos et al. 1992). Sixteen hours after transfection, GFP-AFVY is present in a reticulate structure that most probably represents the ER and perhaps the Golgi complex (punctate structures, Fig. 3). After 24 h, the pattern is different and the protein is mainly detected in the large central vacuole of transfected cells (Fig. 3). At the same time points, no fluorescence is detectable in cells transfected with mGFP5 (data not shown), which is consistent with previous microscopical observations (Boevink et al. 1999) and with our biochemical data indicating that the protein is efficiently secreted in the incubation medium. Therefore GFP-AFVY is delivered to the vacuole.

Discussion

We provided biochemical and microscopical evidence that the AFVY tetrapeptide is able to redirect an otherwise secreted reporter protein to the plant vacuole. This makes AFVY the shortest natural plant vacuolar sorting signal described so far.

It is possible that mGFP5 has anyway a slight tendency to be delivered to the vacuole. GFP is not a natural secretory protein, and it is possible that a small proportion of molecules that fail to fold properly in the environment of the ER are delivered to the vacuole by some form of quality control. Recently, it has been shown that GFP, when fused to the signal peptide of tobacco chitinase, is completely targeted to the vacuole in yeast (Kunze et al. 1999). The situation in our case is clearly different, as the majority of mGFP5 is secreted. However, we have recently shown that a relevant proportion of a hybrid immunoglobulin A/G, which is instead a secretory protein, is also delivered to the vacuole in transgenic tobacco (Frigerio et al. 2000). Nevertheless, our data show that AFVY dramatically changes the intracellular fate of GFP, leading to its virtually complete vacuolar delivery. The fact that vacuolar GFP-AFVY is fluorescent strongly suggests that the protein is correctly folded, thus effectively ruling out the possibility that vacuolar delivery is in this case the result of disposal of a misfolded protein.

The terapeptide IAGF is part of the vacuolar determinant of Brazil nut 2S albumin and resembles AFVY, but it is not sufficient to deliver secretory invertase to vacuoles (Saalbach et al. 1996). It is possible that in spite of their similarity the two propeptides differ in their ability to promote interactions with the as yet unknown sorting machinery for C-terminal signals. Indeed, binding of the vacuolar sorting receptor BP-80 to an affinity column indicated that the 2S albumin propeptide is only part of a larger sequence-specific vacuolar sorting determinant (Kirsch et al. 1996). It is also possible that surface exposure of the tetrapeptide is not optimal when placed at the C-terminus of invertase, because of the folding of the reporter protein (Saalbach et al. 1996). There may be a few amino acid residues at the end of GFP that are highly exposed, as they are not resolved in crystal structures. These could contribute part of the minimal length required for the interaction with a putative sorting receptor for C-terminal sorting determinants. Alternatively, the size of the reporter protein may be critical for C-terminal vacuolar sorting signals. Invertase is a much larger protein than GFP. We have recently provided evidence that sorting of phaseolin to the vacuole is probably receptor-mediated, due to the fact that vacuolar sorting can be saturated at high levels of protein expression (Frigerio et al. 1998), however, it has been suggested that aggregation may play a role in the sorting of storage proteins (Hinz et al. 1999, Saalbach et al. 1991). The presence of a large, normally non aggregated, polypeptide could unfavour aggregation driven by the sorting signals. This hypothesis

can be tested by fusing reporter proteins of different sizes to the same sorting signal.

The *Arabidopsis* protoplasts used in this study differ from tobacco mesophyll protoplasts in targeting GFP with either sequence-specific or C-terminal vacuolar sorting determinants to the central vacuole, which appears thus to be hybrid in nature (Hernández Felipe, unpublished observation). Further work will establish whether the determinants of phaseolin and 2S albumin target reporter proteins to the same type of vacuoles as the propeptides of chitinase or aleurain, i.e. to an acidic or neutral vacuolar compartments, in cells that possess identifiable separate vacuoles.

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