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University of Neuchâtel
Botany Institute
Biochemistry department

TARGETING OF SOLUBLE PROTEINS TO TWO DIFFERENT VACUOLES IN PLANT CELLS

Ph.D. thesis of Gian Pietro Di Sansebastiano

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Targeting of soluble proteins to two different vacuoles in plant cells

de M. Gian-Pietro Di Sansebastiano

UNIVERSITÉ DE NEUCHÂTEL

FACULTÉ DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel sur le rapport des membres du jury,

MM. J.-M. Neuhaus (directeur de thèse), E. Martinoia, P. Schürmann et C. Hawes (Oxford Brookes Uni., UK)

autorise l'impression de la présente thèse.

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Le doyen:

F. Stoeckli

F. Stoeckli

Abbreviations

CCV	clathrin coated vesicle
DV	dense vesicle
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EtOH	ethanol
GFP	green fluorescent protein
GUS	ß-glucuronidase
LV	lytic vacuole
MOPS	3-[N-Morpholino]propanesulfonic acid
NR	neutral red
O.N.	over night
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene-glycol
pps	protoplasts
PSV	protein storage vacuole
R.T.	room temperature
SDS	sodium dodecyl sulphate
SV	small vacuole
TIP	tonoplast intrinsic proteins
TCA	trichloracetic acid
VSD	vacuolar sorting determinant
VSR	vacuolar sorting receptor

Abbreviations used only in single subchapters are explained in the text.

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Chapter 1

GENERAL INTRODUCTION

Vacuoles are typical compartments of plant cells, their origin, evolution and function have been studied for over half a century. The vacuoles are derived from the endoplasmic reticulum (ER), where its protein components, both soluble and membrane-bound, are synthesised. Thus, the vacuoles belong to the secretory pathway of plants. The plant vacuoles share many general characteristics with homologous compartments in mammalian cells (lysosomes) and fungi but they fulfil a variety of original functions.

This thesis investigates the mechanisms involved in the vacuolar targeting of soluble proteins. The main tool to elucidate the different targeting systems to the vacuole is the use of reporter proteins.

Because of their multiple functions, the plant vacuoles present higher complexity than equivalent compartments in the others eukaryotes and the following paragraphs will focus on the plant cell to provide an overview as complete as possible about the present state of knowledge.

THE SECRETORY PATHWAY.

All eukaryotic cells have many specialised compartments, defined by a complex membrane system. The targeting of many different kinds of molecules to different compartments requires many elaborate systems. The secretory pathway is one of the most complex among these transport routes. Trafficking between the various organelles is thought to be primarily mediated by small carrier vesicles. The basic molecular components involved in the formation of these vesicles have been defined in yeast and mammalian cells and related components have been then identified in plants.

The basic organisation of the plant secretory system is morphologically similar to that of other eukaryotes but there are a number of features that distinguish it. For example, the *de novo* assembly of the cell-plate during plant cell division has no counterpart in other systems.

The basic secretory pathway includes several discrete organelles, among them the ER and the Golgi apparatus, involved in the assembly, post-translational modification, trafficking and correct localisation of newly synthesised proteins to the plasma membrane and vacuoles. Each

of these organelles is the subject of current investigations and appears more complex than originally expected.

In this introduction I focus on the plant secretory system. For additional discussion on yeast and mammalian secretory pathway, I refer the reader to recent reviews (Bryant and Stevens, 1998; Harter and Wieland, 1996; Schekman and Orci, 1996).

THE ENDOPLASMIC RETICULUM.

For proteins and lipids the secretory pathway begins with the ER. In general, soluble proteins that enter the secretory pathway are inserted across the membrane of the ER during their synthesis (co-translationally) on membrane-bound ribosomes.

Once in the ER lumen, a newly synthesised protein will be retained there, or directed to a specific organelle within the pathway depending upon information contained within its polypeptide chain. This information is encoded either by the primary structure or by determinants formed from its three dimensional structure as the protein folds into a stable conformation with the assistance of ER chaperone proteins. An other process selects certain proteins for export from the ER (Balch *et al.*, 1994; Barlowe *et al.*, 1994) and concentrates them into vesicles that transport them to cis-Golgi cisternae (Staehelin and Moore, 1995) (see Figure 1).

Proteins are co- and post-translationally modified in the ER, before they acquire the correct conformation required for further transport steps. First, the small signal peptide necessary to enter the ER is co-translationally cleaved by a signal peptidase. Then, many post-translational modifications can occur: N- and O-glycosylations, prolyl hydroxylation and disulphide bond formation. Finally proteins acquire their final conformation, assisted by chaperone proteins resident of the ER (endoplasmins, e.g. calreticulin, calnexin, BiP, PDI) (Bednarek and Raikhel, 1992; Denecke, 1996). All these processes are conserved among eukaryotes, and in plants the main components have also been identified (Gomord *et al.*, 1997; Welters, 1996).

After leaving the ER, a soluble protein that lacks specific information for retention or sorting to a particular organelle of the secretory pathway will pass through the Golgi complex and be packaged into vesicles that fuse with the plasma membrane and release the protein to the cell exterior, by the so-called default pathway (Denecke *et al.*, 1990).

Some proteins that should not to leave the ER have a signal containing this information. The proteins BiP and PDI, mentioned above, reside in the ER and have at their C-terminus a tetrapeptide which is highly conserved through all eucaryotes. Both tetrapeptides HDEL (usual in

yeast) and KDEL (usual in animals) are sufficient for ER retention in plants. The C-terminal peptide HDEF of the tomato RNAse LX, is also necessary and sufficient to direct this and other proteins to the yeast ER (Kaletta *et al.*, 1998). The retention mechanism may involve vesicles recycling between ER and Golgi. This theory is supported by evidence in yeast and animals (Ferro-Novick and Jahn, 1994; Hong and Tang, 1993). In plants, even the existence of such a retrograde transport from Golgi to ER is still discussed (Gomord *et al.*, 1997; Klausner *et al.*, 1992) but the discovery of a receptor for H/KDEL (Bar-Peled *et al.* 1995; Welters, 1996) in *A. thaliana* supports the existence of a retrograde transport.

THE GOLGI APPARATUS.

An overview of the function and structure of the Golgi stacks is essential to our comprehension of protein targeting.

The Golgi apparatus of plant cells is a very dynamic organelle engaged in the synthesis of complex polysaccharides of the cell wall matrix, the processing of N-linked glycans of glycoproteins and O-glycosylation of cell wall proteins.

The Golgi is composed of a number of flattened membranous cisternae. Unlike animal cells in which the stacks are interconnected by tubular elements and clustered around the nucleus (Mellman and Simons, 1992), plant cells contain up to several hundred individual Golgi stacks (or dictyosomes) dispersed in the cytoplasm (Harris, 1986).

Each higher plant Golgi stack consists of 3 to 10 cisternae displaying a morphological polarity from the *cis* to the *trans* face. Morphological parameters such as width of the cisternae, the spacing between the cisternae, the staining of cisternal membranes and contents, as well as the location of intercisternal elements and of the *trans* Golgi network (TGN), permitted to define precisely Golgi cisternae (Staehelin and Moore, 1995).

A recent major controversy about the functioning of the Golgi apparatus concerns the progression of the secretory material and membrane from the ER to the *cis*-face of the stack, across to the *trans*-face of the stack and before exit. Two basic models have been proposed:

The vesicle shuttle model suggests that there is a sequential vesicle-based transport of products through the stack from cisterna to cisterna.

The cisternal maturation/progression model suggests that the functional gradient of the cisternae in the stack is generated by a progressive maturation of the *cis* cisternae into *trans* cisternae. These two models do not have to be mutually exclusive.

The presence of a vesicle-based pathway for protein transport from ER to the Golgi has recently been supported by evidence showing that the fungal metabolite brefeldin A (see below), which inhibits the formation

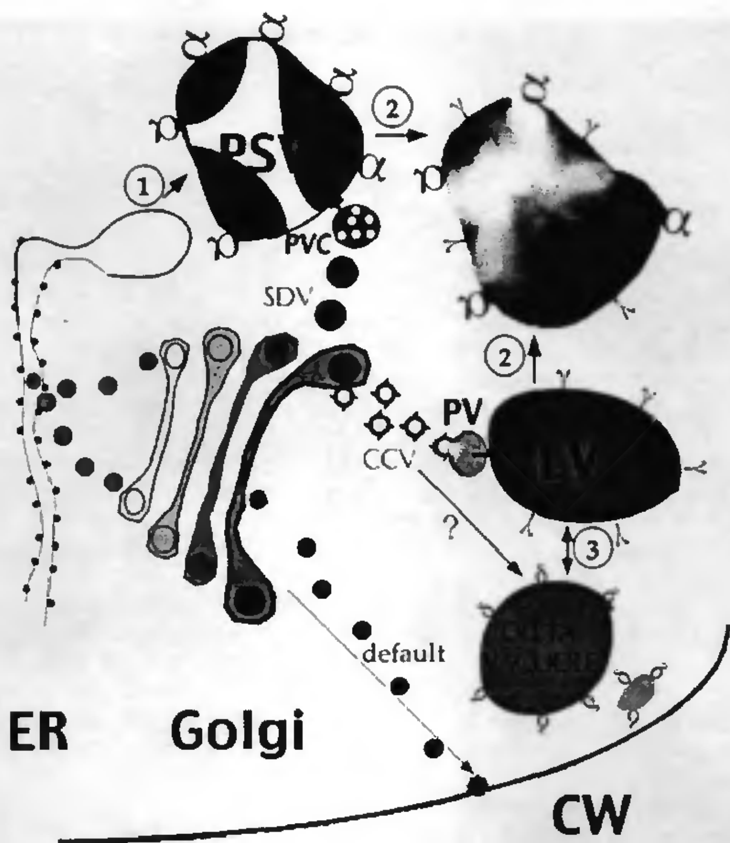


Figure 1: The plant secretory pathway. *Drawn by J.C. Rogers*
PSV: Protein Storage Vacuole
PVC: Pre-Vacuolar Compartment
LV: Lytic Vacuole
PV: Pre-Vacuole
SDV: Smooth Dense Vesicles
CCV: Clathrin Coated Vesicles
α, γ, δ: TIPs

of certain classes of vesicles, also blocks the transport of sporamin (a sweet potato storage protein) from ER to the Golgi apparatus in transgenic tobacco cells (Holwerda *et al.*, 1992). Small coated vesicles can be seen in many electron micrographs of plant Golgi. It was originally assumed that these coated vesicles were the plant equivalent of the coatomer-coated COPI vesicles first described for mammalian cells by Rothman (1994). There is now evidence that COPI vesicles may be primarily involved in retrograde transport from the Golgi to the ER (Letourneur *et al.*, 1994). A new class of coated vesicles (discovered in yeast), the COPII vesicles, may in fact mediate the anterograde transport of material from ER to Golgi (Schekman and Orci, 1996). The initial stage in the binding of COPII-vesicle coat components to the cytosolic face of the ER is facilitated by small GTP-binding proteins that interact with ER resident proteins (Davies, 1994; Lin *et al.*, 1992; Schekman and Orci, 1996).

A vesicle-based flow of membrane from the ER into the *cis*-Golgi should be balanced by a secretory vesicle-based loss of membrane from the *trans* side of the stack.

The cisternal maturation model proposes a *de novo* synthesis of cisternae on the *cis* face and decay of old cisternae on the *trans* side by vesiculation. A single cisterna will thus appear to traverse the Golgi stack (Mironov *et al.*, 1997). The strongest support for this model has come from ultrastructural studies on scale-forming unicellular protists, which have Golgi stacks composed of numerous cisternae in which the scales form and mature without ever leaving the cisternae (Becker *et al.*, 1995). A recent work in animal cells has shown that procollagen type I was secreted by moving along the Golgi stacks without leaving the lumen of the cisternae (Bonfanti *et al.*, 1998). It is possible that cisternal maturation may coexist with other intra-Golgi traffic mechanisms, such as anterograde carrier vesicles. Anterograde cargo proteins have been immunolocalized within COPI vesicles near the Golgi apparatus in animal cells (Orci *et al.*, 1997). In any case the cisternal maturation mechanism seems involved in the transport of supramolecular aggregates.

THE PLANT CELL VACUOLES.

Plant cell vacuoles have many different functions (Wink, 1993): they may contain hydrolytic enzymes that function in an environment with an acidic pH (Boller and Kende, 1979); they may contain high concentrations of secondary metabolic products such as alkaloids, glycosides, organic acids and anthocyanins (Wink, 1993), or they may store proteins. Vacuolar storage proteins are most prominent in seeds but also may occur in many different vegetative tissues (Herman *et al.*,

1994). Vacuoles with proteases active at an acidic pH (so called lytic or vegetative vacuoles) and protein storage vacuoles are structurally and functionally distinct organelles (Hoh *et al.*, 1995; Paris *et al.*, 1996). The secretory pathway of the plant cell is more complex than in yeast, because plant cells can store proteins within a vacuolar compartment. To keep storage proteins separated from active proteases in distinct vacuoles, plant cells must generate at least two biochemically and structurally distinct types of tonoplast membranes. These different kinds of tonoplasts must form separate vacuoles with distinct transport pathways that allow sorting of soluble proteins to their different destinations.
Two general models have been offered for vacuole biogenesis (Robinson and Hinz, 1997): in one model vacuoles are proposed to originate from a Golgi associated tubular network of membranes (Marty *et al.*, 1980). An alternative model suggests that vacuoles originate directly from the ER (Hilling and Amelunxen, 1985).
Studies of vacuole function and biogenesis were greatly assisted by the finding that plant vacuole tonoplast contain abundant integral membrane proteins, the Tonoplast Intrinsic Proteins (TIPs) (Johnson *et al.*, 1989). TIPs have six membrane-spanning regions and belong to a family of proteins that mostly function as aquaporins, channels that facilitate water transport (Chrispeels and Maurel, 1994) (see below). Aquaporins are required to maintain vacuole function (Chrispeels and Maurel, 1994) and all TIPs could have this same function. However TIPs are very abundant proteins, they seem to be in excess of the amount needed for water transport and could thus also have a structural function PROGCOMP ENRfu . (Hoh et al., 1995; Jauh et al., 1998; Paris et al., 1996) .
Plant cells have several functional types of vacuole and a specific isoform of TIP is associated with a specific type. It has been demonstrated that a TIP isoform, the _-TIP PROGCOMP ENRfu (Johnson *et al.*, 1989), is specifically present in protein storage vacuole (PSV) tonoplast, while the TIP-Ma27 isoform (Marty-Mazars *et al.*, 1995), later called γ-TIP, was identified in vacuoles with active proteases and acidic pH, termed lytic or vegetative vacuoles (LV) both in pea and barley (Hoh *et al.*, 1995; Paris and Rogers, 1996). The α-TIP is abundant in seeds, reflecting the abundance of PSV, but it is also present in PSV in root tip cells that contain barley lectin as a storage protein (Paris *et al.*, 1996). Interestingly, a single cell in pea or barley root tips could contain vacuoles labelled separately with α-TIP or γ-TIP (Paris *et al.*, 1996). But there are more than two TIPs and the situation is not yet clear. TIP gene family members that are expressed at measurable levels in *Arabidopsis* include α, γ and δ-TIP and DIP, a

similar transmembrane protein expressed in dark-grown seedlings (Culianez-Macia and Martin, 1993). While α-TIP is associated with PSV and γ-TIP with LV (Hoh *et al.*, 1995; Paris *et al.*, 1996), δ-TIP seems to determine the transformation of a degradative vacuole into a storage vacuole in vegetative tissues (Jauh *et al.*, 1998) indicating that TIP isoforms may determine vacuole identity. Classification of vacuole types based on their complement of TIP proteins is not a simple task when we consider that vacuoles can also be identified where α- and γ-TIP, γ and δ-TIP, and α- and δ-TIP are both present in the same tonoplast (Paris *et al.*, 1996).
A recent study confirmed that there are two separate pathways to vacuoles for membrane proteins (Jiang and Rogers, 1998) and thus probably for tonoplast formation. The cytoplasmic tail of α-TIP but not γ- TIP could prevent the traffic of a reporter protein fused to BP-80 (see later), affecting a pathway via the Golgi to the LV, while a direct ER to PSV pathway was not affected.

SORTING OF SOLUBLE PROTEINS TO VACUOLES

THE VACUOLAR SORTING DETERMINANTS

In mammals, soluble proteins targeting to the lysosome, the lytic compartment, is frequently mediated by mannose 6-phosphate residues in glycosyl side chain of the glycoproteins (Kornfeld, 1990). While some acid hydrolases in plants are glycoproteins (Gaudreault and Beevers, 1983) they were found to contain no mannose 6-phosphate, nor could an M-6-P receptor be detected (Gaudreault and Beevers, 1984; Leborgne and Hoflack, 1997). Soluble protein targeting in plants is instead mediated by short peptide sequences within amino-terminal or carboxy-terminal propeptides of a propeptide, or inside the protein sequence (Matsuoka and Neuhaus, 1999).
One group of VSD was termed originally NTPP (N-terminal propeptide), examples of which were found in the N-terminal propeptides of sweet potato prosporamin (Matsuoka and Nakamura, 1991) and barley proaleurain (Holwerda *et al.*, 1992). The second group of VSD was originally termed CTPP (C-terminal propeptide) as they were identified in the C-terminal propeptides of barley lectin (Matsuoka *et al.*, 1995) and tobacco chitinase A (Neuhaus *et al.*, 1991). The third group of internal determinants was described for certain seed storage proteins (Chrispeels, 1994; Nakamura *et al.*,1993).
The vacuolar sorting determinants have recently been reviewed (Matsuoka and Nakamura, 1991; Neuhaus and Rogers, 1998) and a new nomenclature was proposed. The determinants found in N-terminal propeptides require a conserved amino acid sequence probably

recognised by a sorting receptor. They also function when placed elsewhere in the protein (Koide *et al.*, 1997); for these reasons they have been termed "sequence-specific VSD" (ssVSD); in contrast, the C-terminal propeptide determinants have little discernible requirement for a conserved sequence, but they must be placed at the C-terminus of a protein; they have thus been renamed "C-terminal VSD" (ctVSD); finally it has not been possible yet to define any conserved amino acid sequence involved in the function of the internal determinants of storage proteins, they have been termed "physical structure VSD" (psVSD) but doubts about the separate identity of this category persist. In the following paragraphs the new classification will be used.

THE SEQUENCE SPECIFIC VACUOLAR SORTING DETERMINANTS (ssVSD)

The ssVSD was first defined in the propeptides of sweet potato prosporamin and barley proaleurain. Sporamin is an abundant storage protein in sweet potato tubers. It is stored in vacuoles with characteristics of LVs rather than PSVs (Matsuoka *et al.*, 1990). After cleavage of the signal peptide, prosporamin carries a 16 amino acid N-terminal propeptide that is removed to form mature sporamin in sweet potato tubers. When expressed in tobacco suspension culture cells, sporamin is sorted to the vacuole (Nakamura *et al.*, 1993). Expression of a mutant lacking the 16 amino acid propeptide resulted in secretion of sporamin, demonstrating that the propeptide contains the essential vacuolar sorting determinants (Matsuoka and Nakamura, 1991).

Barley aleurain is a cystein protease closely related to mammalian cathepsin H (Holwerda and Rogers, 1993). It is synthesised as a proenzyme and transported to an acidified, post-Golgi compartment where it is processed to the mature form (Holwerda and Rogers, 1990). In aleurone cells aleurain was localised by immune-electron microscopy to compartments morphologically and physically distinct from protein storage vacuoles (Holwerda and Rogers, 1990). These facts all argue strongly that proaleurain is stored in an acidic vacuolar compartment with active proteases, so aleurain has been used as a marker to define lytic vacuoles (Paris *et al.*, 1996).

Comparison of the prosporamin and proaleurain VSDs demonstrated the presence of a conserved central motif NPIR (one letter code for amino acids). The presence of an essential conserved sequence within sorting determinants from two completely different types of proteins suggests that the sequence might be recognised by a sorting receptor. The motif SNPIR in the propeptide of barley aleurain was sufficient to redirect a secreted protease to the vacuole of tobacco cells (Holwerda *et al.*, 1992), although the efficiency of targeting depended on the presence of other contiguous determinants (SSSSFAD<u>SNPIR</u>VTDRAST). The sorting

efficiency of the sporamin propeptide is sequence dependent. Replacement of Ile within the NPIR motif by a Gly, caused 95% of prosporamin to be secreted (Nakamura *et al.*, 1993). It can also function as a VSD when fused to an unrelated protein such as the barley lectin or moved to the C-terminus (Koide *et al.*, 1997).

A VACUOLAR SORTING RECEPTOR

As clathrin-coated vesicles (CCVs) were known to function in traffic from Golgi to the lysosome/vacuole in mammalian and yeast cells, a systematic work was done to search for a ssVSD-binding receptor in membranes purified from CCVs from developing peas. A single ~80kDa protein was identified that bound to the propeptide of proaleurain in a pH-dependent manner. It was named BP-80 (Kirsch *et al.*, 1994).

Subsequent studies used different affinity columns to assess binding of BP-80 to the amino-terminal targeting propeptide of prosporamin from sweet potato (high affinity) and to the ctVSD of barley lectin (little affinity) (Kirsch *et al.*, 1996). These results also confirmed the crucial role of a four amino acid motif (NPIR) in aleurain and sporamin propeptides.

Shimada at al. (1997) identified two proteins of 72 and 82 kDa in preparations of (storage protein) "precursor accumulating vesicles" from developing pumpkin cotyledons. These proteins are homologues of BP-80.

Using amino acid sequence data from the N-terminus and two internal tryptic peptides from BP-80, an Arabidopsis EST clone was identified, representing another BP-80 homologue. This clone served as a probe for the isolation of the BP-80 cDNA and three homologues from developing peas, and for another homologue from Arabidopsis. Database searches also identified EST clones encoding homologues from rice and maize (Paris and Rogers, 1996; Paris *et al.*, 1997). As of spring 1999, six different homologues have been sequenced in *Arabidopsis* alone (EMBL databases). This protein family has been termed VSR (Vacuolar Sorting Receptor) family (Paris *et al.*, 1997). The VSR proteins represent a new gene family with the following novel characteristics: the first ~400 amino acids represent a unique region without homologies in the yeast or mammalian gene databases. This unique region is followed by three Cys-rich repeats (EGF repeats), one of which is predicted to co-ordinate calcium ions. Then a short Ser and Thr-rich sequence follows, preceding the transmembrane domain. The cytoplasmic domain sequences are highly conserved for the first three-quarters and then diverge. All, however, contain a form of the Tyr-motif (Tyr-X-X-Ø, where Ø is a large, hydrophobic amino acid)), YMPL; this motif has been demonstrated to mediate incorporation into CCVs in mammalian systems (Boll *et al.*,

1996; Ohno *et al.*, 1995). Thus it is likely that all of the members of the VSR family are assembled into CCVs. Consistent with this prediction, BP-80 is highly enriched in preparations of pea CCVs lacking detectable storage proteins, while pea smooth dense vesicles preparations are highly enriched in storage proteins but have little detectable BP-80 (Robinson *et al.*, 1998a; Robinson *et al.*, 1998b).
Immunogold electron microscopy using the antibodies directed against an N-terminal BP-80 propeptide demonstrated specific labelling of Golgi and of ~250 nm compartments that were found often adjacent to large vacuoles. Some of these compartments appeared to be in the process of fusing with the large vacuoles (Paris *et al.*, 1996). The large vacuoles were labelled in their tonoplast by TIP-Ma27 and therefore were lytic vacuoles. These observations are consistent with what is known of protein traffic to the vacuole in yeast. There, the vacuolar sorting receptor Vps10p (Cooper and Stevens, 1996) binds its ligand in the Golgi and delivers it to an acidified prevacuolar compartment that contains active proteases (Piper *et al.*, 1995).
However the plant VSR proteins and yeast Vps10p share no homology. Probably plants require a specialised receptor because of the complex pattern of protein sorting to different vacuoles in plant cells (Paris *et al.*, 1996). The observation that several different genes for VSR proteins are expressed in the same plant tissue (at least four in developing pea seeds) can be explained by the necessity to express *different* VSRs *with different ligand binding specificities.*

THE C-TERMINAL VACUOLAR SORTING DETERMINANTS (ctVSD)
Several C-terminal propeptides have been positively identified: in barley lectin (Dombrovski *et al.*, 1993), in a chitinase, a glucanase and an osmotin from tobacco (Melchers *et al.*, 1993; Neuhaus *et al.*, 1991; Sticher *et al.*, 1992), in the 2S albumin storage proteins from Brazil nut (Saalbach *et al.*, 1991) and pea (Higgins *et al.*, 1986) and in phaseolin (Frigerio *et al.*, 1998).
Deletion of these propeptides from the precursor proteins result in a secreted form of the protein. In the case of tobacco chitinase A, the fusion of the C-terminal propeptide to several reporter proteins (cucumber chitinase, hen egg white lysozyme, rat ß-glucuronidase, GFP) indicated that it is both necessary and sufficient for vacuolar targeting (Di Sansebastiano *et al.*, 1998; Freydl *et al.*, 1995; Neuhaus *et al.*, 1991). In contrast to sequence-specific VSDs, no essential motif was common to the studied ctVSD. In the case of the C-terminal propeptide (GLLVDTM) of tobacco chitinase A, all partial deletions strongly reduced the percentage of intracellular accumulation, only the terminal Met was dispensable (Neuhaus *et al.*, 1994). Single and multiple replacements

affected sorting to varying degrees, but no general rule could be deduced from them. When the length of the ctVSD was kept constant, no replacement of a single amino acid was sufficient to cause complete secretion, whether the charge was reversed, or several hydrophobic residues were replaced by hydrophilic residues or vice versa. The single most effective replacement was unexpected, in fact Gly at the place of the terminal Met reduced the sorting efficiency by more than 50% while, as mentioned, the complete deletion of Met had no effect on retention (Neuhaus *et al.*, 1994).

Partial deletions of the propeptide of barley lectin established that no single portion of its 15 amino acids is essential (Dombrovski *et al.*, 1993). Instead, the first four or the last four residues each were sufficient. Furthermore, a minimal length of three amino acids beyond the processing site was sufficient for a significant vacuolar targeting. Again, C-terminal Gly were most detrimental to vacuolar sorting, as was a terminal N-glycosylation site.

Quantitative analysis of sorting efficiency indicated that there are differences depending on the sequence but many random sequences also showed varying sorting efficiencies. Sorting determinants without strongly conserved motif are also known for the ER-targeting signal peptides and for the transit peptides of mitochondrial or chloroplastic proteins. (Chrispeels, 1991; Glick *et al.*, 1992; Schnell *et al.*, 1994)

A more precise model for the preferences of a sorting system with no conserved motif can be obtained by compiling the sequences of as many examples as possible from natural proteins, an approach that was successful for ER-targeting signal sequences (von Heijne, 1986) and mitochondrial transit peptides but it is severely limited by the small number of protein families were such a C-terminal VSDs has been identified.

A low sequence specificity for a short peptide would predict a low binding affinity for a receptor protein, which could be functionally acceptable for the vacuolar sorting system. A low affinity may be compensated by the high surface to volume ratio in the Golgi apparatus. Differently from ssVSDs, C-terminal VSDs can be easily acquired or lost by point mutations or small insertions or deletions without affecting the proper folding of the protein. These facts may explain why several attempts to identify a receptor have failed so far. Screening of expression libraries with a labelled peptide, anti-idiotypic antibodies, affinity columns (J-M. Neuhaus, personal communications), as well as the yeast two-hybrid system (D. Humair, personal communications), did not detect any receptor when attempted in this laboratory or other laboratories.

An alternative explanation for ctVSD function is still possible. The

final step in protein folding may involve binding of the C-terminus to the surface of the folded core (Fedorov and Baldwin, 1997). If the three-dimensional structure of proteins is a determinant for sorting them into vesicles in the PSV pathway, removal of C-terminal propeptide could substantially affect that determinant. This kind of mechanism is however difficult to reconcile with very short ctVSDs such as those of chitinases and with the successful fusion of the VSD to reporter proteins (Di Sansebastiano *et al.*, 1998).

THE PHYSICAL STRUCTURE VACUOLAR SORTING DETERMINANTS (psVSD)

There is a third heterogeneous group of proteins for which neither of the two first categories of VSD seem to apply. They are known or supposed not to have propeptides, or if propeptides are present, they have been shown not to be required for vacuolar sorting. The sorting determinant must somehow be carried within the mature polypeptide. An internal ssVSD is possible, but for several seed storage proteins, another sorting system is likely to be involved. These are the vicilin-like and legumin-like proteins that accumulate into dense vesicles at the *trans* side of the Golgi and are transported without involvement of clathrin-coated vesicles (Robinson *et al.*, 1998b). They accumulate in protein storage vacuoles distinct from the lytic vacuoles that pre-existed in these cells. As proposed by Vitale and Chrispeels (Vitale and Chrispeels, 1992), aggregation is a possible sorting mechanism. Sorting by aggregation is known to occur in animal cells, where it is possibly induced by the lower pH of the secretory granules (Castle *et al.*, 1997). Determinants for aggregation would likely be associated with hydrophobic regions on the surface of the molecule, formed by folding of the three dimensional protein structure. In this regard, it is interesting that the precursor to the pea storage protein legumin, prolegumin, when isolated from ER and Golgi vesicles, is much more hydrophobic and binds much more tightly to membranes than the mature protein (Hinz *et al.*, 1997)

EVIDENCE FOR TWO FUNCTIONALLY DISTINCT SORTING PATHWAYS

The existence of more than one vacuolar compartment has been observed in many cell types and different functions have been proven for these compartments. There are at least two types of vacuoles in root tip cells, one being the target compartment of ssVSD-targeted proteins such as aleurain, while the other compartment is the destination of ctVSD-targeted proteins such as barley lectin (Paris and Rogers, 1996). In developing pea cotyledons it is also clearly possible to distinguish storage vacuoles from lytic vacuoles as well as the corresponding dense vesicles and clathrin coated vesicles (Robinson *et al.*, 1998b). In mature

plant tissues however, barley lectin and sporamin were found together in aggregates in the central vacuole of transgenic tobacco (Schroeder *et al.*, 1993). This finding indicates that pathways for sorting proteins with the two types of VSD may end in separate vacuoles or ultimately converge on the central vacuole (Paris *et al.*, 1996).
Matsuoka et al. (1992) developed a system in tobacco suspension culture cells (BY2) to test the effects of compounds known to affect sorting processes in yeast and mammalian cells. Two reporter proteins were used: sporamin and barley lectin with either their own propeptide or the propeptide of the other protein. Thus, the functions of the prosporamin ssVSD and barley lectin ctVSD were tested separately on two different proteins. Without any propeptide both sporamin and barley lectin were secreted from the cells, while with either propeptide the reporter proteins were efficiently sorted to the vacuole. But even if proteins were found in the same compartment, sorting mechanisms depended on the VSD, as it was possible to inhibit selectively one of the two pathways. Wortmannin, an inhibitor of phosphatidylinositol 3- and 4-kinase in mammalian cells and of phospholipid synthesis in plant cells (Matsuoka *et al.*, 1995), caused almost complete inhibition of ctVSD-mediated transport to the vacuole at a concentration, 33μM, that had little or no effect on ssVSD-mediated transport to the vacuole (Matsuoka and Nakamura, 1992).
It is known that the proteins with a ssVSD are transported by CCVs in a wortmannin-insensitive manner, but we don't know yet whether the wortmannin-sensitive pathway (ctVSDs) is identical to the pathway involving smooth dense vesicles (psVSDs), as there are no reports yet of its effects on sorting of proteins with a psVSD.

SORTING OF ONE PROTEIN TO BOTH PSV AND LV COMPARTMENTS

The barley aspartic proteinase is present in both PSVs and LVs (Paris *et al.*, 1996). Two potential VSDs are present in the proenzyme sequence. The sequence NPLR is found in the N-terminal propeptide, a motif that functions as well as NPIR when placed in the prosporamin ssVSD (Koide *et al.*, 1997). Additionally, in comparison to yeast and mammalian aspartic proteases, the barley enzyme and other plant homologues have a central insert of 104 amino acids that closely resembles the sequence of mammalian saponins (Guruprasad *et al.*, 1994). Saponins interact with certain lysosomal enzymes and are thought to be involved in the membrane-associated mannose-6-phosphate-independent pathway for targeting proteins to lysosomes (Staab *et al.*, 1994). Experiments in tobacco suspension culture cells have shown that, while the intact proenzyme was efficiently targeted to the vacuole, a mutated form with an intact N-terminal propeptide, but where the "saponin" insert had

been deleted, was secreted from the cells (Tormakangas, 1997).
It is possible that the topological distribution of different sorting mechanisms within the Golgi may be important in determining how a protein with two possible VSDs is sorted; in fact some proteins with potential ssVSDs could take part in an aggregation process in the cis-Golgi and would thus have little opportunity to interact with the receptor if its site of action is located in the trans-Golgi (Neuhaus and Rogers, 1998).
The importance of VSD position has bean shown as well by the observation that a same propeptide may function with two sorting systems. This was found when the propeptide of sporamin was moved to a C-terminal position (Koide *et al.*, 1997). Mutation of the Ile to Gly had much *less effect on vacuolar sorting* when the propeptide was at the C-terminus but wortmannin only then strongly inhibited vacuolar transport when the Ile to Gly mutation was present in the propeptide at the C-terminus. Thus this mutation appeared to reveal functional characteristics of a ctVSD in this sequence when placed at the C-terminus. The ER retention signal may also mediate vacuolar sorting. When sporamin carrying an HDEL extension was expressed in tobacco BY2 cells, a significant fraction escaped from the ER and was recovered in the vacuole (Gomord *et al.*, 1997). In fact the HDEL peptide resembles a ctVSD. A function of HDEL as a ctVSD would enable plant cells to send ER proteins to a vacuole if they escaped the retrieval system or as a means to change the localisation during development.

TRANSPORT OF INTEGRAL MEMBRANE PROTEINS TO VACUOLES

Since 1992 it is known that clathrin coated vesicles play a direct role in the sorting of soluble proteins in yeast but with the first evidence of the role of CCV, evidences appeared as well for the existence of an independent pathway for integral membrane proteins. By using yeast strains with a temperature-sensitive allele of clathrin heavy chain, Seeger and Payne (1992) showed the importance of clathrin for sorting of soluble proteins but at the same time they showed the normal targeting to the vacuole of proteins associated to tonoplast, such as alkaline phosphatase. To determine whether a similar situation is found in plant cells the effect of two drugs, BFA and monensin, was studied on tobacco protoplasts (Gomez and Chrispeels, 1993). These drugs block protein transport by interfering with specific vesicle transport steps (see below). Neither drug could block the delivery of α-TIP to the tonoplast at concentrations that effectively inhibited transport of phytohemagglutinin (PHA), a soluble protein.
Jiang and Rogers (1998) confirmed recently that α-TIP targeting is not sensitive to BFA but also showed that targeting of other membrane

proteins like BP-80 (see below) or γ-TIP were drastically affected. In this study a reporter protein was used which is able to acquire Golgi-specific modifications of Asn-linked glycans, proving the transit of BP-80 and γ-TIP through the Golgi. It seems that two separate pathways to vacuoles are possible for membrane proteins: a direct pathway from ER to PSV, and an indirect pathway via the Golgi to the LV. However, α-TIP was detected in dense vesicles along with storage proteins, indicating that it must have passed through the Golgi.

While α-TIP is a specific marker for PSV, it is not clear whether α-TIP passes through a small PSV before arriving in the central vacuole. It is possible that cells establishing and maintaining separate PSVs and LVs need not only the specific TIPs but also several other proteins characterising the two types of tonoplast.

The molecular mechanisms involved in targeting of TIPs to their destinations are essentially unknown. Targeting of integral membrane proteins to the appropriate tonoplast may depend on processes similar to those involved in vacuole biogenesis (see later).

ENDOCYTOSIS AND VACUOLES.

Markers internalised by endocytosis have been observed in a variety of vesicles and endomembranes near the Golgi apparatus and the vacuole (Low and Chandra, 1994). On the basis of immunocytochemical studies in meristematic cells it has been recently hypothesised that a minority of tonoplast proteins can follow an alternative exocytic-endocytic route to the vacuole (Robinson *et al.*, 1996). According to this suggestion, a few newly synthesised proteins destined to the tonoplast, including the two H^+proton pumps (V-ATPase and H^+PPase) and the aquaporin, γ-TIP, can escape the direct intracellular pathway to the vacuole and first move to the plasma membrane by exocytosis and are then targeted to the tonoplast (Matile, 1997) in a way which resembles the endocytic pathway to lysosomes in animal cells (Kornfeld and Mellman, 1989).

Although they already contain a large central vacuole, cells from sycamore (Aubert *et al.*, 1996), tobacco (Moriyasu and Ohsumi, 1996) and rice (Chen *et al.*, 1994) are capable, in starvation conditions, of reinitiating complete sequences of autophagy in their peripheral cytoplasm as yeast cells do (Baba *et al.*, 1994).

Conventional electron microscopy and subcellular fractionation studies provide evidence for plant cell structures morphologically and functionally homologous to compartments that were shown to perform endocytosis in animal cells (Low and Chandra, 1994).

There is a general agreement on the loading of small vacuoles with molecules internalised by endocytosis (Owen *et al.*, 1991), but results

differ concerning the convergence of endocytotic compartments in the central vacuole (Fowke *et al.*, 1991; Low *et al.*, 1993).
It is clear that endocytic vesicles do belong to the vacuolar apparatus but their direct contribution in the construction of the central vacuole remain a subject of debate.

VACUOLE BIOGENESIS

It is still not clear how the vacuoles are generated. In different tissues vacuoles and their tonoplast can have different components and characteristics. Vacuole development has been investigated using different approaches: transmission electron microscopy (Buvat, 1982), biochemical studies of the changes accompanying cell enlargement (Maeshima, 1990), and studies of the mechanisms by which proteins are targeted to the vacuole (Bednarek and Raikhel, 1992; Hofte *et al.*, 1991).
A complementary and novel approach is to study vacuole reformation in evacuolated cells. Vacuoles may be removed from plant protoplasts by high-speed centrifugation through a continuous density gradient (Lörz *et al.*, 1981). Evacuolated protoplasts are viable and can regenerate vacuoles and cell walls in culture (Wu and Tsai, 1992), thus they provide a convenient *in vitro* system in which to study synchronous development of vacuoles in large numbers of cells. Vacuole regeneration in evacuolated tobacco and petunia protoplasts has been shown to occur after culture for 12-44 h (Erdmann *et al.*, 1989). Reappearance of the vacuole in tobacco is accompanied by increased levels of hydrolytic enzymes and tonoplast H^+-PPiase activity, neutral red uptake (indicating the acidic nature of the developing vacuoles), and the reappearance of a 41 kD vacuolar protein of unknown function (Hörtensteiner *et al.*, 1992). Inclusion of bafilomycin A, a specific inhibitor of the vacuolar H^+-pumping ATPase (Sze *et al.*, 1992), in the culture medium decreased uptake of neutral red, but did not prevent vacuole regeneration (Hörtensteiner *et al.*, 1992). Vacuole formation in evacuolated petunia protoplasts is associated with the accumulation of flavonoids, followed by synthesis of vacuole-associated ethylene-forming activity (Erdmann *et al.*, 1989). Protoplasts cultured in the presence of cycloheximide failed to develop vacuoles, showing that protein synthesis is required. Hörtensteiner *et al.* (Hörtensteiner *et al.*, 1994) found that the strongly inhibitory effects of 10-100 µg/ml cycloheximide were completely reversible when evacuolated protoplasts were washed with inhibitor-free medium.

DISSECTION OF THE PLANT SECRETORY PATHWAY WITH BREFELDIN A

Brefeldin A (BFA) is a lipophilic fungal toxin that has become a major tool for cell biologists interested in studying vesicle-mediated

trafficking in animal and plant cells. Differential BFA sensitivity of different steps of the secretory pathway could allow the dissection of the pathway in a reversible way. More than other drugs affecting membrane trafficking, BFA needs here a thorough introduction.

In plants as in animal cells, BFA exerts its primary effects through perturbations of vesicular transport in the secretory pathway.

In BFA-sensitive mammalian cells (cells that respond to 1-10µg/ml BFA), the block in secretion has been traced to the ER-to-Golgi transport step and has been shown to be caused by the disassembly of the Golgi complex and the subsequent redistribution of resident Golgi enzymes into the ER (Klausner *et al.*, 1992).

In plant cells, a treatment with 2.5 to 10 µg/ml BFA both blocked secretion and altered glycosylation patterns of glycoproteins and complex polysaccharides, but did not cause any breakdown of Golgi stacks in sycamore maple cells. Instead, a loss of TGN cisternae, an increase in the number of *trans*-like Golgi cisternae, and the accumulation of large numbers of *trans*-Golgi derived vesicles in the adjacent cytoplasm were seen. At 50 µg/ml BFA the Golgi stacks disintegrated. These two types of BFA responses have been confirmed in other plant systems and should be viewed as part of continuum of BFA effects.

BFA can probably block vesicular transport in at least two sites, between the ER and the Golgi, and between the Golgi and the TGN (Driouich *et al.*, 1993; Staehelin and Driouich, 1997). BFA inhibition of vesicular transport "upstream" from the Golgi stacks leads to inhibition of secretion and to the disintegration of Golgi stacks, whereas inhibition of the "downstream" site causes inhibition of secretion without Golgi breakdown.

The effects of BFA have been attributed to at least two mechanisms. One is the release of coat proteins, including the coatomer (a major protein complex involved in coat protein I (COPI)-coated vesicle formation) and the small GTP-binding protein ARF (ADP-ribosylation factor) from Golgi membranes (Chardin and McCormick, 1999).

The gradual effect of BFA has been explained in animal cells by the fact that mammalian cells contain at least three classes of Arf exchange factors with high, moderate, or low sensitivity to BFA. The collapse of the Golgi complex caused by BFA is most likely due to the inhibition of Arf exchange factors; however, it is not clear which ones are the most important targets that explain the morphological effects.

Sec7, a protein required for ER through Golgi transport in yeast, displays guanine nucleotide exchange activity for Arf1 and has been used for detailed biochemical studies. The result of these studies is that BFA is an uncompetitive inhibitor that binds to the transient

complex formed between Arf-GDP and Sec7, leading to an abortive Arf-GDP:BFA:Sec7 domain complex. BFA causes thus endogenous Arf1-GDP to block activation of other Arf1 molecules by this particular exchange factor (Chardin and McCormick, 1999).
The second recently discovered mechanism could explain the complexity of the action of BFA. It consists in the activation of the endogenous ADP-ribosylation of two cytosolic proteins of 38 KDa (glyceraldehyde-3-phosphate dehydrogenase, a multifunctional protein involved in several cellular processes), and 50 KDa (BARS, a protein of unknown function) (Spanò *et al.*, 1999).

OTHER TRANSPORT PROCESSES ACROSS THE VACUOLAR MEMBRANE

TRANSPORT OF CYTOPLASMIC PROTEINS TO VACUOLES

Several plant proteins reech the vacuoles by different processes completely independent from sorting of soluble proteins described so far. Some proteins synthesised on free ribosomes are transported directly from the cytoplasm to the vacuole without entering the secretory pathway, e.g. a soybean lipoxygenase (Tranbarger *et al.*, 1991), as well as some Late Embryogenesis Abundant (LEA) proteins (Marttila *et al.*, 1996).

VACUOLAR PROTON PUMPS

Transport processes across membranes depend in many cases on the protonmotive force (DpH and DΨ) generated by the pumps residing in the respective membrane. The tonoplast contains two different type of proton pumps, PPases (Rea *et al.*, 1992) and ATPases (Sze *et al.*, 1992). Vacuolar-type ATPases (V-ATPases) are multimeric complexes present on the endomembrane system of eucaryotic cells (Sze *et al.*, 1992). They show some homologies to the F-ATPases present on the plasma membrane of eubacteria and on the inner membrane of mitochondria and chloroplasts. Like the F-ATPases the V-ATPases form a characteristic "head and stalk" structure. The V-ATPases can be distinguished from other ATPases through their highly specific inhibition by some antibiotics such as bafilomycin. V-ATPases are also found on other plant endomembranes such as the Golgi apparatus or the endoplasmic reticulum. However, it is still a matter of discussion whether the V-ATPase on these membranes is functional. The tonoplast H^+-PPase is found widely distributed in higher plants as well as in algae, liverworts, mosses and ferns. Homologous proteins are found in some bacteria, however none has so far been found in animals or fungi. The

tonoplast H^+-PPase is a monomeric enzyme of about 87 KD that is strictly dependent on Mg^{2+} and is activated by K^+ (Rea and Poole, 1993).

CHANNELS AND CARRIERS

Plant vacuoles may exercise different roles depending on the nutritional state of a plant or the function of the considered tissue. Therefore transport mechanisms may differ for different types of vacuoles. A substrate for which different vacuolar transport mechanisms have been described in different plants is sucrose, which is accumulated within sugarbeet tuber vacuoles by an H^+ antiport mechanism, while in barley or tomato, as well as in sugar cane stalk tissue, facilitated diffusion is observed. Similar differences as for sucrose may exist for glucose. In contrast to the members of the fructan family, which are not transported across the tonoplast, some members of the raffinose family are taken up by an H^+ antiport mechanism in plants accumulating these sugars (Pollock and Kingston-Smith, 1997). Amino acid concentrations are often lower in the vacuole that in the cytosol. A transporter that is modulated by free ATP catalyses the transfer of most amino acids across the tonoplast. A similar transport system has also been described for the exchange of peptides, polyamines, cations and inorganic anions. Since the free ATP level is low in the cytosol and therefore only a very low transport activity of this transport system can be observed under physiological conditions, this transport system may play a role either in cytosolic homeostasis or as H^+-shunt mechanism (Allen and Sanders, 1997). Inorganic and organic anion uptake is driven by the DΨ (see above). Malate and citrate are the main organic acids accumulated in large quantities within the vacuole. Inhibition experiments suggest that one carrier or channel is responsible for the uptake of most di- and tricarboxylates. Patch clamp studies indicate that a separate channel is involved in the export of organic acids. Furthermore inorganic anions such as chloride and nitrate most probably use a different transport system. In the case of nitrate it must be postulated that an additional force is driving its vacuolar uptake, since the observed membrane potential difference would not allow to drive the concentration difference observed between the cytosol and the vacuole. In stomata vacuoles evidence was shown that chloride channels are activated by a protein kinase (Allen and Sanders, 1997). Several cation permeable channels have been observed in the vacuolar membrane. The so-called slow vacuolar channel opens mainly at cytosolic positive membrane potentials, shows a permeability for potassium, and is activated in the presence of Ca^{2+}. The fact that calmodulin antagonists inhibit SV channels indicates that the effects of Ca^{2+} are mediated by calmodulin. In contrast, the channels known as

fast vacuolar channels have a higher open probability at cytosolic negative membrane potentials and are activated by low cytosolic Ca^{2+} concentrations. Additionally, in guard cells a K^+ channel thought to have a role in facilitating vacuolar K^+ release was described. For different plants a Na^+/H^+ exchange mechanism which may play a crucial role in salt tolerance, has been demonstrated, while a K^+/H^+ antiport also seems to exist in some cases but has not been described in detail (Blumwald and Gelli, 1997). Three different calcium channels have been reported to reside on the vacuolar membrane: 1) an IP_3-dependent channel that allows the release of calcium after activation with IP_3, 2) a cyclic ADP-ribose (cADPR) dependent channel which is also responsible for the release of Ca^{2+} from the vacuole. 3) A Ca^{2+} channel which is activated on membrane hyperpolarization. This channel is potently inhibited by La^{3+} and Gd^{3+}. Vacuolar calcium uptake is mediated by a Ca^{2+}/H^+ exchange mechanism and by a Ca^{2+}-ATPase (Blumwald and Gelli, 1997).

OTHER FUNCTIONS OF THE PLANT VACUOLES

THE VACUOLE AS WATER RESERVE: THE AQUAPORINS.

Due to its large size, the central vacuole of plants plays an important role as water reserve and hence in water stress. For a long time water fluxes across biological membranes were thought to occur simply through the lipid bilayer. However, during the last years it was shown that in many cases special proteins, so called aquaporins, mediate the exchange of water between the extracellular medium and the cell as well as within the cell. For vacuoles different types of aquaporins have been described. The best knowns are the α- and the γ-TIPs (tonoplast intrinsic proteins; see below). γ-TIPs can be found mainly in the large central vacuole and are strongly expressed in young, expanding tissue as well as in vacuoles of moving cells like *Mimosa* pulvini. α-TIPs are preferentially present in storage vacuoles where they are probably involved in dehydration. Interestingly, water permeability of α-TIPs, but not γ-TIPs, is induced by phosphorylation (Chrispeels *et al.*, 1997; Maurel *et al.*, 1997).

THE VACUOLE AS DETOXIFICATION COMPARTMENT: THE ABC TRANSPORTERS.

A classical function of the central vacuole is the role as storage compartment for potentially toxic metabolites, like phenols or alkaloids which may serve as repellents for herbivores or which are toxic for micro-organisms. Beside such plant-born substances, potentially toxic chemicals or pesticides (xenobiotics) can be modified by the plant and stored within the vacuole. An efficient detoxification step requires transport mechanisms that enable a strong accumulation of the potentially toxic compound within the vacuole. In several cases, e.g. *for coumarylglucosides* or *flavonoids, it* has been shown that proton antiport mechanisms drive the vacuolar accumulation. Furthermore, trapping mechanisms like protonation or conformational changes have been observed to be involved in vacuolar accumulation of secondary compounds. Metabolism of xenobiotics to glucosides or to glutathione conjugates is usually considered a detoxification process (Kreuz *et al.*, 1996), but these products may exert other biological activities. Vacuolar transport of glutathione conjugates is not energised by the vacuolar proton gradient but directly by ATP. On the molecular level, it has been shown that several proteins can mediate glutathione conjugate transport in plants. These proteins belong to the ABC (ATP binding cassette) family and are highly homologous to those encoding glutathione conjugate transporters of fungi and animals (Rea *et al.*, 1998). It is interesting to note that in animals glutathione conjugates are transported through the plasma membrane and are excreted in the extracellular fluid while in plants these conjugates are transported into the vacuole through the tonoplast. Direct energization by ATP allows a plant to create a high gradient between the cytosol and the vacuole and it is therefore supposed that the plant uses this type of transporter where the potential toxicity of a product is very high.

THE ROLE OF VACUOLES IN SENESCENCE

Plant cell senescence is subject to hormonal regulation, and the potential role of vacuoles in the compartmentation of calcium ions, signal transduction and gene expression must be considered. Moreover the temporary storage of intermediary metabolites such as organic acids and amino acids continues to function in vacuoles as cells are induced to senesce. The compartmentation of unspecific hydrolases has been proposed in the past (Matile, 1997) as the function of vacuole in cell senescence but the mechanism and subcellular organisation of protein breakdown in senescent cells have so far not been elucidated.

THE USE OF REPORTER PROTEINS

Studies of the timing and cellular localisation of a gene expression as well as protein localisation *in vivo* are often essential to understand the integration of a gene's function at the level of the organism. The first reports pertinent to the localisation of gene expression used immunocytochemistry with fluorescence-labelled protein-specific antibodies. Later, thanks to the development of molecular techniques, fluorescence *in situ* hybridisation (FISH) made it possible to determine precisely in which cells the gene under study is transcribed. More recently, reporter gene systems have dramatically changed the approach for *in situ* localisation of gene expression, since detection of expression of a reporter gene is much simpler than either immunocytochemical or FISH detection techniques. Among the reporter genes described so far, the *lacZ* and *uidA (gusA)* genes from *E. coli*, coding for ß-galactosidase and ß-glucuronidase (GUS) respectively, are the most widely used in molecular research. Because of the presence of endogenous ß-glucuronidase in animals and of endogenous ß-galactosidase in plants, *lacZ* is used in animals, while *uidA* is mostly used in plants. Although GUS has already proven to be a valuable marker gene for higher plants, there are other markers that may prove to be more useful in the future, as for example the green fluorescent protein (GFP) of *Aequoria victoria* (Chalfie, 1995).

THE GUS GENE IN THE PLANT CELL.

In plants, the *lacZ* system (Helmer *et al.*, 1984) is often not useful, because of the high endogenous ß-galactosidase activity. In contrast, the low level of endogenous GUS activity in nearly all higher plants has made the *gus* reporter gene a powerful tool for the assessment of the gene activity in transgenic plants (Jefferson, 1987). However, several groups have recently expressed concerns that GUS histochemistry is prone to certain types of artefacts.

The major advantage of the GUS reporter system lies in its sensitivity, simplicity and flexibility, due to the range of substrates available. The histochemical GUS assay proposed (Jefferson, 1987) uses such a substrate, namely 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-glu, also abbreviated X-gluc or X-glucU), which is hydrolysed in presence of GUS to yield a colourless soluble intermediate that undergoes oxidative dimerization to the final product (Guivarch *et al.*, 1996). The end product, diX-indigo, forms blue crystals that are visible by light microscopy, are stable through fixation, dehydration and embedding and thus are compatible with preparation of permanent slides. X-glu localisation is prone to obvious sources of artefact that can also affect other techniques, such as irregularity of substrate penetration, but also to the diffusion of the intermediate into other compartments before the

insoluble end-product is formed. Another potential flaw with any reporter gene is that in certain cases the reporter coding sequence itself can affect the expression pattern.

THE GFP IN THE PLANT CELL.

The jellyfish (*Aequorea victoria*) green-fluorescent protein (GFP) possesses a number of desirable traits as a universal reporter in living tissues as it requires only blue or UV light for green fluorescence emission without any exogenous substrate (Chalfie *et al.*, 1994; Heim *et al.*, 1995; Heim *et al.*, 1994; Heim and Tsien, 1996)). Indeed the protein itself is fluorescent, and thus all problems related to substrate penetration or reaction product diffusion are eliminated.
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.
In addition, fusion proteins can be easily made to provide a fluorescent tag without disturbing the native function and compartmentation (Grebenok *et al.*, 1997); the GFP tolerates both N- and C-terminal extensions, and thus can be used in subcellular localisation studies in confocal microscopy of living tissues.
Although the expression of the jellyfish GFP has been successful in many heterologous systems, its application in higher plants and especially in the secretory system required a few modification of the coding sequence of the gene.
A modified version of the GFP (mGFP4) has been produced where the AU content of the mRNA was decreased in order to eliminate an aberrant splicing (Heim *et al.*, 1994) occurring in the plant cell. Further mutations were made to improve the thermostability (Siemering *et al.*, 1996), increasing the protein stability in the secretory pathway at the normal physiological conditions.
Genetic manipulations were performed also in attempts to alter the fluorescent properties of GFP. Random and site-directed mutagenesis produced potentially useful mutants with single and multiple amino acid substitutions that exhibit excitation and emission spectra different from those of wtGFP. Among the most promising are brighter mutants, such as S65T (Heim *et al.*, 1995) or the similar GFPmut1 (F64L/S65T) (Cormack *et al.*, 1996), and mutants with shifted spectral peaks, such as P4 (Y66H) and P4-3 (Y66H/Y145F) (Heim and Tsien, 1996). Mutants with shifted absorbance and emission peaks allow the multiple labeling necessary for protein colocalisation experiments (Rizzuto *et al.*, 1996).

OUTLINE OF THE THESIS

This thesis was built around three main groups of results, which will be reported in chapters 2, 3 and 4. Other results are reported in the form of annexes; these may be helpful for further developments but remained at the level of preliminary observations.

Chapter 2 reports the discovery of two different vacuolar compartments in tobacco protoplasts, using green fluorescent protein (GFP) as reporter protein. This chapter was already published as a paper (Di Sansebastiano *et al.*, 1998). You will find annexed to chapter 2 a report of results obtained with another reporter protein, rat ß-glucuronidase, both in protoplasts and transgenic tobacco plants; these results confirm and support results with GFP.

Chapter 3 characterises more completely the vacuolar compartments previously identified, using new fusion proteins as reporters and investigating the biogenesis of the observed compartments. This chapter is going to be submitted for publication soon.

Annexed to chapter 3, you will find preliminary results of the immunological characterisation of the vacuoles described in this chapter.

Chapter 4 presents data about molecular sorting and processing of the GFP fusion proteins described in the previous chapters. It includes the evidences of a different sensitivity of vacuolar proteins sorting to the drug BFA that affects specific membranes.

Chapter 5 describes the sorting of reporter GFPs in transgenic plants of two different species: *Nicotiana tabacum* and *Arabidopsis thaliana*. It includes a very preliminary description of Arabidopsis plants that we intend to use as model plants for the further study of vacuolar targeting.

Chapter 6 describes material and methods important for the experiments reported in this thesis. Full detailed protocols are reported especially for methods optimised during the experimentation.

Chapter 7 presents a general discussion of the results of this thesis.

Chapter 2

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

Gian-Pietro Di Sansebastiano, Nadine Paris, Sophie Marc-Martin and Jean-Marc Neuhaus, The Plant Journal (1998) 15, 449-457

Summary

The green fluorescent protein (GFP) from *A. 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 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 (18-24h) 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 characterise the vacuolar compartment to which GFP is specifically targeted as non-acidic since it does not accumulate neutral red, while acidic vacuoles did not accumulate GFP.

Introduction

The secretory pathway is one of the most complex transport routes in eucaryotic cells. Studies of vesicular trafficking *in vivo* can be addressed in real time by using the reporter protein GFP. The jellyfish (*Aequorea victoria*) green-fluorescent protein (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 merker 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 recently produced 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) (Neuhaus and Rogers, 1998): (1) a sequence-specific signal mostly found in N-terminal propeptides es in sporamin or aleurain, (2) internal surface determinants as in phytohemegglutinin and (3) 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 VSDs strongly suggests that the sorting system for C-terminal propeptides differ from the sorting system for the other VSDs (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 phosphatidyl-inositol 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 immunolabeling 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 visualise 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).

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 contains also 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 were accumulating 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 optimise 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 homogenous way in the whole cell as shown in the Figure 2b 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 of the emitted green light resulting in a lower intensity of the GFP signal of compartments located below them.

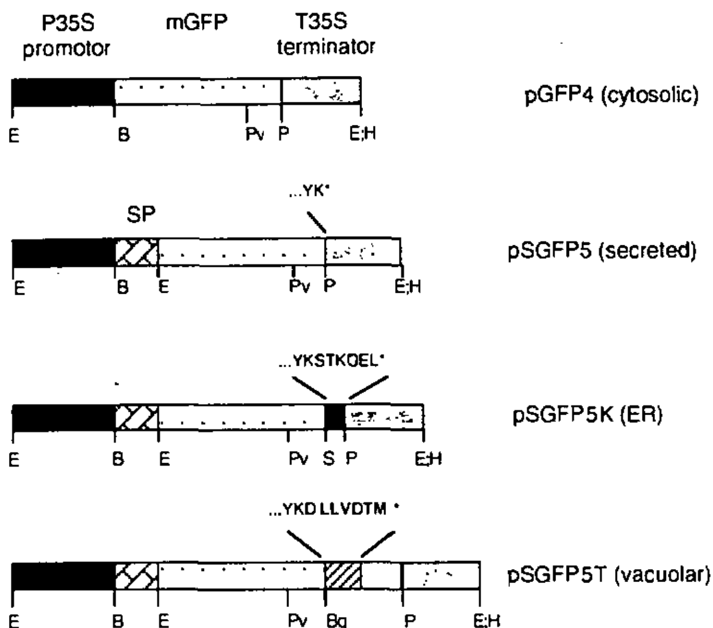


Figure 1: Schematic representation of GFP constructs.
GFP4 and GFP5 are plant-adapted GFP sequences (Haseloff *et al.*, 1997). The C-terminal amino acid sequences of the fusion proteins are presented; (*) stop codon; bold: C-terminal VSS of tobacco chitinase.
B: BamHI. Bg: BglII. E: EcoRI. H: HindIII. P: PstI. Pv: PvuII. S: SalI.

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 (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 further confirm the localisation of SGFP5T, we isolated vacuoles from protoplasts which had been incubated in presence of a cell wall *synthesis inhibitor, dichloro-benzonitrile.* The protoplasts were lysed by a thermal shock and vacuoles were harvested by floatation (Gomez and Chrispeels, 1993). Among the isolated vacuoles we could observe not only the large vacuoles of ~50 mm (Figure 3b, left) of cells as shown in Figure 2a, but also the small fluorescent vacuoles less than 10 mm (Figure 3b, right) 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 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 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 ten chloroplasts and probably derived from the spongy mesophyll. We found

Figure 2: Protoplasts expressing the vacuolar SGFP5T.
Pictures a and c show true colour fluorescence obtained with UV light; pictures 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 yields a yellow colour. **a+b**) Chloroplast-rich cells, on the left a transformed cell, on the right an untransformed cell. **c+d**) Chloroplast-poor cell. Magnification X230.

Figure 3: Vacuolar localisation of SGFP5T.
a) Bursting protoplast with an evaginating fluorescent vacuole.
b) Isolated fluorescent large and small vacuoles. Magnification X230.

Figure 4: Confocal microscope images from single protoplasts, emphasising the role of tha ER. **a)** and **b)** Consecutive sections close to the surface of a protoplast expressing the ER-retained SGFP5K for 24 hours. **c)** and **d)** Consecutive sections close to the surface of a protoplast expressing the vacuolar SGFP5T for six hours. 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 hours. 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 X1400 (a to d) and X2350 (e).

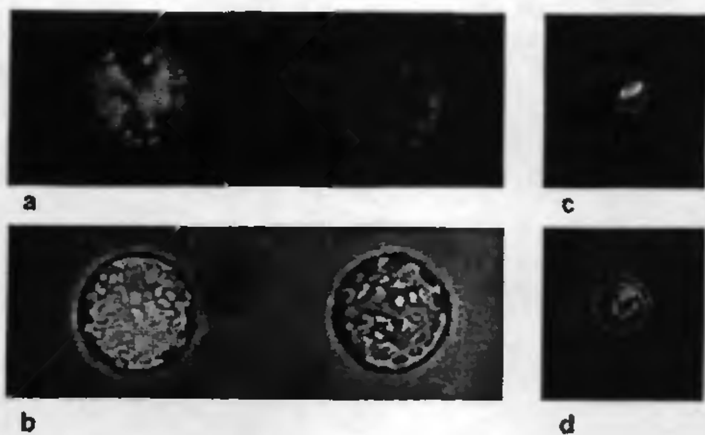


FIGURE 2

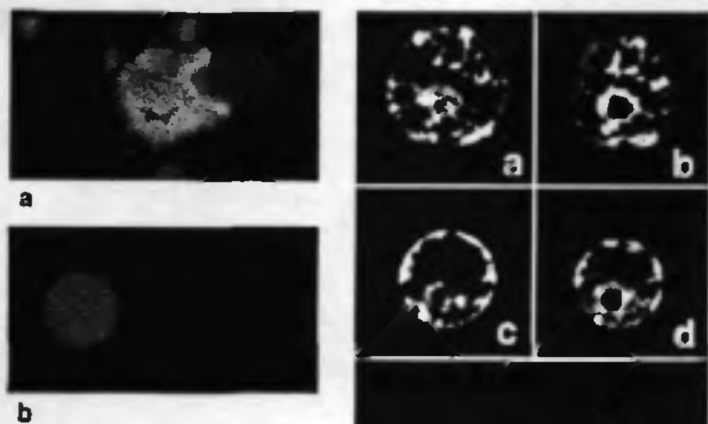


FIGURE 3

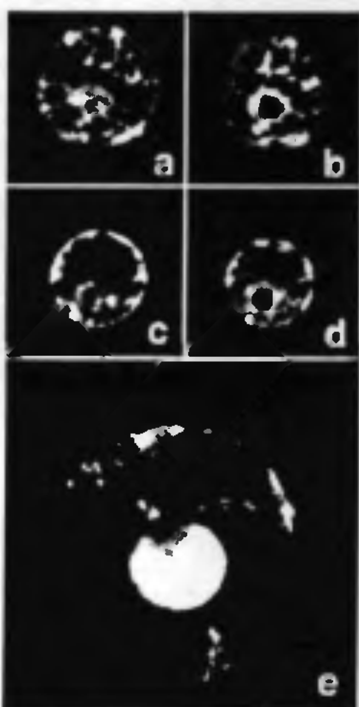


FIGURE 4

a correlation between the pattern of vacuolar accumulation of GFP and the cell type 18-24h after transformation. As shown in Table I, 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 that was far from occupying the entire internal space (Figure 4e). 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 thus clearly associated with cells from palisade mesophyll while smaller GFP accumulating vacuoles were mainly found in spongy mesophyll cells.

TABLE I: GFP patterns in two protoplast subpopulations expressing SGFP5T.
After 24 hours 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 in 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).

Localisation / Subpopulation	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)

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 (Figure 4c and d and 5a), as shown by the similarity with the pattern observed with the ER-retained SGFP5K for six to 24 hours (Figure 4a and 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 (not shown), as did also the ER-specific dye $DiOC_6(3)$ (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 II, this ER pattern was present after 6 hours in a large majority of both cell types expressing the vacuolar SGFP5T.

Between six and twelve hours, 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 hours, some chloroplast-rich cells already contained a much larger fluorescent vacuole (Figure 5e).

After 18 hours, we quantified the GFP accumulation patterns for both cell types (Table II) and we 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 hours, while the large vacuole remained unstained (Table II). At this stage, the ER pattern was still highly represented in both cell types.

From 24 hours 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, Table II) and 75% of chloroplast-poor cells contained a small green vacuole (Figure 5c, Table II) that is typical for this cell type (Figure 2d). We followed for up to 2 hours the evolution of selected cells with a small vacuole 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 II).

The transient expression system allowed the observation of some steps of protein transport. Since GFP synthesis continued during the incubation it is reasonable to detect some ER staining while GFP is 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 which is the final destination for SGFP5T and a large vacuole which remained unstained.

TABLE II: Localisation of vacuolar SGFP5T in tobacco protoplasts after various time of transient expression.
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 in percentage, 100% corresponding to the number (n) of GFP-accumulating cells in each subpopulation.

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

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 extensively used 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 (see also Figure 6c and 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 further asked whether the GFP-accumulating compartments were also accumulating NR. We therefore stained tobacco protoplasts with NR after 24 hours 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. We selected under UV light 51 cells with a GFP-accumulating large vacuole such as in Figure 6a and we found after switching on to the visible light 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 6c (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 localisation of SGFP5T and NR, we isolated vacuoles from NR stained protoplasts expressing SGFP5T. Already during lysis we could observe separate green (Figures 7a and 7b, left) and red (Figures 7a and 7b, right) vacuoles emerging from lysing protoplasts. Some of the membranes from which the vacuole emerges fluorescence in Figure 7a shows a more intense green fluorescence, a hint to the presence of some SGFP5T in other compartments than the large vacuole also 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 vacuole (Figure 7d, left) were invisible in UV (Figure 7c, left) since they did not contain any GFP.
These results showed 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 (10-50mM) or monensin (5-10 mM). 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 that 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 mM. GFP no longer accumulated in either large or small vacuoles but was visible in the endoplasmic reticulum 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 hours was affected. The dose which 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 is wortmannin-sensitive as typical for a C-terminal VSS-dependent pathway.

Figure 5: Time course of the transiently expressed SGFP5T in chloroplast-poor cells (b and c), compared to chloroplast-rich cells (a, d to f) under UV light.
a) 5-6 hours after transformation, the ER contained most of the GFP. **b)** 12-15 hours after transformation, in cells with few chloroplasts small compartments were filled with GFP. **c)** After 24 hours or more, chloroplast-poor cells reached a final state with a small green vacuole. **d)** 12-15 hours after transformation, in chloroplast-rich cells, GFP appeared limited to small vacuoles, as in the chloroplasts-poor protoplasts. **e)** After 18 hours larger vacuolar compartments became visible. **f)** After 24 hours GFP occupied the large central vacuole. Magnification X230.

Figure 6: Distinct accumulation of SGFP5T and neutral red in protoplasts expressing SGFP5T.
a and b correspond to c and d, respectively, under different illumination: UV light (a and c); visible light (b and d). **a+b)** A chloroplast-rich cell accumulated GFP in the large vacuole and NR was not visibly accumulated. **c+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). Magnitication X230.

Figure 7: Specific accumulation of SGFP5T and neutral red in distinct vacuoles.
Cells were lysed by a thermic shock and vacuoles were purified. Pictures a and c were obtained with UV light, b and d with visible light. a+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+d)** Shows the same field containing two purified vacuoles under UV light (c) and under visible light (d). *Magnification X230.*

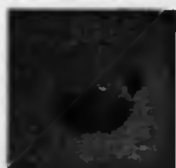
a b c

d e f

FIGURE 5

a

c



b

d

FIGURE 6

a

c

b

d

FIGURE 7

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, characterised 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 synthesised 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 VSD mediated vacuolar sorting (Matsuoka *et al.*, 1995). A current model (Okita and Rogers, 1996; Paris and Rogers, 1996) proposes that sequence-specific VSDs target soluble proteins to a lytic compartment while C-terminal VSDs direct proteins to a storage vacuole. We predict that the sorting system based on sequence-specific VSDs 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 characterised 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 chloroplasts-poor cells a small green fluorescent vacuole* coexisted with a large vacuole which occupied most of the cell volume

and accumulated NR. This lead 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 and Rogers, 1996). In these particular protoplasts the presence of two distinct vacuolar compartments, an acidic (lytic) and a non-acidic (storage) vacuole, allows us to test if this type of cells are able to properly sort soluble proteins carrying VSDs to their specific vacuolar destination. Here we showed that it is the case for C-terminal VSDs 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 do 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 VSDs 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 pBIN-mGFP4 and pBIN-mGFP5-ER encoding a cytosolic and an ER-targeted GFP respectively, and adapted for expression in plants, were kindly given 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 pBIN-mGFP4 was isolated as a BamHI-SacI fragment, blunt ended with Klenow polymerase, cloned into the SmaI site of pGY1, producing plasmid pGFP4, which encodes a cytosolic GFP (E. Freydl, Zurich). The 3' BamHI site was destroyed by partial BamHI digestion, filling up and religation. A NheI 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 NheI 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 cacaa tccca ctatc cttcg ca) and primer 4 (gttct gcgct agcag aaagc agtag g). This allowed to construct plasmid pSGFP4, encoding a GFP fused to the signal sequence of chitinase A. Furthermore a BglII 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 BglII 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 BglII site in the same reading frame, (Neuhaus *et al.*, 1994) resulting in plasmid pSGFP4T. In another construction, a C-terminal STKDEL sequence contains 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 SalI and SacI sites within the coding sequence and a PstI site after the stop codon in the resulting plasmid pSGFP4K.

The BamHI-PvuII fragments from pSGFP4, pSGFP4T and pSGFP4K were then replaced by the corresponding fragment from pBIN-mGFP5-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 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 (Nagy and Maliga, 1976), cultured and rinsed using the indicated media and transformed by PEG-mediated direct gene transfer essentially as described (Freydl *et al.*, 1995; Negrutiu *et al.*, 1987). Ten micrograms plasmid were used for the transformation of 600'000 protoplasts. After two hours 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 (Gomez and Chrispeels, 1993). The protoplast suspension was supplemented with the cell wall synthesis inhibitor dichlorobenzonitrile at 2 mg/ml (not 2mg/ml as erroneously printed in the reference). After 24 hours incubation, protoplasts were pelleted and resuspended in a lysis buffer (0.2M mannitol, 10% Ficoll 400, 20mM EDTA, 2mM DTT, 5mM HEPES pH8, 150mg/ml

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, 10mM HEPES pH7.5, 150mg/ml 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 the concentration of 10 mg/ml. Final concentration for staining was 1 mg/ml. Cells were incubated with NR for 30 min. at room temperature. One volume of osmoticum was added, calls were centrifuged 5' at 80g, 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.

NH_4Cl (Merck, Darmstadt, Germany) was filter sterilised as a 1M stock solution and added to the protoplast suspensions at concentrations of 5, 10, 20 and 50mM (Ehara *et al.*, 1996). Monensin (Sigma, Buchs, Switzerland) was dissolved in ethanol and used at the final concentrations of 5 and 10mM (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 10mM 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 EliteII 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.

Annexe to chapter 2

Rat ß-glucuronidase as a reporter protein for the analysis of the plant secretory pathway.

The results in this chapter have been useful to confirm and support results reported in chapter 2 end anticipated results reported in chapter 4 .

Introduction

Analysis of soluble vacuolar proteins in plants has revealed the existence of three classes of vacuolar sorting signals (VSD) (Matsuoka and Neuhaus, 1999; Neuhaus and Rogers, 1998): (1) a sequence-specific signal mostly found in N-terminal propeptides as in sporamin or aleurain, (2) internal surface determinants as in phytohemagglutinin and (3) 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).

The vacuolar targeting was proposed to use (at least) two pathways; one would be mediated by a C-terminal VSD and would lead to a storage compartment while the other would be mediated by a sequence-specific VSD and would lead to a lytic compartment.

To investigate the dynamics of vacuolar sorting along a C-terminal VSD-mediated pathway, before that the GFP system was functional, we decided to use a secreted form of glucuronidase (GUS) fused to the ctVSD from tobacco chitinase A. This VSD has been shown to be sufficient to redirect a secreted form of chitinase to the vacuole (Neuhaus *et al.*, 1991).

E. *coli* GUS was found not to be a good reporter enzyme for secretion studies because of the position of its cysteine residues and of a cryptic N-glycosylation site (Piñeiro *et al.*, 1995). A related enzyme was thus chosen as a new secretory reporter protein: rat preputial ß-glucuronidase (RGUS), an enzyme that is targeted to the lysosome in animal cells by means of mannose-6-phosphate residues on some of its glycan side chains (Powell *et al.*, 1988). RGUS should thus be well

suited for the secretory pathway also in plants and could be targeted to different compartments of plant cells by different targeting propeptides, to study vesicular trafficking *in vivo* and the effect of drugs and chemicals known to effect proteins sorting.

Chemical tests on the sorting machinery

In this work I tested the effect of wortmannin, brefeldin A (BFA), bafilomycin A1 (BafA1), monensin and NH_4Cl on the sorting of RGUS fusion proteins.

The fungal metabolite wortmannin is a potent and specific inhibitor of mammalian PI 3- and 4-kinase (Arcaro and Wyman, 1993; Yano *et al.*, 1993). It has been demonstrated that phosphatidyl inositol (PI) 3 and 4 - kinase activity is involved in vesicle transport and membrane structure in various cell types (Hong and Verma, 1994; Schu *et al.*, 1993) and thus wortmannin is expected to disrupt membrane transport and structure. In tobacco cells wortmannin had no effect on protein synthesis up to 33 μM, while 100 μM caused a decrease of protein synthesis by ~70% (Matsuoka *et al.*, 1995).

Brefeldin A (BFA) is a lipophilic fungal toxin with an effect on different steps of the secretory pathway. In plants as in animal cells, BFA exerts its primary effects through perturbations of vesicular transport in the secretory pathway, interfering with the coat assembly (see general introduction).

Bafilomycin A1 (BafA1) is a potent inhibitor of the vacuolar ATPase (V-ATPase) in the tonoplast (Bowman *et al.*, 1988). In yeast cells, there is evidence that the acidification by the V-ATPase of vacuoles or of prevacuolar compartments is required for the sorting of several soluble and some membrane proteins to the vacuoles (Rothman *et al.*, 1989; Morano and Klionsky, 1994; Yaver *et al.*, 1993). In plant cells, the possible requirement for the acidification of the Golgi complex in the sorting of proteins has been suggested by many investigations (Gomez and Chrispeels, 1993; Hasenfratz *et al.*, 1995; Matsuoka *et al.*, 1997). Proton pumps have a great importance, of which plant cells contain three different kinds, namely the plasma membrane-type ATPase (P-ATPase), the H^+-pyrophosphatase (H^+-PPase) of the tonoplast and a vacuolar ATPase nitrate-sensitive (V-ATPase) that is found in a wide variety of endomembranes, such as those of tonoplast (Hurley and Taiz, 1989; Matsuura-Endo *et al.*, 1992) the Golgi complex (Herman *et al.*, 1994; Oberbeck *et al.*, 1994), clathrin-coated vesicles (Oberbeck *et al.*, 1994), ER (Herman *et al.*, 1994) and plasma membrane tonoplast (Hurley and Taiz, 1989). BafA1 was found to affect the transport and processing of the sporamin precursor, a vacuolar protein with a ssVSD (Matsuoka *et al.*, 1997).

However the V-ATPase is not essential for maintenance of vacuolar acidity (Brauer *et al.*, 1997).
Monensin is a ionophore which destroys the concentration gradient of protons, potassium or sodium ions across membranes. It induces the elevation of the pH in the vacuole to slightly above 7, the pH of the cytoplasm. Ammonium salts (NH_4Cl) have also a strong effect on vacuolar pH (Ehara *et al.*, 1996).

Results

Transient expression of RGUS fusion proteins.

To assure the vacuolar targeting of this new reporter protein, we tested by transient expression, different constructs in tobacco protoplasts, after a PEG-mediated direct gene transfer. We tested a non-modified RGUS protein, a RGUS where 15 amino acids were deleted at the C-terminus (RGUSD15) and a RGUS were the C-terminal 15 amino acids were substituted by the C-terminal propeptide of tobacco chitinase A: LLVDTM (Freydl *et al.*, 1995; Matsuoka *et al.*, 1995) (RGUSD15+T).
The RGUS sequence was cloned behind the 35S promoter in the plant expression vector pGY1 and used for transient expression in tobacco protoplasts. After an expression time from 18 to 24 hours, protoplasts were harvested and separated from the culture medium. After cell lysis, the soluble fraction and an aliquot of the culture media were used to measure the glucuronidase activity on the substrate 4-methylumbelliferyl ß-D-glucuronide (4-MUG).
To determine the distribution of RGUS in the different cell compartments and to correct for the variable yield of vacuoles, we selected an endogenous enzyme for comparison. We chose the α-mannosidase and we measured the activity of this vacuolar enzyme with the substrate, 4-methyl-umbelliferyl α-D-mannopyranoside (4-MUM), a molecule producing the same fluorescent hydrolysis product (4-methyl-umbelliferone) as the ß-glucuronidase substrate 4-MUG. In our experiments we determined that 85 to 95% of the activity of α-mannosidase was intracellular. The enzymatic activity found in the extracellular fraction could come from cells lysed early in the experiment releasing their vacuolar content before they produced notable amounts of glucuronidase. The soluble fraction of lysed cells contains not only the soluble proteins from vacuoles but also from other compartments, so we determined whether the measured activity was mainly due to vacuolar proteins. We isolated vacuoles from gently lysed protoplasts and compared the ratio of glucuronidase to mannosidase activity in the total intracellular fraction and in the vacuolar fraction. The ratio was constant or even increased in the vacuolar fraction,

showing that all glucuronidase activity resided in the vacuolar compartment. We performed preliminary experiments using only the total intracellular fraction because it was a more abundant and simple source of the reporter enzyme.

Differential accumulation of RGUS forms.
The original rat GUS (RGUS) enzyme is recognised by the plant cell essentially as a vacuolar protein (Neuhaus, personal communication). Since it has C-terminal propeptide, this could be involved in vacuolar targeting in the plant cell. The C-terminus of rat ß-glucuronidase has no clear homology with plant C-terminal VSDs. In tobacco protoplasts a large part of the RGUS was sorted by the default pathway to the culture medium, so that only 65% of the RGUS activity was detected inside the cells, in the soluble intracellular fraction. Deletion of the whole propeptide (19 amino acids) resulted in. a secreted glucuronidase but with in a much reduced expression level. Deletion of the last 15 amino acids, leaving 4 amino acids of the propeptide, resulted in a perfectly active enzyme. This new form of rat ß-glucuronidase (RGUSD1S) was no more recognised by the vacuolar sorting system, as only 7% of the total activity was recorded in the intracellular soluble fraction. This low residual activity is probably not vacuolar since it was undetectable in purified vacuoles. From this deleted version of RGUS, the third and most important construct for this work was obtained by fusion with the ctVSD (6 amino acids) from tobacco chitinase A (RGUSD1S+T). It was expected to be vacuolar and 86% of the total ß-glucuronidase activity was found in the intracellular fraction and after vacuole isolation showed to be specifically localised in this compartment (Table I).

TABLE I:
Intracellular retention of different RGUS fusion proteins.
In parenthesis we report the observed minimal and maximal percentage in the independent experiments (min-max). n= number of independent experiments.

	RGUSD15+T n=16	RGUSD15 n=9	RGUS n=6
Enzyme retention	86% (59-100)	7% (0-15)	65% (36-86)

Effects of drugs on vacuolar sorting of RGUS.

Having set up this model system to study vacuolar targeting of soluble proteins, we decided to trace the pathway followed by the sorting of proteins with a ctVSD like tobacco chitinase A.

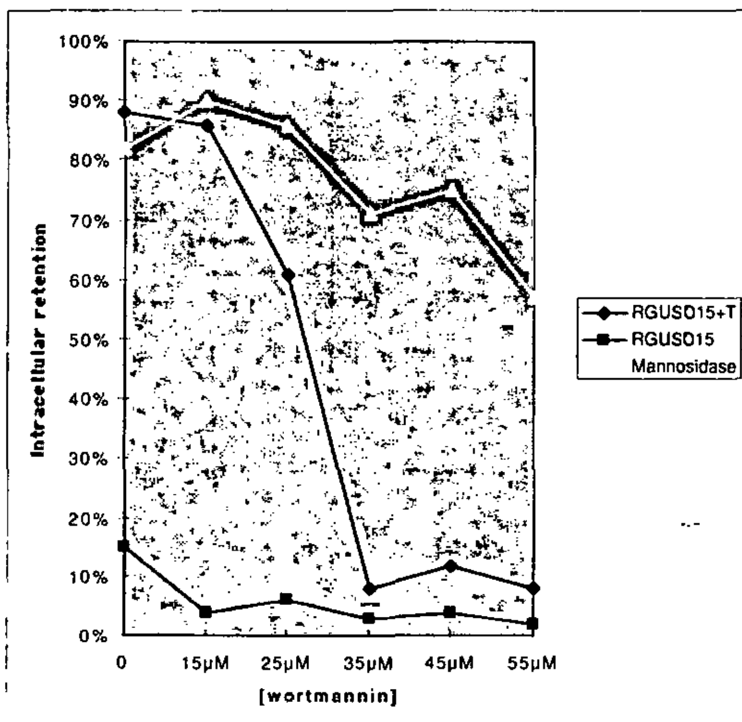
It was also important to test if RGUSD15+T was a valid reporter for the entire category of proteins with ctVSDs. To verify how general this pathway is, we tested the effect of wortmannin. This drug affects the sorting of chitinase A and barley lectin, both with a ctVSD, and does not affect other proteins with ssVSD like sporamin (Matsuoka *et al.*, 1995). To verify the importance of the Golgi apparatus for soluble protein sorting we found in brefeldin A a valid tool that can block Golgi traffic. Finally we tested the importance of the pH gradient across the tonoplast, using drugs that affect proton transport (monensin and bafilomycin A) or chemicals with a direct effect on proton distribution (NH_4Cl).

Effect of wortmannin.

As expected wortmannin affected the vacuolar targeting of RGUSD15+T. While transient expression of RGUSD15+T for 24 hours without drug treatment resulted in the detection of 88% of the enzymatic activity in the intracellular fraction, addition of wortmannin to the culture medium from the beginning of the expression caused a mistargeting of glucuronidase to the medium of the cells, strongly reducing the enzymatic activity detected inside the cell. A drug concentration of 15 μM did not cause a significant reduction of intracellular enzymatic activity but a concentration of 25 μM reduced the intracellular retention of glucuronidase to 65%. Drug concentrations higher than 35 μM caused the almost complete missorting of the enzyme, as only 8% of total activity was detected inside the cell, probably as a fraction of proteins not yet arrived to their final destination, as also observed for the secreted form of the enzyme in the absence of wortmannin.

The activity of the control enzyme α-mannosidase suffered only a very low effect from the drug treatment, probably because the vacuole already contained high amounts of this protein before the treatment began. Only newly synthesised proteins could be missorted in presence of the drug (Figure 2).

FIGURE 2: Enzyme retention in presence of wortmannin.
Variation of the intracellular retention of the vacuolar RGUS (RGUSD15+T in blue) or of the secreted RGUS (RGUSD15 in violet), in transiently transformed tobacco protoplasts. In the same cells the constitutively expressed mannosidase (in yellow) was only weakly missorted.



Effect of brefeldin A.

From previous studies and published observations (Driouich *et al.*, 1993; Staehelin *et al.*, 1997), we knew that a dose of 100 µg of BFA per ml of culture medium would block any Golgi trafficking. We added BFA from the beginning of transient expression and measured enzymatic activity 24 hours later.

BFA affected the secreted as well as the vacuolar form of glucuronidase. In cells treated with 100 µg/ml BFA, the secreted glucuronidase (RGUSD15) was retained inside the cell at 29%; the vacuolar form (RGUSD15+T) in contrary reduced its intracellular activity to 55%.

These results would need further investigation because the effect of this drug concentration appeared more extreme than the effect of wortmannin on resident mannosidase, the intracellular activity of which was reduced to 54%.

Effect of pH variations.

The ionophores used here should elucidate the importance of acidification throughout the secretory pathway for protein sorting.

The role of the vacuolar ATPase was tested using its potent inhibitor bafilomycin A1 (BafA1). Protoplasts were cultivated in the presence of 10 µM BafA1 for 24 hours. Enzymatic activity was then measured as usual in the intracellular soluble fraction and in the culture medium and used to obtain a retention value. Secreted RGUSD15 continued to be secreted, being detectable inside the cell only as 6% of total activity. Vacuolar RGUSD15+T was missorted, as we found only 47% inside the cell. Control cells from the same experiment showed as usual 88% of intracellular enzymatic activity. Mannosidase localisation in these cells was only weakly affected.

To test the importance of the concentration gradient of protons, potassium or sodium ions across membranes we used monensin. concentrations of monensin from 5 to 10 µM did not affect RGUSD15+T at all, nor did NH_4Cl. NH_4Cl is a weak base which has a strong effect on vacuolar pH. NH_3 diffuses through membranes and is trapped as NH_4^+ after reprotonation in the vacuole, causing a pH increase inside the compartment (Ehara *et al.*, 1996). Concentrations of NH_4Cl from 5 to 50 mM had no effect in RGUS or mannosidase vacuolar targeting even when the vacuolar pH was raised to 7.

All these chemicals had no effect on RGUSD15 secretion (Table II). The efficacy of treatments was controlled by the observation of neutral red accumulation (Chapter 2; Di Sansebastiano *et al.*, 1998).

TABLE II:
RGUS retention after treatment with various drugs.

	RGUSD15+T	RGUSD15	Mannosidase
No treatment	90%	3%	96%
Wortmannin 35μM	8%	4%	71%
Brefaldin A 100μg/ml	55%	29%	54%
Bafylomicin A1 10μM	47%	5%	80%
Monanain 10μM	90%	3%	95%
NH_4Cl - 50mM	96%	3%	96%

Stable expression of RGUS fusion proteins in tobacco plants.
RGUSD15 and RGUSD15+T were introduced into tobacco by Agrobacterium-mediated transformation. The transformation efficiency was very low, so we had to repeat the experiment several times.
Out of 26 primary transformants, both with RGUSD15 and RGUSD15+T, only two clones produced a T2 generation with good glucuronidase expression level (in both cases the vacuolar form).
The T1 (progeny) of the first regenerated plant (expressing RGUSD15+T) segregated kanamycin resistance as a hemizygote. We have now both homozygous and heterozygous T2 seedlings. The second T1 plants segregated kanamycin resistance 1 to 1; T2 plants showed segregation as homozygous, or as normal heterozygous or with a proportion of 5:1, demonstrating the presence of at least two inserts. The presence of the transgene was tested only by PCR and no Southern analysis was performed so that this interpretation remains speculative. The important point to consider here is the reporter protein distribution in these two plants, both expressing the vacuolar form of glucuronidase, RGUSD15+T.

Stable expression of RGUSD15+T.

The transformant generated with a single insert was further characterised and showed no visible physiological alteration.

Histochemical GUS assays confirmed the expression of the reporter protein in all plant cells but its accumulation was not homogenous.

If tissues were infiltrated with the X-Gluc solution for less then 6 hours, GUS activity produce a visible staining only in few cells (Figure 3c). Over 10 hours incubation time was necessary to reveal a blue staining in all tissues, with compartments in which the indigo crystals were more or less concentrated, as shown in the figure 3.

This coloration was slower than expected, which could be due to a low enzymatic activity in the tissues, as found when tested by the enzymatic test used for protoplasts (3-20 times lower than in the transient expression essay).

We had no doubt of the presence of the reporter gene, confirmed as well by PCR analysis. We suppose that the turnover of ß-glucuronidase in differentiated cells was faster than in protoplasts.

Surprisingly, when protoplasts were prepared from transgenic plants, a very high enzymatic activity were detected (see also similar results with GFP in chapter 5). In protoplasts from transgenic plants RGUSD15+T was retained at 95%.

Wortmannin affected the sorting of RGUS in transgenic plants in the same way as for the α-mannosidase reference enzyme: a 25 µM concentration was necessary to detect a small reduction to 90% of enzyme retention, 35 µM caused a reduction to 77%, while 45 µM caused a reduction to 73%.

As observed for mannosidase activity in protoplasts, detection of so limited variations is due to the presence of ß-glucuronidase accumulated in the vacuole prior to the treatment, which hides the *possible effects on sorting of newly* made *ß-glucuronidase.*

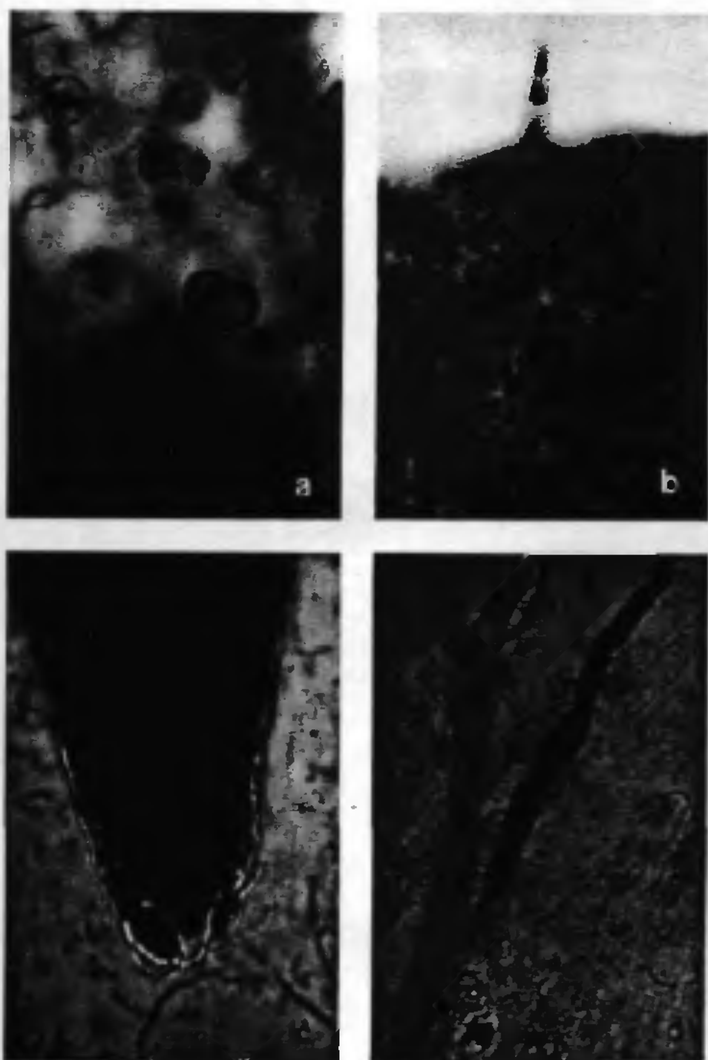


FIGURE 3:
RGUSΔ15+T staining in different tissues of a transgenic plant.
a) Leaf epidermis; b) mesophyll and one tricome; c) root tip; d) root hair.
Magnification X172.

Discussion

Rat preputial ß-glucuronidase (RGUS) was found to be an additional useful reporter to study the plant secretory pathway. Deletion of 15 aa from its C-terminal propeptide produced a secreted RGUS form, representing a tool for the study of secretion. Fusion to this secreted RGUS with the ctVSD from tobacco chitinase A produced a vacuolar RGUS form suitable for the study of vacuolar targeting. These reporter proteins proved to be good tools for our study in the system of transient expression in tobacco protoplasts.

We were able to confirm the efficacy of the ctVSD selected for our study and the sensitivity of its sorting to different drugs. RGUSD15+T sensitivity to wortmannin allowed to an assimilation of its sorting pathway, to the sorting pathway of tobacco chitinase A and possibly to the sorting pathway of other storage proteins rather than to sorting pathways characteristic of vacuolar proteases. Wortmannin affected the vacuolar targeting of RGUSD15+T at the same concentration affecting targeting of barley lectin (Matsuoka and Nakamura, 1992). Brefeldin A caused a general disturbance on the secretory pathway, affecting the vacuolar as well as the secreted RGUS.

When we tested the importance of vacuole acidification in the targeting of RGUSD15+T, we confirmed a possible role of the V-ATPase but not of the vacuolar pH itself. Indeed bafilomycin A1, acting directly on the V-ATPase, induced missorting of RGUSD15+T but the ionophore monensin or the weak acid NH_4Cl that both destroy the pH gradient across the tonoplast had no effect.

These observations encouraged the use of the transient expression in protoplasts as a tool for our research but also revealed the limits of reporter enzymes. Because they cannot be localised visually, fractionation is necessary to identify the targeted compartments. The vacuoles are however so fragile that it is very difficult to avoid the contamination of other fractions by vacuolar enzymes.

We faced major difficulties in obtaining transgenic plants with a useful expression level of RGUS to study its localisation in different tissues. Staining of transgenic tissues needed a stronger treatment than usually needed to detect the *E. coli* GUS, which indicates a much lower level of enzyme accumulation and raises doubts about the correspondence of X-Gluc staining with GUS activity.

The vacuole in which this enzyme is accumulated is lytic in the differentiated cells and reduces the enzyme activity, but from the very beginning of protoplast generation, hormonal induction determines the formation of a new separated vacuole with less lytic activity (see chapter 3). Indeed it is the use of GFP fusion proteins that elucidated

the reasons of this rapid increase of detectable vacuolar proteins in protoplasts compared to plant tissues.
Many considerations in the previous paragraphs were possible only after the characterisation of the protoplast system using the GFP reporter; at the same time this work was useful to confirm the basis on which the further work was based and to give a parallel approach to the investigation of the fate of other reporter proteins in the differentiated tissues.
From histochemical GUS assays, we think that RGUSD15+T, like SGFP5T, is present in all vacuoles of a differentiated tissue, but is degraded because these vacuoles resulted of the fusion of the lytic compartment with the storage vacuole where the reporter proteins appear to be targeted in the protoplasts.
We consider the reported results as an important step in the establishment of the transient expression system we then used with GFP. We demonstrated here that the sorting of fusion proteins with the ctVSD can be followed in protoplasts and that drugs can be used to affect the protein sorting in these living cells.

Experimental procedures

Protoplast transient expression.
Nicotiana tabacum cv. SR1 protoplasts were isolated following the protocol of Nagy and Maliga (Nagy and Maliga, 1976), cultured and rinsed using the indicated media and transformed by PEG-mediated direct gene transfer essentially as described (Freydl *et al.*, 1995; Negrutiu *et al.*, 1987). Ten micrograms plasmid were used for the transformation of 600'000 protoplasts. After two hours protoplasts were rinsed to remove the PEG, resuspended in 2 ml culture medium and incubated at 26°C in the dark.

Inhibitors
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.
NH_4Cl (Merck, Darmstadt, Germany) was filter sterilised as a 1M stock solution and added to the protoplast suspensions at concentrations of 50mM (Ehara *et al.*, 1996). Monensin (Sigma, Buchs, Switzerland) was dissolved in ethanol and used at the final concentrations of 10 µM (Ehara *et al.*, 1996). Bafilomycin A1 (Calbiochem, Lucerne, Switzerland) was dissolved in DMSO and used at concentrations of 10 µM (Matsuoka *et al.*, 1997). Wortmannin (Sigma, Buchs, Switzerland) was dissolved in DMSO in a 10mM stock solution. Final concentrations of 35 µM were used (Nakamura *et al.*, 1993). Brefeldin A (FLUKA, Buchs, Switzerland) was dissolved in methanol and used at the final concentration of 100µg/ml.

Protein extraction from protoplasts and enzymatic tests.
Protoplasts were harvested by centrifugation, resuspended in 0.1 M Na-acetate pH 5 and lysed by 3 cycles of freezing (in liquid nitrogen) and thawing.
The soluble proteins were separated from insoluble residues by centrifugation. The extraction buffer (0.1 M Na-acetate pH 5) was directly used to measure enzymatic activity

of RGUS and α-mannosidase (the constitutive enzyme used as internel control). Measurements were made in a microplate fluorescence reader FL500 (Bio-Tek Instruments).
The reaction substrate was 4-Methyl-Umbelliferyl-ß-D-Glucuronide (BIOSYNTH, Staad, Switzerland) to test RGUS activity and 4-Methyl-Umbelliferyl-α-D-Mannoside (SIGMA, Steinheim, Germany) to test mannosidase activity. Tests in normal condition were normelised by comparing RGUS activity to the intemal control (α-mannosidese).

Plant transformation.
Nicotiena tabacum plents (cv. SR1), were trensformed with the *Agrobacterium tumefaciens* strain PM6000 (a sub-clone of GV3101, from F. Meins, FMI, Basel) and the plant binary transformation vector pCIB200 (maintainable in E coli under kanamycin selection).
The procedure is similar to thet published by Burow et al. (1990) with minor modifications to speed up the method as suggested by Fisher and Guiltinan (1995).
A. tumefaciens GV3101 wes transformed by triparentel mating using the *E. coli* helper strain HB101 pRK2013.

Histochemical RGUS staining.
Tissues from transgenic plants were vacuum infiltrated in 1 ml X-Gluc buffer (10 mg X-Gluc (5-Bromo-4-chloro-3-indolyl-ß-D-glucuronic acid Cyclohexylammonium; BIOSYNTH, Staad, Switzerland) were dissolved in 100µl dimethyl formamide and diluted in 10 ml of 100mM Na phosphate buffer, pH 7) and incubated 10-16 hours at R.T. The X-Gluc buffer was removed and the stained samples were fixed by incubating 10 min in 1 ml FAE (5% formaldehyde, 5% acetic acid, 20% ethenol in water) at R. T. Chlorophyll was eliminated by washing first in 50% ethenol, then in 100% ethanol, untill complete chlorophyll elimination.

Chapter 3

The central vacuole regenerated by miniprotoplasts is a lytic vacuole, not a storage vacuole. Identification through the use of GFP targeted to either type of vacuole.

Gian-Pietro Di Sansebastiano, David Humair, Nadine Paris, Sophie Marc-Martin and Jean-Marc Neuhaus. Manuscript in preparation.

Summary

Vacuole development has been investigated using several approaches. A complementary and novel approach is the study of vacuole regeneration in evacuolated cells, providing a convenient *in vitro* system in which to study synchronous development of vacuoles in large numbers of cells.

Using the knowledge accumulated on the sorting of soluble proteins in plants, we revisited the regeneration of vacuoles in tobacco miniprotoplasts. Two different VSDs were compared: a C-terminal VSD from tobacco chitinase A and a sequence-specific VSD from barley aleurain, which target proteins to a storage and lytic vacuole respectively. Both VSDs were fused to the reporter protein GFP and visualised by confocal microscopy. The trafficking and final location of the two GFPs differed and the newly formed vacuoles of miniprotoplasts were differently labelled.

Introduction

In contrast to yeast or mammalian cells, plant cells may contain separate vacuoles. Some vacuoles have storage or digestive functions, others have not yet a clear function but all of them seem to be characterised by the presence of specific tonoplast intrinsic proteins (TIPs). Protein storage vacuoles (PSVs) are marked by the presence of α-TIP, lytic or degradative vacuoles (LVs) are marked by the presence of γ-TIP (Paris *et al.*, 1996) and a third kind of vacuole used to store pigments are marked by δ-TIP (Jauh *et al.*, 1998). Vacuole development has been investigated using several approaches: transmission electron microscopy (Buvat, 1982), biochemical studies of the changes accompanying cell enlargement (Maeshima, 1990), and studies of the mechanisms by which proteins are targeted to the vacuole (Bednarek and Raikhel, 1992; Höfte and Chrispeels, 1992).

A complementary approach is the study of vacuole regeneration in evacuolated cells. Vacuoles can be removed from plant protoplasts by high-speed centrifugation through a continuous density gradient (Lörz *et al.*, 1981). Evacuolated protoplasts (miniprotoplasts) are viable and can regenerate vacuoles and cell walls in culture (Wu and Tsai, 1992), thus providing a convenient *in vitro* system in which to study synchronous development of vacuoles in large numbers of cells. Vacuole regeneration in evacuolated tobacco and petunia protoplasts has been shown to occur after culture for 12 to 44 h (Erdmann *et al.*, 1989). Reappearance of the vacuole is accompanied by increased levels of hydrolytic enzymes, tonoplast H^+ Pyrophosphatase activity, neutral red uptake (indicating the acidic nature of the developing vacuoles), and the reappearance of a 41 kD vacuolar protein of unknown function (Hörtensteiner *et al.*, 1992). Inclusion of bafilomycin A, a specific inhibitor of the vacuolar H^+-pumping ATPase, in the culture medium decreased the uptake of neutral red, but did not prevent vacuole regeneration (Sze *et al.*, 1992). Vacuole formation in evacuolated petunia protoplasts is associated with the accumulation of flavonoids, followed by the synthesis of vacuole-associated ethylene-forming activity (Erdmann *et al.*, 1989). Protoplasts cultured in the presence of cycloheximide failed to develop vacuoles, showing that protein synthesis is required, but the strong inhibitory effect of cycloheximide was completely reversed when evacuolated protoplasts were washed with inhibitor-free medium (Hörtensteiner *et al.*, 1994).

The sorting of a number of vacuolar proteins has been investigated and their vacuolar sorting determinants (VSDs) have been determined. Coupling the knowledge of such sorting determinants to the use of reporter proteins, it is possible to revisit some of the observations about vacuole regeneration and vacuolar sorting. In this paper two

different VSDs are compared: a C-terminal VSD from tobacco chitinase A and a sequence-specific VSD from barley aleurain, which target proteins to a storage and lytic vacuole respectively (Neuhaus and Rogers, 1998). Both VSDs were fused to the reporter protein GFP. The differences in localisation were visualised by confocal microscopy of living protoplasts and miniprotoplasts expressing transiently the hybrid GFPs. The trafficking and final location differed and only one of the two reporters labelled the newly formed vacuole of miniprotoplasts.

Results

Two different vacuolar sorting determinant target GFP to different compartments: comparison between SGFP5T and AGFP5/6.

Expression of a GFP (SGFP5T) targeted to the vacuole by the C-terminal propeptide of tobacco chitinase A revealed that the reporter protein was sorted to pH-neutral vacuoles but not to acidic vacuoles. This was interpreted as reflecting the existence of two different types of vacuoles, the pH-neutral vacuole corresponding to a storage vacuole, while the acidic vacuole would correspond to a lytic vacuole. The size of the two vacuoles differed in different protoplast types: chloroplast-rich protoplasts accumulated SGFP5T mainly in the large vacuole, chloroplast-poor protoplasts accumulated SGFP5T in small vacuoles (Di Sansebastiano *et al.*, 1998). The patterns observed with SGFP5T in both cell types are shown in figure 1.

To label lytic vacuoles, we fused the same GFP5 variant (Siemering *et al.*, 1996) with the 150 amino acids long N-terminal propeptide of barley aleurain, a thiol protease targeted to the lytic vacuole (Holwerda *et al.*, 1992). The resulting GFP (AGFP5) was only visible in compartments smaller than 2 µm and often clustered around the nucleus (not shown). No vacuoles were visible as with SGFP5T, the GFP targeted to the storage vacuole. Since the fluorescence intensity was much smaller for AGFP5 than for SGFP5T, we quantified by immunoblots the GFP in the medium and in total protoplast extracts. AGFP5 was never found in quantities comparable to vacuolar SGFP5T or secreted SGFP5 (not shown). We suspected that AGFP5 was rapidly degraded in its final target compartment and was thus only visible in the small unidentified compartments described above which could be intermediate in the transport pathway. To increase the detection limit of this reporter protein we introduced into AGFP5 the mutations F64L/S65T (Cormack *et al.*, 1996). The resulting brighter GFP was named AGFP6. Now we could visualise fluorescence in a higher number of protoplasts in the same small compartments (Figure 2a,c) seen with AGFP5. Many protoplasts

TABLE I:

GFP patterns in two protoplast subpopulations expressing SGFP5T or AGFP6.

After 24 hours of expression, SGFP5T was observed in a large vacuole (LV), in a small vacuole (SV, Figure 1b) or in ER-like structures (ER); AGFP6 was observed in small compartments (SC, Figure 2a) or in a large vacuole (LV).

Results are expressed in percentage within each population analysed, 100% corresponding to the total number of cells observed in either chloroplast-rich or chloroplast-poor protoplasts for three independent transformations. In parenthesis we report the observed minimal and maximal percentage in the independent experiments (min-max.).

	AGFP6		SGFP5T	
	Chlor.-rich n=154	Chlor.-poor n=192	Chlor.-rich n=227	Chlor.-poor n=110
LV	**18%** (12-25)	**15%** (14-17)	**75%** (58-88)	**6%** (0-15)
SC (AGFP6) SV&ER (SGFP5T)	**82%** (75-88)	**85%** (83-86)	**25%** (12-42)	**94%** (100-85)

FIGURE 1: Fluorescence patterns of GFP targeted to a storage vacuole (SGFP5T). Confocal images (1µm section) of tobacco protoplasts expressing SGFP5T for 24 hours. **a)** and **b)** Chloroplast-rich protoplasts; **c)** and **d)** chloroplast-poor protoplasts; a) and **c)** more frequent patterns; **b)** and d) less frequent patterns (see Table I). Bar= 10µm.

FIGURE 2: Fluorescence patterns of GFP targeted to a lytic vacuole (AGFP6). Confocal images (1µm section) of tobacco protoplasts expressing AGFP6 for 24 hours. a) and **b)** Chloroplast-rich protoplasts; c) and **d)** chloroplast-poor protoplasts; a) and **c)** more frequent patterns; **b)** and d) less frequent patterns (see Table I). Bar= 10µm.

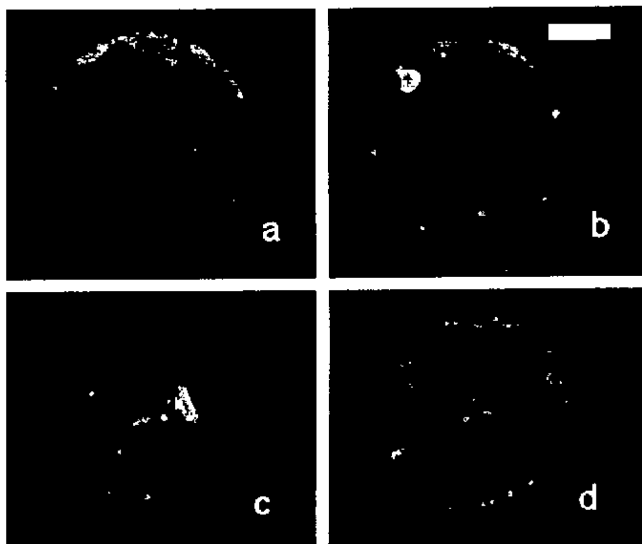


FIGURE 1

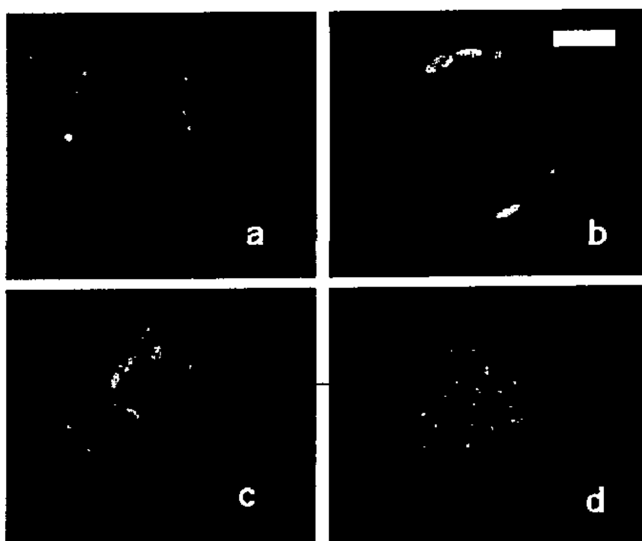


FIGURE 2

also showed a large green vacuole (Figure 2b, d). Contrary to the SGFP5T there was no apparent difference in pattern distribution between chloroplast-rich and chloroplast-poor protoplasts. When a large fluorescent vacuole was visible in a chloroplast-poor cell transformed with AGFP6, it was often also possible to identify several non-fluorescent compartments (Figure 2c). Apart from the nucleus, these areas could correspond to the smaller vacuoles that were found to accumulate SGFP5T in the same cell type (Figure 1d). We quantified the distribution of large vacuoles in cells transformed with AGFP6 and found 18% of chloroplast-rich protoplasts with a large fluorescent vacuole (Table I). This percentage was approximately complementary to the percentage (75%) of large vacuoles observed in protoplasts transformed with SGFP5T under the same conditions. Chloroplast-rich protoplasts come presumably all from palisade mesophyll and are thus probably a single cell type.

In contrast in chloroplast-poor protoplasts there was no such complementarity, but we could see large vacuoles accumulating AGFP6 in 15% of these cells, more than twice the frequency observed with SGFP5T. Chloroplast-poor protoplasts could include cells from the epidermis, trichomes, guard cells, and mesophyll, they present thus a complex mixture to analyse, but we still could observe significant difference in the distribution of large fluorescent vacuoles between the two reporters. It was still clear that ctVSD and ssVSD targeted soluble proteins to different vacuoles.

In our normal experimental conditions we were never able to visualise AGFP6 in the ER. We incubated protoplasts at low temperature to slow down AGFP6 transit through the ER. After incubation at 14°C for 8 hours, the nuclear envelope became partially fluorescent and smaller compartments became visible in the neighbouring cytoplasm (Figure 3a). The ER was labelled by AGFP6 in a pattern very similar to the pattern observed for SGFP5T under the same conditions (Figure 3b). This indicates that AGFP6 leaves the ER at normal temperatures much faster than SGFP5T, possibly too fast for the fluorophore to form.

FIGURE 3: Fluorescence patterns of vacuolar GFPs in cells incubated at 14°C. Confocal images (1μm section) of tobacco protoplasts cultivated in the same conditions, expressing vacuolar GFPs for 8 hours. **a)** Protoplasts expressing AGFP6; **b)** protoplasts expressing SGFP5T; in both protoplasts the nuclear membrane and the ER are visible. Note the typical small compartments accumulating AGFP6 in a). Bar= 10μm.

FIGURE 4: Neutral Red staining of protoplasts expressing AGFP6. Confocal images in transmitted light (a and c) or green fluorescence (b and d) of protoplasts expressing AGFP6 and stained by neutral red. **a + b)** The left cell showed no fluorescence and accumulated NR, the right cell was fluorescent and did not accumulate NR; **c + d)** both cells accumulated NR and the cell on the right also showed green fluorescence. Bar= 10μm.

FIGURE 5: Regeneration of a large central vacuole by miniprotoplasts. Confocal images (1μm section) of tobacco miniprotoplasts at different times after evacuolation. **a-c)** SGFP5T expressing miniprotoplasts within 8, 36 or 52 hours after evacuolation; **d-f)** AGFP6 expressing miniprotoplasts 8, 36 or 52 hours after transformation. Bar= 10μm.

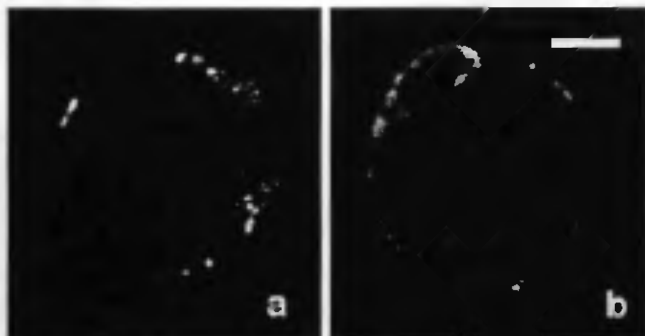


FIGURE 3

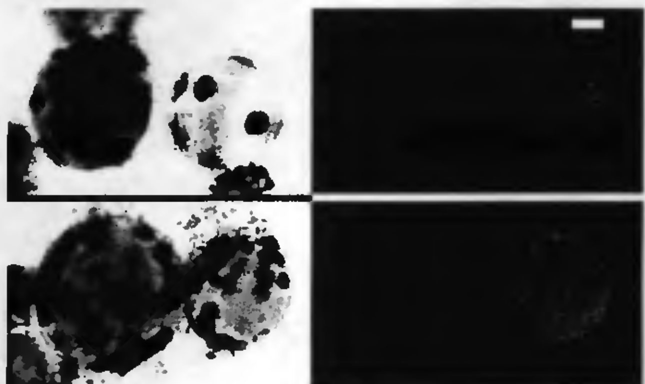


FIGURE 4

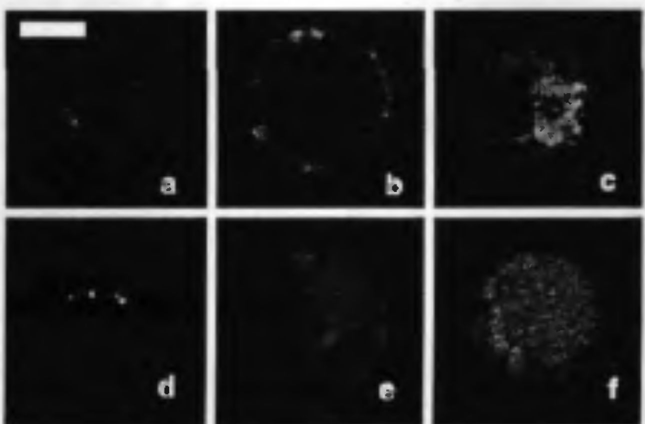


FIGURE 5

The fluorescence enhancing mutations F64L/S65T in GFP6 did not influence GFP sorting but increased the apparent transformation efficiency.

The use of GFP6 instead of GFP5 was necessary to visualise the lytic compartment but it could have altered sorting. We therefore verified with the GFP targeted to the storage vacuole that the mutations did not affect sorting. When comparing SGFP5T and SGFP6T, we observed a 20% increase of the number of fluorescent protoplasts. The pattern of GFP labelling was conserved, excepted that ER labelling was more visible, even after a long expression time. We concluded that the mutations lowered the detection limit of GFP so that residual GFP in the ER was now more often visible. Indeed, after treatment with cycloheximide, the ER fluorescence strongly decreased (not shown). Therefore we chose to continue to use the SGFP5T which gave a sufficient signal for the comparison with AGFP6.

The apparent transformation efficiency depended on the intensity of the obtained fluorescence. With both VSDs, the GFP6 was visible in a higher proportion of protoplasts than the GFP5 and GFP targeted to the storage vacuole was visible in more cells than GFP targeted to the lytic vacuole. Since we used the same vector and the same transformation protocol for both vacuolar GFPs, we expect a similar rate of GFP synthesis.

AGFP6 accumulate in lytic acidic vacuoles.

Throughout the experiments vacuoles containing AGFP6 only showed a faint fluorescence compared to vacuoles containing SGFP5T. The detection must then depend on the stability of the protein. This implies that GFP is rapidly degraded in the compartment that accumulates AGFP6. Indeed the proportion of fluorescent protoplasts in a population expressing AGFP6 decreased from 20% after 24h to only 3% after 48h.

The lower pH of the lytic compartment can also partially explain the difference in visible fluorescence between SGFP5T and AGFP6. pH can influence the quantum yield of GFP and the F64L/S65T GFP (GFP6) is known to be pH sensitive (Cormack *et al.*, 1996). To elucidate the nature of these vacuoles we stained the protoplasts with neutral red (NR) (Di Sansebastiano *et al.*, 1998). In many cases the green fluorescence of AGFP6 was visible in the same compartment as NR staining (Figure4), a *situation never observed in cells expressing* SGFP5T or SGFP6T. Surprisingly we observed that the proportion of stained vacuoles decreased compared to control protoplasts or those expressing SGFP5T. While in these two populations more than 70% of all chloroplast-rich cells (fluorescent or not) showed NR stained large vacuoles, only 48% of corresponding protoplasts transformed with AGFP6 accumulated neutral red. For chloroplast-poor protoplasts, control or SGFP5T expressing

cells accumulated NR at about 95%, this proportion dropped to 74% with AGFP6 expressing cells. It is thus evident that AGFP6 can accumulate in acidic vacuoles from which SGFP5T was excluded. However, overexpression of AGFP6 seems to affect the acidification of the lytic vacuoles.

TABLE II:
GFP accumulation in regenerated large central vacuoles in miniprotoplasts.
Large central vacuoles became visible after 30-36h. The percentage of fluorescent large vacuoles was calculated for cells showing any green fluorescence.

	36h after evacuolation	**48h after evacuolation**
SGFP5T	0% (n=144)	36% (n=245)
AGFP6	97% (n=161)	84% (n=208)

Vacuole regeneration in transformed miniprotoplasts.

Protoplasts are cells from which the cell wall has been removed and to which an important hormonal stimulation has been applied. It cannot be easily decided whether the observed compartments of the secretory pathway remain unchanged during the experiment or whether they changed their properties or even formed *de novo* due to the dedifferentiation process induced by the digestion and culture conditions. To distinguish between targeting to a pre-existing vacuole and formation of a new vacuole in transformed protoplasts, we used evacuolated miniprotoplasts.

Because evacuolation is based on the large density difference between chloroplasts on one side and vacuoles on the other side, it works therefore best with chloroplast-rich protoplasts, while other protoplasts are mostly lost in the procedure. We thus followed vacuole regeneration in a single cell type, which happened to be the cell type that showed the larger difference in accumulation of the two different GFPs in large vacuoles.

In evacuolated protoplasts expressing SGFP5T, fluorescence accumulated within the first 3 to 4 hours in discrete areas of the ER, while after 6-8 hours, it was more evenly distributed throughout the ER and also accumulated in some larger compartments (Figure 5a). Clearly visible large vacuoles only appeared after 24-36 hours of incubation, but they did not contain GFP (Figure 5b). The reporter protein accumulated further in the same compartments seen after 6-8 hours. Only after 48 to 78 hours did we see from 29 to 51% of cells in which GFP now occupied the large central vacuole. We could thus observe that the initial formation of a central vacuole was different from the SGFP5T transport pathway (Table II).

When AGFP6 was expressed, miniprotoplasts started a few hours after evacuolation to accumulate GFP in the small compartments (Figure 5d) described above as the early pattern for normal protoplasts (Figure 2a). After 36-48 hours almost all transformed cells had large central vacuoles labelled with GFP (Figure 5e). In few cells (around 7% after 36 h) it was possible to distinguish a non-labelled vacuole even if there was an intense GFP fluorescence in peripheric small compartments. The proportion of fluorescent cells with large vacuoles excluding GFP was always very low and it was possibly due not to the exclusion of the protein from the central vacuole but to detection problems. With time (over 48 h) AGFP6 fluorescence strongly decreased in all cells.

DISCUSSION

In this paper we describe the sorting of two chimeric proteins obtained by the fusion of GFP to two VSDs, a C-terminal propeptide derived from tobacco chitinase A and an N-terminal propeptide derived from barley aleurain. These two proteins have been selected because they have been shown to follow different pathways (Holwerda *et al.*, 1992).

Confocal laser scanning microscopy was used to visualise the different steps of the two sorting systems. This revealed an interesting difference between the patterns of GFP accumulation for the two constructs. The pattern of SGFP5T accumulation described in a previous paper (Di Sansebastiano *et al.*, 1998) led us to conclude that this GFP fusion protein transited through the ER and was accumulated either in small vacuoles or in the large central vacuole. In contrary AGFP6, described for the first time, after having resided a very short time in the ER, transited through small compartments, not described until now. In some cells the AGFP6 then reached the large central vacuole but presented different distributions in different protoplast subpopulations compared to SGFP5T.

First large vacuoles accumulating AGFP6 were equally rare in chloroplast-rich and chloroplast-poor protoplasts, a very different pattern than observed with SGFP5T. If we consider only chloroplast-rich protoplasts, we can see a clear correspondence in the percentage of cells in which AGFP6 occupied the large vacuole and cells in which SGFP5T was limited to small vacuoles and ER. The two markers can label essentially all large vacuoles.

Second in large vacuoles, the AGFP6 fluorescence was much fainter than SGFP5T. This can be due to the acidic pH of the compartment which is known to influence GFP emission (Robey *et al.*, 1998), or to a reduced concentration of GFP due to faster degradation. This latter possibility is supported by the faster degradation of AGFP6 in cells treated with cycloheximide, an inhibitor of protein synthesis (data not shown). Even without cycloheximide the stability of the two reporters was different. After two days very little fluorescence remained in AGFP6 expressing protoplasts while SGFP5T expressing protoplasts were still fluorescent.

The most important argument for a difference between AGFP6 and SGFP5T vacuoles was neutral red (NR) accumulation. Some protoplasts didn't accumulate NR in their large vacuoles, but were viable as indicated by cytoplasmic movements and NR accumulation in small compartments. These cells corresponded to a fifth of the total population used for transient expression. This proportion was the same in control cells and cells transformed by SGFP5T. The cells with a large

vacuole that did not accumulate NR were all chloroplast-rich and SGFP5T was found to accumulate only in such large neutral vacuoles. The pH of SGFP5T accumulating vacuoles was thus higher than 6, the pH of the medium. Rarely, as reported in table I, some chloroplast-poor protoplasts accumulated SGFP5T in the large vacuole, but when this situation was observed in NR stained cells, the dye never colocalised with GFP. In contrary colocalisation of AGFP6 and NR was found in the majority of cells with a fluorescent vacuole. NR however, seemed to be accumulated less in AGFP6 transformed cells than in SGFP5T transformed cells or in control cells. When NR and GFP fluorescence were colocalised the NR accumulation was often weak and difficult to document in photographs. In comparison with the identity of NR accumulation in SGFP5T and control protoplasts, we can argue that AGFP6 influenced the vacuole acidification, which could explain the reduced NR accumulation. We postulate that the pathway used by lytic enzymes to the lytic vacuole, which is also responsible for acidification, sorted AGFP6. The overload of this pathway with the chimeric protein increased the volume or the buffering capacity of the lytic vacuole without a corresponding increasing rate of acidification. We assume that the AGFP6 is targeted to the lytic vacuole because of three considerations: first the ssVSD derives from a protease targeted to the lytic vacuole, second AGFP6 undergoes a rapid degradation, third low pH is generally associated to the lytic compartment.

Having shown that SGFP5T and AGFP6 are transported through different pathways to different vacuoles, we used these proteins to monitor vacuole regeneration in evacuolated protoplasts. We observed that evacuolated protoplasts regenerate several small prevacuolar compartments at the same time, some containing SGFP5T, while others contained AGFP6. Out of some of these prevacuoles the large central vacuole of the cell was formed after 36 hours. AGFP6 was almost always present in this main compartment and its fluorescence was very bright. SGFP5T remained limited to small vacuoles. While its quantity increased, SGFP5T was not observed in the newly formed large central vacuole before 48 hours after evacuolation, when some large green vacuoles began to appear. We did not observe any intermediate compartment with a volume between the small vacuoles and the large central vacuole, it is thus probable that the large vacuole became fluorescent through fusion with some prevacuole containing a large amount of SGFP5T. After more than 2 days, SGFP5T was found in increasing numbers of large vacuoles. It seems that the vacuole that re-establishes the normal cell volume is generally identical with the lytic vacuole accumulating AGFP6; it always excluded SGFP5T but in a few cells we could not exclude the presence of a third type of vacuole

excluding AGFP6 as well. A few cells in fact were occasionally observed with AGFP6 fluorescence but a non-fluorescent large vacuole.
From the simple comparison of GFP sorting in protoplasts and vacuoles regeneration in miniprotoplasts, we tried to represent the system here described, in a model (Figure 6):
Miniprotoplasts regenerate from the start different kinds of vacuoles. At the beginning of regeneration storage vacuoles and lytic vacuoles both have a small volume. SGFP5T labels small storage vacuoles and the ER, AGFP6 labels a larger number of lytic prevacuoles (Figure 6a).
The miniprotoplasts take from 36 to 48 hours to regenerate a vacuole with a normal volume, but already after 24 hours the lytic vacuole occupies most of the intracellular space. One single lytic vacuole receives all AGFP6 from the characteristic prevacuoles so that these compartments are no more abundant in cells where the large vacuole has formed. In contrary SGFP5T continues to be accumulated in small vacuoles of variable size (Figure 6b). These compartments are true vacuoles because they persist in many protoplasts as compartment for the definitive accumulation of SGFP5T but later and in some cells only, they fuse with each other and with the lytic vacuole. In this case they could be called provacuoles to indicate their potential to become independent vacuoles.
We ignore the criteria by which the miniprotoplasts regulate the fusion of the large lytic vacuole with the smaller storage provacuoles. Our data indicate that this fusion happens in around 36% of cells (Figure 6c).
In the end, miniprotoplasts regenerated two types of vacuolar complexes: in more than the half of the cells the lytic central vacuole remained separate from the surrounding storage provacuoles, in the other cells the lytic vacuole and the storage provacuoles fused to a unique compartment.
The fusion between vacuoles must be regulated by some determinants on their tonoplasts. Because the vacuoles are competent for fusion only some time after their formation, the fusion determinants will either need time to accumulate sufficiently or will arrive to the tonoplast through an independent pathway.
The situation observed in miniprotoplasts is well comparable with the situation observed in normal protoplasts transiently expressing GFPs. SGFP5T labels initially the storage provacuoles (Figure 6d); if the pre-existing vacuole is a storage vacuole, the provacuoles will fuse with it (Figure 6e); if the pre-existing vacuole is a lytic one, the provacuoles will become separate storage vacuoles (Figure 6f). In normal protoplasts we can not exclude the existence of a third kind of vacuole from which both SGFP5T and AGFP6 are excluded (Figure 6g). This third

kind of vacuole could be typical of most chloroplast-poor cells, but rare in chloroplast-rich cells, from which the miniprotoplasts were derived. Anyway, each time we define the identity of a vacuole on the basis of the GFP accumulation, we have to consider the possibility that GFP is rapidly degraded so that it is not visible. Vacuoles from which AGFP6 is apparently excluded could simply be more degradative than others. It is possible that lytic vacuoles have different degradative activity just as they have variable pH as visualised by NR.

On the basis of the suggested model we intend to complete immunological characterisation of tonoplast membranes and to investigate by the use of drugs, the mechanisms responsible of vacuoles fusion. Comparing our results with published results (Jauh *et al.*, 1998), we think it will be possible to associate early SGFP5T vacuoles with α-TIP (see annexe) and AGFP6 vacuoles with γ-TIP, while the central vacuole formed by the fusion of the two types of vacuoles after more than 48 h could be associated with δ-TIP.

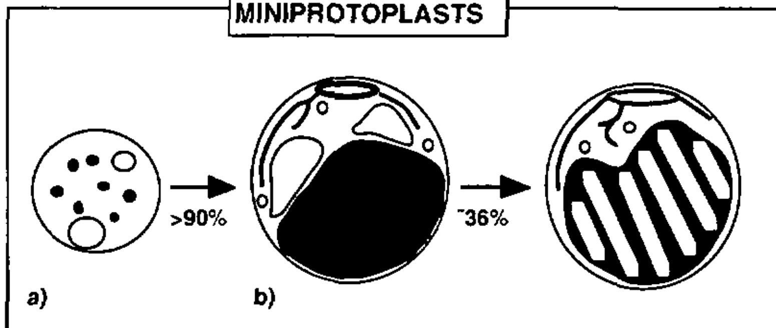
FIGURE 6: Model of vacuole biogenesis in miniprotoplasts and protoplasts. SGFP5T labelling is indicated in light green, AGFP6 labelling is indicated by dark green. The percentage of cells with each pattern is reported in black for chloroplast-rich cells and in red for chloroplast-poor cells.

Situation in miniprotoplasts:

a) 8 hours after evacuolation no large central vacuole is visible. SGFP5T labels ER and small provacuoles, AGFP6 labels small lytic prevacuoles. **b)** 36 hours after evacuolation the large central vacuole is a lytic vacuole labelled by AGFP6; c) 52 hours after evacuolation around 36% of cells have fused the lytic central vacuole with the storage provacuoles.

Situation in normal protoplasts:

d) At the beginning of transient expression GFP has not reached yet the pre-existing central vacuole: AGFP6 is in the lytic prevacuoles while SGFP5T is in newly formed storage provacuoles; **e)** if the pre-existing vacuole is a storage vacuole, it will rapidly fuse with the newly formed storage provacuoles while lytic prevacuoles will accumulate in the cytoplasm; **f)** if the pre-existing vacuole is a lytic vacuole it will fuse with the *lytic prevacuoles* and will *remain separate from* the newly formed storage vacuoles; **g)** a third kind of vacuole could be incompetent for fusion with either newly formed storage provacuoles or lytic prevacuoles.



PROTOPLASTS

Lytic pre-vacuoles

Storage pro-vacuoles

Pre-existing storage vacuole

75% 6%

d)

e)

Pre-existing lytic vacuole

18%
15%

Fusion-incompetent vacuole or 3d type of vacuole

7%
79%

f)

g)

SGFP5T AGFP6

Experimental procedures

Fusion gene constructs

The pSGFP5 and pSGFP5T plasmids have been described (Di Sansebastiano *et al.*, 1998). GFP6 was obtained by directed mutagenesis of F64 to L and S65 to T (ctcact).

We introduced by PCR a NhaI site at the beginning of the coding sequence for GFP5 or GFP6 (gctagcgcaatg) and at the 3' end of the Aleurain N-terminal fragment (ccgctagcc) with appropriate primers. pAGFP5 plasmid was obtained by the substitution of the signal sequence of pSGFP5 with the first 431 bases (143 amino acids) of Barley aleurain cDNA (Rogers *et al.*, 1985) as a fragment BamHI/NheI.

Plasmids were isolated by alkaline lysis in presence of SDS (Sambrook *et al.*, 1989) and purified on an ethidium bromida-CsCl density gradiant.

Protoplast and miniprotoplast transient expression

Nicotiana tabacum cv. SR1 protoplasts were isolated, transformed and stained with neutral red (Fluka, Buchs, Switzerland) as described (Di Sansebastiano *et al.*, 1998).

Protoplasts were evacuolated 2 hours after transformation essentially as described by Newell et al. (1998) and purified as described by Hörtensteiner et al. (1992) with few modifications. Miniprotoplasts were centrifuged 10 min at 200 g in a Percoll step gradient 1,5 ml 80% Percoll solution (Mannitol0.5 M, $CaCl_2$1 mM, MES10 mM), 5 ml 40% solution, 1,5 ml 20% solution, finally the 2ml of evacuolation solution containing the evacuolated protoplasts. Miniprotoplasts were washed and incubated in the same conditions for normal protoplasts.

Confocal laser scanning microscopy and data collection

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 while chlorophyll epifluorescence was detected with the filter set for TRITC.

The stored digital images were pseudocolored as red or green images, using Photoshop 4.1 (Adobe, Mountain View, CA) corresponding to the real red or green colours. Negative cells did not show any green fluorescence for the setting at which images were usually collected, except chlorophyll epifluorescence.

Annexe to chapter 3

SGFP5T is accumulated in vacuoles marked by the presence of α-TIP

The preliminery results presented in this ennexe encourage further investigation of the TIP composition of tonoplasts, coupled with GFP trensient expression.

Introduction

It is not clear yet how plant cells can regenerate and maintain functionally distinct vacuoles.

Tonoplast membranes contain abundant tonoplast intrinsic proteins (TIPs) that may function as aquaporins, but their abundance seems far in excess of what is required for water transport (Neuhaus and Rogers, 1998). Protein storage vacuoles (PSVs), containing seed-type storage proteins, are marked by the presence of α-TIP, and lytic vacuoles (LVs) are marked by the presence of γ-TIP (Hoh *et al.*, 1995; Paris *et al.*, 1996). A third type of vacuole differentiates when δ-TIP is inserted into the tonoplast of a lytic vacuole (Jauh *et al.*, 1998).

These observations indicate that a specific TIP isoform correlates with a specific vacuole function (Jauh *et al.*, 1998; Jiang and Rogers, 1998). To characterise the separate vacuoles that a tobacco protoplast is able to generate, we want to identify each tonoplast with anti-TIP antibodies. We present here some preliminary results indicating the applicability of this strategy as well as the limits of our material.

Results

Anti α-TIP antibodies label selectively one of the regenerating vacuoles in miniprotoplasts

Evacuolated protoplasts (see chapter 3) were harvested and fixed at different time intervals after the start of vacuole regeneration. These cells have regenerate vacuoles and thus probably different kinds of tonoplast membranes well separated. We labelled with antibodies against α-TIP fixed cells in which SGFP5K, SGFP5T, SGFP6T or AGFP6 were expressed.

At the initial stages of regeneration, after 24 hours, α-TIP antibodies labelled specifically the tonoplast of the vacuoles containing SGFP5T or SGFP6T while AGFP6 containing vacuoles were generally not labelled (Figure 1). The presence of this epitope confirmed the identification of the SGFP5T containing vacuole with the PSV.

Discussion

We performed some preliminary experiments and we planned to investigate the tonoplast constitution of the observed compartments using antibodies against TIPs. So far we confirmed that the regenerating vacuoles containing SGFP5T contained α-TIP, while this epitope was excluded from tonoplast of regenerating vacuoles containing AGFP6. We believe that the SGFP5T accumulating compartment is the storage vacuole, while the AGFP6 accumulation compartment is the lytic vacuole. At the same time it is not possible to exclude that in protoplasts from the leaf blade, these definition do not correspond exactly to the functions of the two vacuoles till now identified. In fact lytic and storage vacuoles have been usually characterised in specialised cells like barley aleurone cells (Holwerda *et al.*, 1990) or root tip cells (Paris *et al.*, 1996). The PSV of these cells could share some function with the LV remaining separated.

We will continue our immunological characterisation but we evidenced the limits imposed by our material are evident. Protoplasts from green tissues have such a density of chloroplasts that the red fluorescence of chlorophyll almost completely prevents the use of rhodamine as label for immunofluorescence, why the use of fluorescein is prevented by the fluorescence of GFP itself. Chloroplasts represent a problem, disturbing the recognition of the tonoplast and confusing with their autofluorescence due to the chlorophyll, the antibody fluorescent signal. Other cell types have to be prepared and evacuolated, more suitable for immunological labelling.

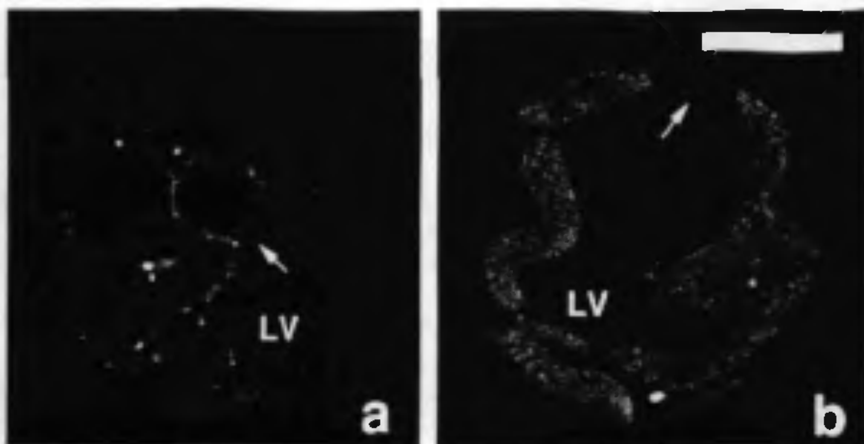


FIGURE 1: Anti α-TIP antibodies label the tonoplast of SGFP5T accumulating vacuoles.
Cells were fixed 24 h after the beginning of regeneration.
a) The immunolabelled membrane (arrow) defines the vacuole containing SGFP5T; b) the AGFP6 accumulating vacuole (LV) is not labelled by the α-TIP antibodies that immunolabel a second compartment (arrow). Bar= 10μm.

Experimental procedures

Immunofluorescence staining

Miniprotoplasts were fixed at various times of vacuole regeneration and immunolabelled, using the methods described by Paris et al. (1996).
Primary antibodies anti α-TIP antibodies were a kind gift of John Rogers (Johnson *et al.*, 1989); anti-rabbit secondary antibodies were conjugated to lissamine Rhodamine.

Chapter 4

Characterisation and comparison of the two vacuolar sorting systems in tobacco cells

The results reported here have been presented in part as a poster:

Di Sansebastiano G.P., Paris N., Marc-Martin S., Neuhaus J-M.
March 1999; 6th International Botanical Microscopy Meeting Plant Cell Biology; Royal Microscopical Society. St Andrews, UK.

Summary

We have shown that the N-terminal propeptide of the barley aleurain and the C-terminal propeptide from tobacco chitinase A mediate transport to different vacuoles. We report now that the transport system also differ markedly as indicated by the differential effects of Brefeldin A and by the difference in glycosylation of GFPs targeted to the two vacuoles. The two sorting machineries have different intermediate compartments as evidenced by brefeldin A (BFA) differential effect and confocal imaging. BFA (from 10 to 100 µg/ml) had a strong effect on SGFP5T pattern distribution. In presence of the drug SGFP5T did not arrive to the final vacuolar destination. The BFA dose necessary to complete disruption of Golgi was 50µg/ml, as the Golgi was no more detectable immunologically in protoplasts using JIM84 antibodies. GFP fused to the N-terminal vacuolar sorting determinant of barley aleurain (AGFP6) appeared to be targeted to the central vacuole or restricted to small compartments and BFA had no effect of these two patterns even at highest doses which disrupted completely the Golgi apparatus. BFA can probably block vesicular transport in at least two sites, between the ER and the Golgi, and between the Golgi and the TGN. BFA inhibition of vesicular transport "upstream" from the Golgi leads to inhibition of secretion and to the disintegration of Golgi stacks, whereas inhibition of the "downstream" site causes inhibition of secretion without Golgi breakdown. Differential sensitivity of GFP fusion proteins to this drug could

represent the occasion to further elucidate proteins sorting in the secretory pathway and especially the role of the Golgi.

Introduction

Vacuolar proteins can have three different kinds of vacuolar sorting determinants (VSDs). These determinants have been shown to be necessary to target proteins to the different types of vacuoles that can coexist in a single plant cell. Very little is known about mechanisms of sorting and the intermediate compartments involved. In mammalian and yeast cells clathrin coated vesicles (CCVs) are known to function in traffic from Golgi to the lysosome/vacuole. In analogy, a receptor for the ssVSD (see general introduction) has also been identified in CCVs in plants. From CCVs from developing pea (*Pisum sativum*) a single ~80kDa protein was purified by affinity binding to the propeptide of proaleurain and was named BP-80 (Kirsch *et al.*, 1994). Partial peptide sequence allowed the isolation of the cDNA from pea and from other species. This protein family was named VSR (vacuolar sorting receptors) (Paris *et al.*, 1997). By electron microscopy (EM) it was possible to see CCV assembly in the TGN (Satiat-Jeunemaitre and Hawes, 1993) but it is not yet clear where the interaction of the VSR with the VSD takes place. Much less is known about the sorting system for proteins with ctVSDs. They need an accessible C-terminus to be recognised by their sorting system (Neuhaus *et al.*, 1994). Despite many attempts, it was not possible yet to identify a receptor in any of the intermediate compartments (Golgi, CCVs, and dense vesicles) supposed to be involved in the sorting. Dense vesicles containing storage proteins were seen forming from the Golgi stacks by EM, they were shown to accumulate proteins sorted according to the third type of VSD, the structure-dependent VSD of seed storage proteins, which causes aggregation of proteins after some maturation steps occurred in the Golgi (Robinson *et al.*, 1998a).
Drugs affecting the membrane organisation or membrane proteins interactions are convenient tools to investigate the molecular aspects of the process. It has been demonstrated that phosphatidyl inositol (PI) 3-kinase activity is involved in vesicular transport in yeast and animals (Hong and Verma, 1994; Schu *et al.*, 1993). In plant cells (tobacco BY2) treated with 33 μM wortmannin, a PI-3-kinase inhibitor, sorting of proteins with ctVSDs was severely affected. Almost all the newly synthesised reporter protein was secreted. No effect was recorded at this dose on sorting of proteins with ssVSDs (Matsuoka *et al.*, 1995). Another drug was found to affect vesicle formation: Brefeldin A (BFA) is a lipophilic fungal toxin that has become a major tool of cell biologists interested in studying vesicle-mediated trafficking in animal and plant cells (Staehelin *et al.*, 1997; Chardin *et*

al., 1999). In plants as in animal cells, BFA exerts its primary effects through perturbations of vesicular transport at different levels of the secretory pathway. Differential sensitivity of different steps of the secretory pathway to this drug could allow the dissection of the pathway in a reversible way.

Low BFA doses (2.5-10 μg/ml) both blocked secretion and altered glycosylation patterns of glycoproteins and complex polysaccharides in sycamore maple but did not cause any breakdown of Golgi stacks cells. At 50 μg/ml BFA however the Golgi stacks disintegrated (Driouich *et al.*, 1993; Staehelin and Driouich, 1997). These two types of BFA responses have been confirmed in other plant systems and should be viewed as part of continuum of BFA effect. BFA can probably block vesicular transport in at least two sites, between the ER and the Golgi, and between the Golgi and the TGN (Staehelin and Driouich, 1997).

This gradual effect of BFA has been explained in animals cells by the finding that this drug affects some of the proteins that activate different ADP-ribosylation factors (ARFs), inhibiting the guanine nucleotide exchange of this small GTP-binding proteins (see general introduction). It has been shown that mammalian cells contain at least three classes of ARF exchange factors with high, moderate or low sensitivity to BFA (Chardin and McCormick, 1999).

It is known that ARFs regulate the membrane association of coat proteins involved in intracellular membrane trafficking. ARFs control the membrane association of clathrin coat adapters AP-1, AP-3 and the nonclathrin coat COPI, whereas a similar protein, Sar1p, controls the membrane association of nonclathrin coat COPII (Ooi *et al.*, 1998). ARFs could be a molecular switch that acts as a transducer of diverse signals influencing several sites of coat assembly (Ooi *et al.*, 1998), which can thus be affected by BFA. With the use of BFA on our model system in tobacco protoplasts we aim to determine more precisely the transport pathways for proteins with ssVSDs or ctVSDs.

Results

Transport of two differently targeted vacuolar GFPs follows different kinetics.

To investigate the processing of our reporter proteins I extracted from protoplasts the total soluble proteins and I probed these proteins with specific antibodies against GFP. The different GFP fusion proteins can be distinguished by Western blotting according to their size (Figure 1a). Secreted GFP (SGFP5) was the smallest. As expected its size was the same as cytosolic GFP since signal sequence processing happens cotranslationally in the ER and it was thus not possible to observe

accumulation of immature SGFP5. The GFP retained in the ER (SGFP5K), was slightly larger than SGFP5 because of its 6 additional C-terminal amino acids. For the vacuolar isoform SGFP5T, the difference between immature and mature protein was clearly visible and both forms were found after 24 h transient expression. The precursor is longer by 7 amino acids than the secreted isoform and presumably also than the mature vacuolar isoform. The other vacuolar GFP (AGFP6) was found to have a much larger size than the other isoforms as expected for its precursor VSD form, because it includes not only the 15 aa containing the N-terminal vacuolar sorting determinant but also further 143 aa of the propeptide of aleurain that we think to be necessary for the correct exposition of the sorting determinant (Coulombe *et al.*, 1996). Like the other GFPs with sorting determinants, AGFP6 was expected to be processed but only one protein form was observed by western blot. To visualise the maturation process of the two vacuolar GFPs, and in particular of AGFP6, it was necessary to perform pulse-chase experiments. Only when the AGFP6 was labelled by ^{35}S, was it possible to visualise a maturation step from a high MW form to the form observed by western blotting within the first hour after synthesis (Figure 1b). Processing was very fast, as described for aleurain from which the propeptide came (Holwerda and Rogers, 1990). SGFP5T showed a very slow maturation: some of the protein synthesised in the one hour pulse, was still unprocessed 24 hours later (Figure 1c). Thus, while AGFP6 was processed in less than three hours, it took six hours for SGFP5T to be processed to 50%.
All signal and targeting peptides fused to GFP have been recognised and processed as expected with the only exception of the AGFP6 N-terminus. The precursor form of AGFP6 had an apparent size of 42 KD, while the mature of 41 KD. The decrease in size may correspond to the removal of the first 15 aa of the propeptide which correspond to the VSD (Figure 1a). We had found in preliminary experiments that the whole prosegment included in AGFP6 was necessary for the visualisation of this vacuolar GFP. AGFP versions with shortened prosegment were not visible. We are now investigating if the prosegment is necessary to the sorting (Coulombe *et al.*, 1996) or folding of GFP.
These observations of the kinetics of GFP processing, confirmed the differences described in the previous chapter: SGFP5T spend in the ER a much longer time than AGFP6. Indeed AGFP6 was only visible in the ER if sorting was slowed down by incubation of the cells in the cold (14°C). No differences were observed neither in size nor in kinetics between GFP5 and GFP6 versions of the same chimeric construct.

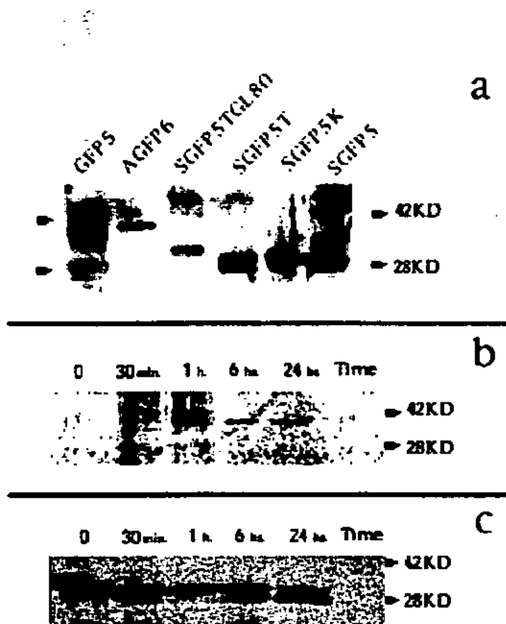


FIGURE 1: Immunological comparison of the differently targeted GFPs. a) Western blot analysis of GFP in protoplast extracts after 24 h expression. The cytosolic GFP5 (first lane) and the secreted SGFP5 (last lane) have an apparent size of 28 kD; the vacuolar AGFP6 has an apparent size of 41 kD; SGFP5K, retained in the ER, has a slightly larger size than the secreted form due to the additional 6 C-terminal amino-acids; for SGFP5T two forms are visible, the precursor with seven aa more than the secreted form, an other one reduced to the same size of secreted form, and the mature form, processed to the size of the latter; the SGFP5TGL80 contains a glycosylation site and is glycosylated, as indicated by the higher apparent size. b) Pulse-chase analysis of the processing of AGFP6: a 2 hours pulse was followed by a chase for the indicated time. 30 min after the start of the chase, AGFP6 has still its N-terminal VSD, after 1 hour around 50% of the protein has been processed, after 6 hours only the mature form is detectable. c) Pulse-chase analysis of the processing of SGFP5T: 1 hour pulse was followed by a chase for the indicated time. 24 hours after the star of chase, a part of the protein produced during the pulse is still unprocessed.

Lack of competition of two proteins in the sorting system for ctVSDs.
AGFP6 sorting was characteristic of a vacuolar protein specifically recognised by its receptor, we tried to test the kind of interactions responsible of SGFP5T sorting. We co-transformed protoplasts with a constant quantity of plasmid RGUSD15+T (see chapter 3), encoding the easily measurable vacuolar RGUS, and different quantities of plasmid SGFP5T. We tried to saturate the sorting machinery with SGFP5T to observe a reduction of RGUS activity in the vacuole and an increase of RGUS activity in the extracellular space, due to missorting. RGUSD15+T was sorted to the vacuole with the same efficiency, both in presence of carrier DNA and of SGFP5T. The amount of SGFP5T could unfortunately not be determined and was evaluated only on the basis of the amount of plasmid DNA used. 10μg plasmid DNA coding for RGUSD15+T resulted in the same GUS activity in cotransformation with 50μg carrier DNA as well as with up to 50μg plasmid DNA coding for SGFP5T.

Competition of the two sorting systems for the same protein.
To compare the receptor-mediated sorting of AGFP6 with the sorting of SGFP5T, we produced a GFP reporter fused to both N-terminal and C-terminal VSDs (AGFP6T). The result showed a complete prevalence of ssVSD. AGFP6T was not distinguishable from AGFP6, both in terms of kinetics and of final localisation.
This result indicates either dominance of the sorting system based on ssVSD if both sorting events occur in the same compartment, or the occurrence of this sorting event at an earlier step of the secretory pathway.

Effects of brefeldin A on protein sorting.
Comparison between the two sorting machineries was also possible with drugs affecting different steps of the secretory pathway.
We used BFA, a drug affecting the formation of different coated vesicles. A BFA concentration known to be sufficient to affect steps both up-stream and down-stream of the Golgi apparatus was first tested in our transient expression system. Protoplasts were then fixed and immunolabeled with JIM84 antibody, known to label the Golgi. We confirmed that 50μg/ml of BFA induced the complete disorganisation of Golgi (Figure 2).

We then treated tobacco protoplasts expressing transiently the reporter proteins with 3 differant BFA concentration: 10, 50 and 100 μg/ml.
SGFP5T was not sorted any more in the way described in the previous chapters but accumulated in small compartments characteristically induced by BFA (Figure 3). The lower dose was just as effective as higher doses but its effect was more easily reversed after cell washing with drug free medium.
Sorting of AGFP6 was not affected by the drug, up to 100μg/ml (Figure 4a). Only in very rare occasions did some cells show a complete reorganisation of endomembranes and a redistribution of AGFP6 in the ER. BFA reduced the maturation of SGFP5T proform already at low doses, while in contrast it had no visible effect on AGFP6 maturation (Figure 5).

FIGURE 2: *Immunolabeling of the Golgi compartment of tobacco* protoplasts with JIM84 antibodies.
a) In control cells the antibodies reveal small structures proposed to be the Golgi stacks; b) in cells treated with 50 μg/ml of BFA, Golgi structures are no more detectable.

FIGURE 3: SGFP5T fluorescence in a cell treated with 50 μg/ml BFA (a) compared to a non-treated control cell (b).

FIGURE 4: AGFP6 fluorescence in a cell treated with 50 μg/ml BFA (a) compared to a non-treated control cell (b).

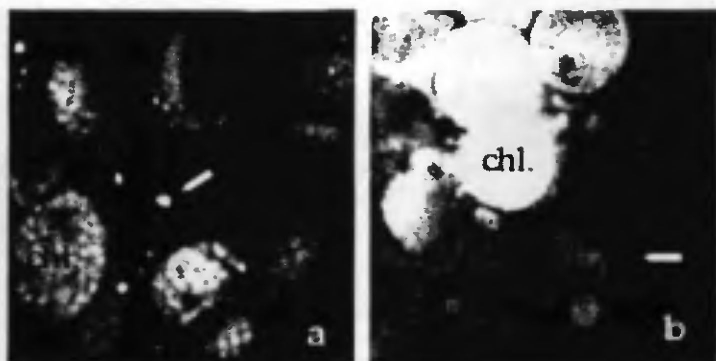


FIGURE 2

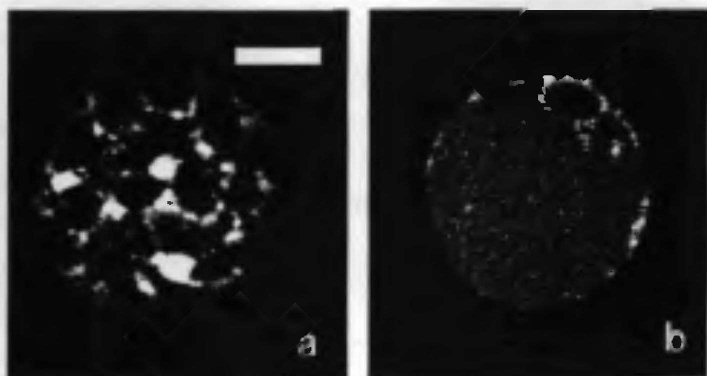


FIGURE 3

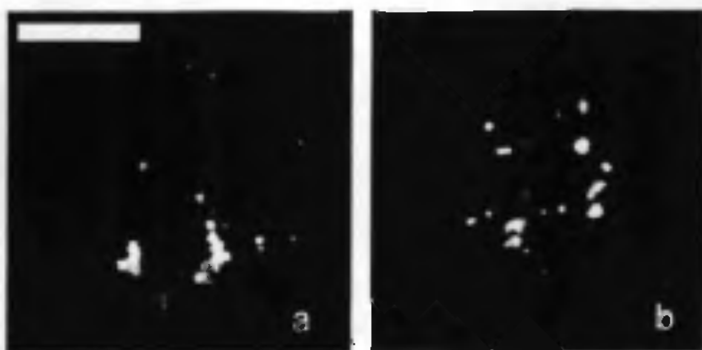


FIGURE 4

Production of glycosylated GFPs as reporters of processing events in the Golgi

While the signal peptides of our reporter GFPs were processed during the synthesis in the ER, the VSDs were probably processed after arriving in the vacuole. To investigate GFP transit through the Golgi, we introduced N-glycosylation sites in different positions of the GFP sequence. N-glycans are added to proteins in the ER and processed to a high-mannose form (6-9 Man). High mannose glycans can be modified in the Golgi to complex glycans, which are resistant to endoglycosydase H (Endo H). Endo H resistance is thus a marker of passage of a glycoprotein through the Golgi. Endo H sensitivity however is less informative, as not all N-glycans are modified in the Golgi (Lerouge *et al.*, 1998), unless the same protein targeted to a different compartment can be found to became resistant.

We expected that a glycan added in the ER would be then modified when (if) the GFP would transit through the Golgi (Figure 6).

GFP has a barrel structure formed by a ß-pleated sheet. Both termini are on one side of the barrel. We prepared GFP variant with a glycosylation site exposed on the same side (GL80) and another GFP with a glycosylation site on the opposite side of the barrel (GL133) (Figure 7). The GL80 and GL133 sites were glycosylated (GL133 only partly) when inserted in SGFP5T but not glycosylated when inserted in AGFP6. It is possible that the GL80 site was not accessible for modifying enzymes in the presence of the large N-terminal prosegment of AGFP6, but I have no explanation for the absence of glycosylation from site GL133. Only if it was possible to produce a glycosylated AGFP6, it could be possible to observe if this protein transits through the Golgi.

As a test of glycan modification in SGFP5TGL80, I tested the resistance of glycosylation GL80 to the Endo H and I found it to be always sensitive (Figure 8). To become resistant, GL80 would have to come in contact with enzymes located in the Golgi, but we cannot decide whether GL80 was simply not accessible for these enzymes, or whether SGFP6TGL80 was not transiting through the Golgi. To complete the investigation, other glycosylation sites have to be tested and one of them has already been made (GL172).

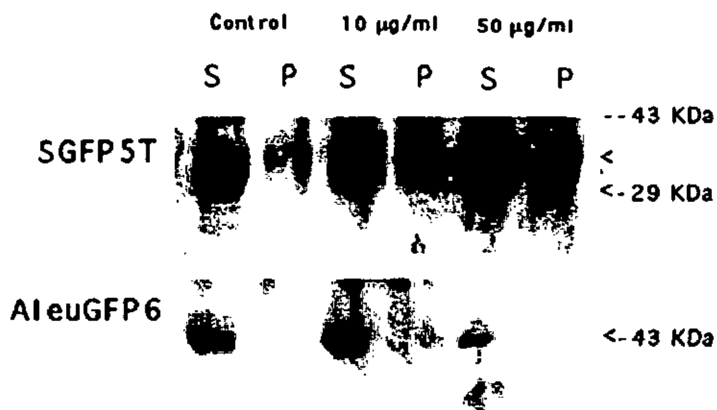


FIGURE 5: Western blot of the soluble content (S) or the microsomal fraction (P) of cells treated with different doses of BFA.

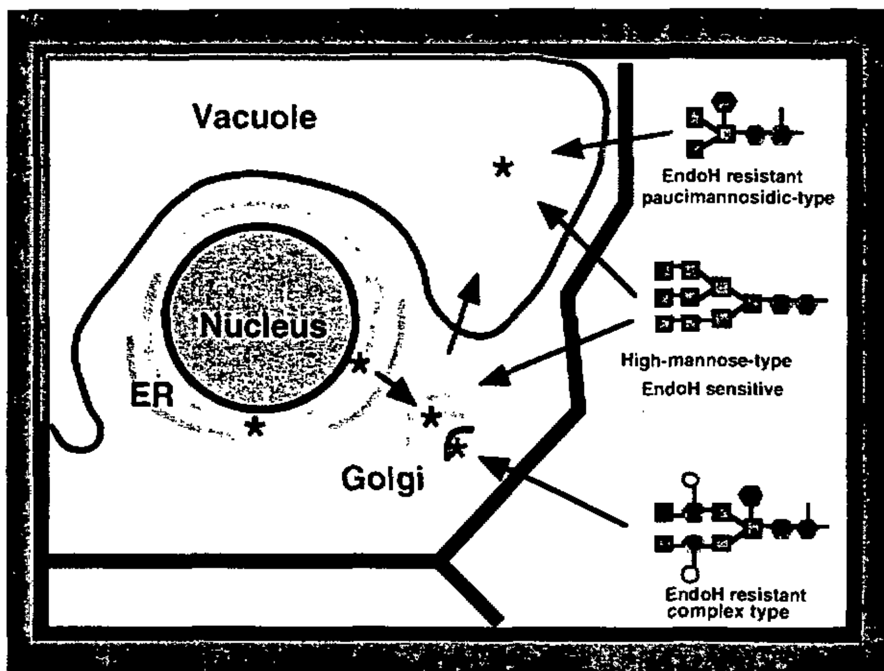


FIGURE 6: Glycosylation can be used as an investigation tool. The N-glycans can be modified in the Golgi and later in the vacuole, becoming resistant to the endoglycosydase H. This modification can be used as a test of transit through the Golgi.

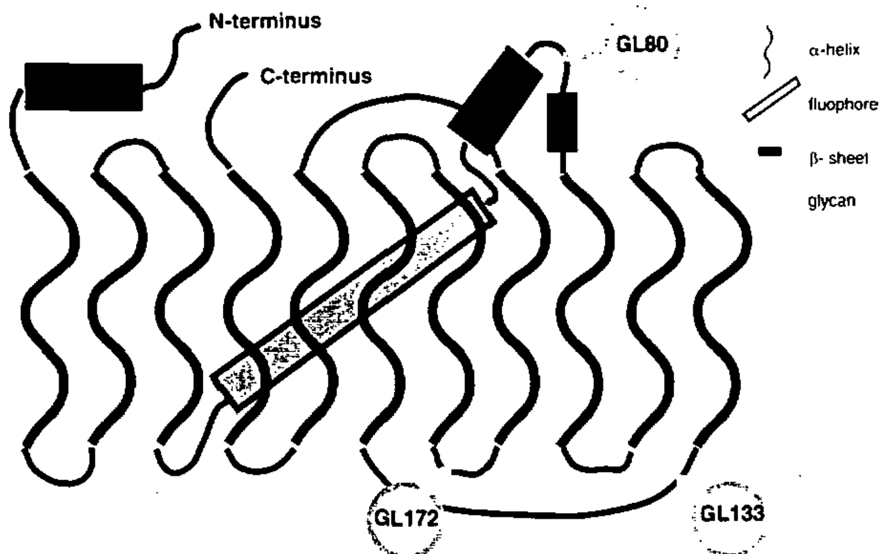


FIGURE 7: The glycosylation sites tested in different GFP constructs are located at 3 alternative positions: aa 80, 133 or 172.

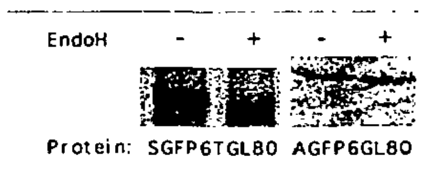


FIGURE 8: EndoH resistance test on glycosylated GFPs. The N-glycan of SGFP6TGL80 glycan is removed by EndoH, therefore it has not been modified; AGFP6GL80 has not been glycosylated at all.

Discussion

In this chapter I completed the characterisation of the different GFPs used in this thesis, confirming the existence of two separate sorting machineries, with different processing kinetics. I identified in BFA an important tool to differentiate the two classes of VSDs and I could propose experiments for further investigations. A large range of alternative explanations is still possible for the mode of action of the two VSDs, especially of the ctVSD.

It was so far impossible to identify a sorting receptor for ctVSDs. It is probably due to the low sequence specificity necessary for the interaction but it could also be due to a receptor-free sorting, mediated by aggregation as hypothesised for psVSD (see general introduction). Aggregation has already been proposed as a non-receptor mediated sorting mechanism (Vitale and Chrispeels, 1992) as in animal cells. In legumes, oligomerization is an important determinant of transport (Vitale *et al.*, 1995). The preliminary experiment I performed to test the possibility to saturate the targeting machinery for ctVSD was not sufficient to give supplementary informations. I verified the difficulties to saturate this system but this difficulty was expected from a receptor with low specificity for the ligand, able to sort many different proteins. In addition, a tetrameric protein like RGUS with four available VSDs may be hard to compete with a monomeric protein like SGFP5T.

The sorting system for ssVSDs is clearly distinct from the system for ctVSD. It is known that ssVSD-carrying proteins are exported from the Golgi apparatus to lytic vacuoles by means of clathrin-coated vesicles. The surprising evidence found until now suggests the need of revisiting Golgi function because its integrity was not necessary for this sorting pathway. In presence of BFA concentrations known to affect the Golgi structure, we observed no alterations in AGFP6 pattern and no change in its maturation.

To answer this question I think we found a promising tool with glycosylation but it will be necessary to produce a glycosylated AGFP6. If a glycosylated AGFP6 is not modified, it will be necessary to induce the modification artificially, to prove that, in presence of the necessary enzymes, it can happen. Indeed we already planned a possible strategy: modification of the glycans could be induced by bringing Golgi enzymes into the ER with high concentrations of BFA.

The current hypothesis to explain the different responses of plants to BFA is the existence of multiple BFA-binding sites at different levels of the secretory pathway. So far we don't know if there is a class of transport vesicles independent from ARFs, which can be formed independently from the correct structure of the Golgi apparatus. To

justify the observation of normal sorting patterns to the lytic vacuole for AGFP6 even in BFA-treated cells, we hypothesise that transport vesicles could be formed on the ER itself, where the Golgi is re-sorbed. Moreover it is important to consider that one of the possibilities explaining the dominant effect of ssVSD on ctVSD is that the most important sorting step for ssVSD proteins happens already in the ER even in normal conditions. It is possible that ARF-independent vesicles forming normally at the Golgi *trans* side, could form elsewhere whenever the concentration of its components is sufficient. In our reporter protein we don't observe alteration of the maturation process because there are no visible maturation steps occurring in the Golgi. To investigate the role of Golgi we have to use a reporter protein that will be modified in the Golgi. A protein with modifiable glycans or sites for Kex-2-like Golgi proteases could possibly show an alteration of these steps without mistargeting. In the case of barley aleurain, it was shown that the proteolytic processing was inhibited by BFA in barley aleurone cells and in tobacco protoplasts (Holwerda and Rogers, 1990). This processing step was supposed to happen in a post-Golgi location but no clear informations indicate any involvment of this step in correct targeting. As reported above I did recover the correct proteolytic cleavage of the aleurain propeptide in AGFP6 expressed in tobacco. I *will discuss these results in the next chapter, where I will show that* processing is different in *Arabidopsis* than in tobacco.
The relations between Golgi structure and vesiculation demand further investigation and in our experimental results we see an invitation to revise the role of the Golgi.

Experimental procedures

Protoplasts preparation

Protoplasts were prepared and transformed as described in chapter 2. Brefeldin A was supplemented to the culture medium immediately after or up to 2 hours after transformation. Reversibility of the treatment was tested harvesting protoplasts after *incubation in presence of BFA for different times and resuspending them in fresh medium for* six hours.

Protein extraction

Plant tissue samples (<0.2 g) were reduced to powder in liquid nitrogen and incubated at 100°C for 30 min in the extraction buffer (TBS 1% and SDS 2%). Insoluble residues were eliminated by centrifugation for 5 min at 14000 g. Total soluble proteins were precipitated by 10% TCA and washed with acetone.

Protoplasts were harvested and resuspended in the extraction buffer (TBS 1% and SDS 2%). Cells were lysed by 3 consecutive freezing-thawing cycles. To separate vacuolar content from most of the microsomal fraction, cells residues were separated by extract from soluble fraction after just one freezing.

Western blotting

The proteins were separated in polyacrylamide gels with SDS (4% stacking gel, 15% separation gel) using the protocol described by Laemmli et al. (1972), in the minigel system "Mini-Protean II Dual Slab Gel System" from Biorad. 200 V were applied and an electrophoresis buffer (25mM Tris; 192 mM Glycine; 0.1% SDS) at pH 8.3 was used.

The protein separated on SDS-gel were electrophoretically transferred on a nitro-cellulose membrane (BA83; 0.2µm; Schleicher and Schuell), it was used a "semi-dry" apparatus (Sartoblot II Sartorius) (Towbin *et al.*, 1979).

Immunolabeling

The western blot was incubated in 100 ml milk-TBS 5% (20 mM Tris-Cl pH7.5; 500 mM NaCl; 5% w/v milk powder) to saturate the nitro-cellulose membrane with proteins for 45 min.

GFP detection was obtained using anti GFP (Molecular Probes A6455) primary antibodies and anti-rabbit secondary antibodies coupled to lissamine rhodamine.

PULSE-CHASE labelling of GFP in the transient expression system.

To perform pulse-chase experiments protoplasts were transformed as usual, except that the amount was double. PRO-MIX (70% L-[^{35}S] methionine and 30% [^{35}S] cysteine; Amersham, UK) cell labelling mix (3 Mbq/ ml) was added 2 h after transformation and chased with 1 mM cold methionine and 0.5 mM cold cysteine.

A 1 hours pulse was sufficient for SGFP5T but not for AGFP6, for which a two hours pulse was necessary. Labelled proteins were immunoprecipitated as described in chapter 6.

Chapter 5

TOBACCO AND ARABIDOPSIS DIFFER IN THE ORGANISATION OF THEIR VACUOLAR SYSTEMS.

This chapter collects the preliminary results on transgenic plants expressing desired reporter GFPs and anticipates further developments on the subject of this thesis.

Summary

Green fluorescent protein (GFP) allows the direct visualisation of gene expression and subcellular localisation of fusion proteins, in living cells. The localisation of different GFP fusion proteins in the secretory system was studied in stably transformed plants.

In this chapter I present some of the preliminary data obtained from the observation of transgenic tobacco and arabidopsis plants. I want to focus the reader's attention on the evident differences in vacuole organisation in tobacco and arabidopsis transformants.

The GFP fusion proteins label the vacuolar complexes of the transgenic arabidopsis and make these plants suitable for mutagenesis and gene-tagging experiments.

Introduction

Reporter proteins for gene expression in plants, like ß-glucuronidase (see annexe to chapter 2) or luciferase, can be identified histochemically in all tissues but they require generally a destructive test or the presence of cofactors. The necessity to destroy the sample for analysis or to use other invasive techniques makes the testing of primary transformants more difficult, makes it difficult to follow the time course of gene expression or of protein sorting in living plants and slows down the screening of segregating populations of seedlings. The green fluorescent protein (GFP) allows the direct visualisation of gene expression and also of the subcellular localisation of fusion proteins in *living cells*, without the need for destructive techniques, as required by ß-glucuronidase, or addition of cofactors as required for the luciferase assay (Haseloff *et al.*, 1997).

GFP has been successfully expressed in protoplasts for the study of the secretory system *in vivo* (chapters 2 and 3) and the study of GFP fusion proteins in different plant systems is on the way in our laboratory since a couple of years. The distinct locations of different GFP fusion proteins in the secretory system of stably transformed plants confirmed the complexity of this membrane system.

In this chapter I present some preliminary data obtained from the observation of transgenic tobacco and arabidopsis. I want to focus the reader's attention not so much on the characterisation of the GFP accumulation in these plants, which is far from being complete, but on the evident differences emerging from this preliminary examination. It seems that lytic and storage vacuoles are differently distributed in different tissues of the same plant and in tissues of different plant species.

GFP accumulation in transgenic arabidopsis makes this plant suitable for the research of sorting mutations and of the responsible genes, using the gene tagging approach. Gene tagging is a technique by which transposons or introduced DNA with a known sequence insert into a desired gene. The inserted foreign DNA both mutates the gene and serves as a vehicle for the isolation of the flanking plant DNA. In *Arabidopsis*, the T-DNA (transfer DNA) of *Agrobacterium tumefaciens* has been used as an insertional mutagen, and several genes have been isolated and characterised this way(Richardson *et al.*, 1998). *Arabidopsis* has many attributes that make it a useful organism for gene-tagging studies: it is a small, rapid cycling, self-fertilising member of the Brassicaceae family (Meyerowitz and Pruitt, 1985); it has a very small genome (estimated to be 70.000 kb) with little repetitive DNA (Leutwiler *et al.*, 1984). The complete genomic sequence will be known in a year or two, but a large part is already available.

It has generally been recognised that the tissue culture process induces e high frequency of somaclonal variants. The whole-plant transformation method originally developed by Feldman and Marks (Feldmann and Marks, 1987) circumvents potential problems related to tissue culture-based transformation methods.

With the recent protocols of *in planta* infiltration (Bechtold *et al.*, 1993; Richardson *et al.*, 1998) it is now feasible to tag all of the genes in the *Arabidopsis* genome. For example, Feldmann et al. (1991) calculated that a collection of 50'000 T-DNA transformants would give a 95% probability of having an insert in an average *Arabidopsis* gene.

The main problem nowadays is the possibility to identify the mutants. For the targeting to the vacuoles, mutations may either prevent normal plant development by disturbing the regulated changes in cell volume or in contrary they may cause no visible phenotype at all if the mistargeted proteins are not essential. It is the use of model plants in which a visible reporter protein (GFP) is expressed that makes possible the screening for otherwise undetectable mutations.

Results

Production of transgenic tobacco plants and GFPs detection

The aim of this work was to produce a few clones expressing the desired transgenes to compare the localisation of GFP in different tissues.

Nicotiana tabacum (cv SR1) plants were transformed by a rapid and efficient protocol (Fisher and Guiltinan, 1995) using a pBIN-derived vector (Haseloff *et al.*, 1997) containing the selection gene NPTII that confers resistance to kanamycin, in the *Agrobacterium tumefaciens* strain GV3101.

In the context of this work tobacco was transformed with four different GFP constructs. We used a pBIN derivative encoding a secreted GFP (pBIN-SGFP5), a GFP retained in the ER (pBIN-SGFP5K), a vacuolar GFP targeted to PSV (pBIN-SGFP5T) and a vacuolar GFP targeted to the LV (pBIN-AGFP6). We observed a very low regeneration rate "per callus", in other words not all independent calli regenerated plants. The regeneration rate appeared dramatically low especially for SGFP5. After this problem of shoot regeneration, we then faced problems in GFP detection. While it was somehow expected that SGFP5 might not be detectable because it has been reported to be unstable in the extracellular space, we were surprised by the total absence of fluorescence in plants transformed with AGFP6.

SGFP5T showed an interesting but also surprising fluorescence distribution: in fact small fluorescent compartments appeared only in

few cell types. One small fluorescent compartment was visible in each *guard cell of stomata (Figure 1a), while a variable number* of small compartments were visible in elongating root cells, epidermis cells at the base of trichomes or other cells from young elongating tissues like hypocotyl (Figure 1b). Plants transformed with SGFP5K showed only a faint fluorescence labelling, essentially the ER.

Expression level and accumulation of GFP in transgenic tobacco plants.
The GFP coding sequence used for our transformations was already plant-adapted (Haseloff *et al.*, 1997) so that the absence of fluorescence had to be due either to a very low expression or to GFP degradation. The calli generated from non fluorescent plants were *fluorescent, both for SGFP5T* and SGFP5K *(Figure 2)*. We tested GFP expression in different plants by RNA blotting (northern blotting), using total RNA extracted from leaves and from calli derived from leaves of the same plants. A comparable GFP mRNA expression was detected in both tissues (Figure 3: two clones with SGFP5T and one with SGFP5K; see also Figure 6: one clone with SGFP5T and one with AGFP6). We confirmed that there was no silencing of the transgene in transformed plants where no fluorescence was visible.
Protoplasts prepared from plants expressing SGFP5T or SGFP5K showed a rapid increase of fluorescence, following the same pattern of GFP accumulation as described for transient expression. Stably expressed *SGFP5K labelled the large vacuoles of these protoplasts more* frequently than in control experiments of transient expression. AGFP6 was no more detectable in protoplasts than in leaves. Considering that this GFP form was detected at a lower level than the other constructs and was more rapidly degraded than the others in transiently transformed protoplasts, it may just be produced at a level too low to allow detection. I have not yet verified other possibilities to explain why these plants did not accumulate AGFP6. I have no proof yet that the gene was not disrupted during insertion.
When SGFP5T was extracted from protoplasts derived from transformed plants it showed a higher proportion of mature protein, than observed in the proteins extracted from leaves (Figure 4). This observation confirms that protoplast production changes the sorting and processing machinery of the differentiated mesophyll cell.

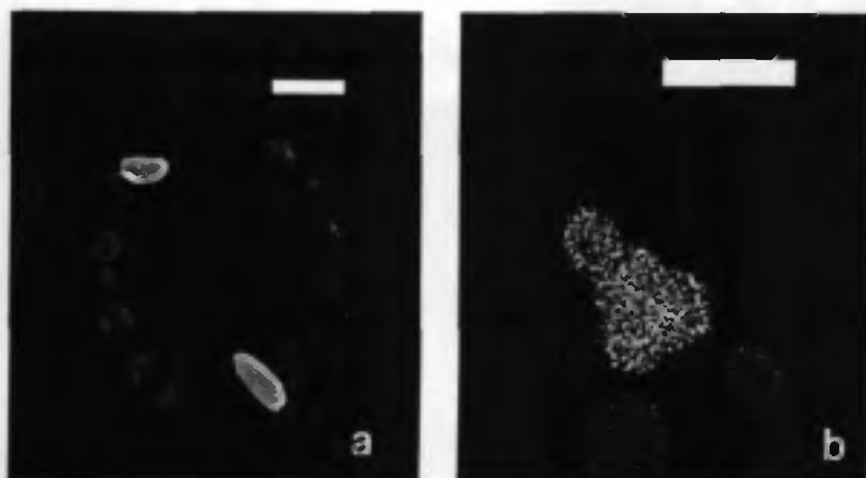


FIGURE 1: SGFP5T accumulation in transgenic tobacco: **a)** stomata; **b)** detail of a small compartment in a cell of the hypocotyle, the same rare compartments are observed in other tissues. Bar= 20µm.

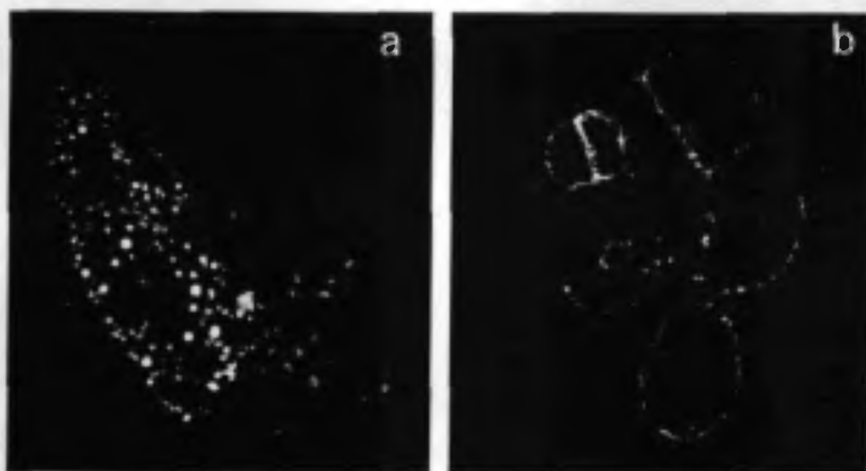


FIGURE 2: Regenerating calli from SGFP5T and SGFP5K transformed tissues. **a)** SGFP5T; **b)** SGFP5K.

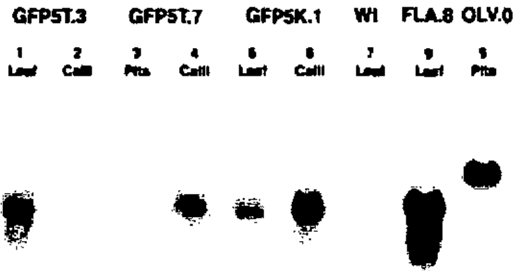


FIGURE 3: Northern blot of total RNA from transgenic tobaccos. For two independent clones transformed with SGFP5T and one with SGFP5T, RNA was extracted from leaves and calli. In the blot there are as well three controls, one negative with a wt SR1 tobacco, two transformants *containing* GFPFLA (*FLA8*) and OLVGFP5 (*OLV0*) (*Grieco, 1999*) *that show* the GFP fluorescence.

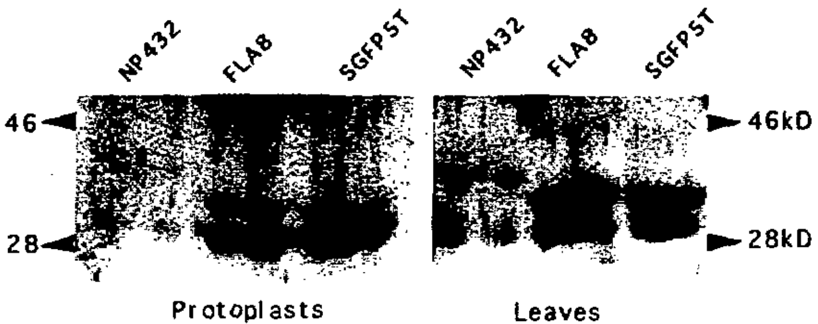


FIGURE 4: *Western blot of different GFP forms.* NP432 and FLA8 are used as controls; SGFP5T is visible in the mature form and in its proform. The proform is more abundant in leaves, the mature form is more abundants in protoplasts.

GFP accumulation patterns in transgenic Arabidopsis plants

Transgenic arabidopsis plants were produced following a standard method of *in planta* infiltration (Bechtold *et al.*, 1993) in collaboration with R. Flückiger. The characterisation and comparison of these plants is the subject of R. Flückiger's diploma thesis (Flückiger, 1999). Contrary to tobacco, transgenic arabidopsis showed a strong fluorescence in most tissues.

We obtained plants expressing the secreted version of the GFP reporter SGFP5 (see chapter 2 and 4) and two vacuolar constructs, SGFP5T (chapter 2, 3 and 4) and AGFP6 (chapter 3 and 4). Homozygous plants expressing these fusion proteins are now being characterised in more detail by R. Flückiger by the simple observation with a low magnification fluorescent microscope. They can be clearly distinguished.

The secreted SGFP5 is characterised by the accumulation of GFP in the intercellular space of all tissues and does not show strong GFP accumulation in young tissues. Two tissues, in which it is easy to identify this secreted GFP are the root (Figure 5a, b) and the leaf epidermis (Figure 7a). In such tissues sample preparation needs no excessive manipulation and differences with vacuolar constructs are evident.

The (storage) vacuolar SGFP5T shows in most tissues an accumulation in small vacuolar compartments, while only fully expanded leaves and stomata exhibit large fluorescent vacuoles. As for the secreted GFP, this fusion protein has a clear and characteristic pattern in the root (Figure 5c, d) and in the leaf epidermis (Figure 7b): in both tissues SGFP5T is accumulated in small vacuolar compartments.

The (lytic) vacuolar AGFP6 is accumulated in large vacuoles in most tissues but it also shows an evolution from small compartments to large vacuoles. For example along the root it is possible to recognise the region of elongation, where AGFP6 is restricted to small vacuoles, whereas in mature roots the large central vacuole is fluorescent(Figure 6). Again, apart from roots, the leaf epidermis shows the most clear and accessible pattern which allows to recognise the differences with the other vacuolar GFP (SGFP5T) and the secreted SGFP5. AGFP6 is accumulated in the large vacuole of epidermis cell, but is not visible at all in guard cells (Figure 7c).

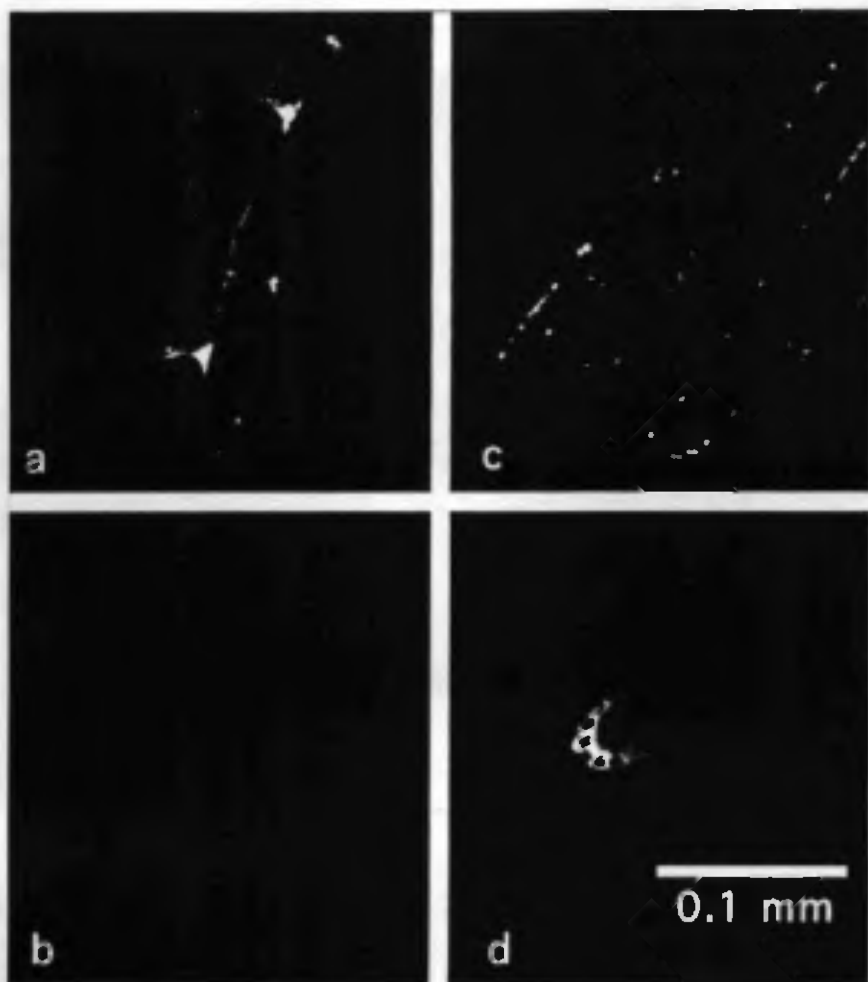


FIGURE 5: Fluorescent pattern in *Arabidopsis* roots of the secreted SGFP5 **(a and b)** and (storage) vacuolar SGFP5T (c **and d)** GFP distribution in mature roots **(a and c)** and root tips **(b and d)** of *Arabidopsis* transgenic plantlets.

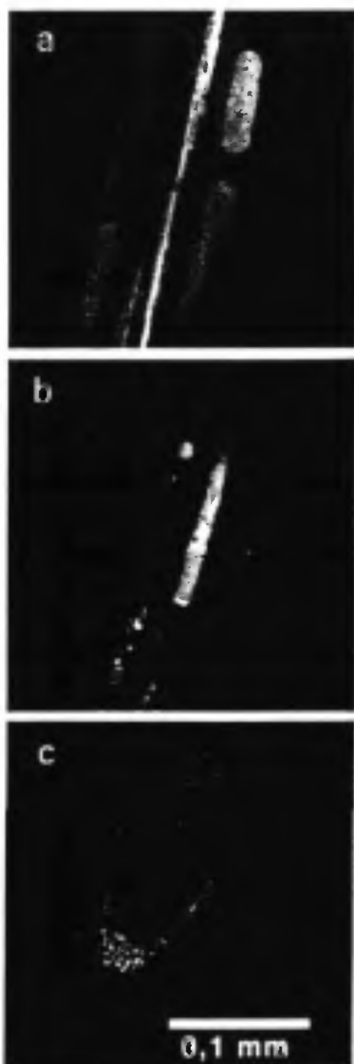


FIGURE 6: AGFP6 distribution in the root of a transgenic *Arabidopsis* plantlet. **a)** Mature root; **b)** elongating root; **c)** root tip.

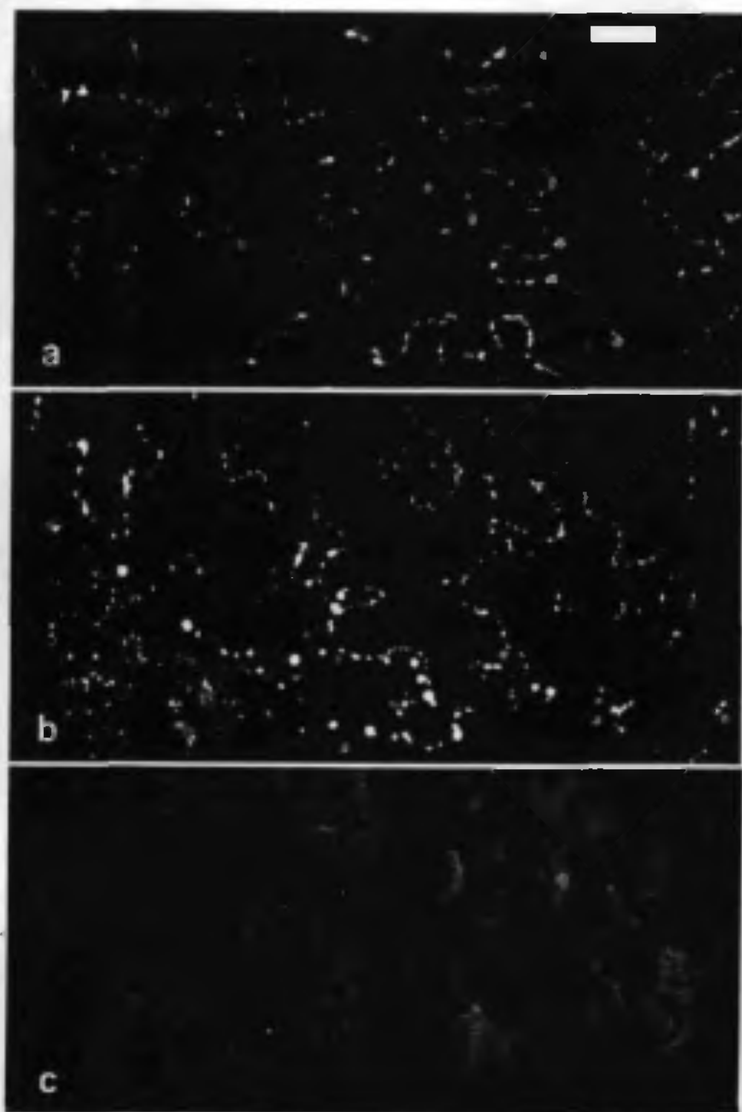


FIGURE 7: GFP fluorescence in the leaf epidermis of transgenic *Arabidopsis* plants. **a)** secreted SGFP5; **b)** vacuolar SGFP5T; **c)** vacuolar AGFP6.

Comparison of fluorescent patterns between tobacco and Arabidopsis
While GFP fluorescence was always visible in arabidopsis, in tobacco it was rarely detectable. At the RNA level, the GFP transgenes were clearly expressed both in arabidopsis as in tobacco plants but differences in the expression levels were detected (Figure 8). AGFP6 was not detectable in protein extracts from tobacco leaves but it was well detectable in extracts from arabidopsis (Figure 9). The AGFP6 detected in arabidopsis showed an apparent molecular weight close to that of the mature SGFP5T, indicating an essentially complete processing of the aleurain propeptide. Proteases present in the vacuole are expected to be responsible for this last processing step, characteristic of the final accumulating compartment. In tobacco protoplasts transiently expressing AGFP6, it was detected with a larger apparent size, indicating that most of the prosegment remained part of the mature GFP (see chapter 4).
A last example of the difference between the vacuolar compartment of tobacco and *Arabidopsis* emerged from this comparison: the clearly different distribution of SGFP5T in guard cells. In tobacco SGFP5T was accumulated in one small vacuole per guard cell while a diffuse fluorescence was visible in the whole arabidopsis guard cells (Figure 10).

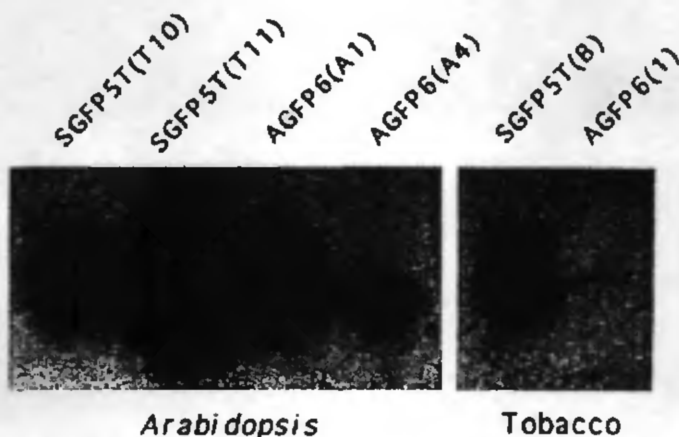


Figure 8: Northern blot of total RNA extracted from Arabidopsis and tobacco transgenic plants. With some variation of quantity; the tobacco clone SGFP5T(B) has a higher GFP expression than the Arabidopsis clone AGFP6(A4), but is not as fluorescent as A4.

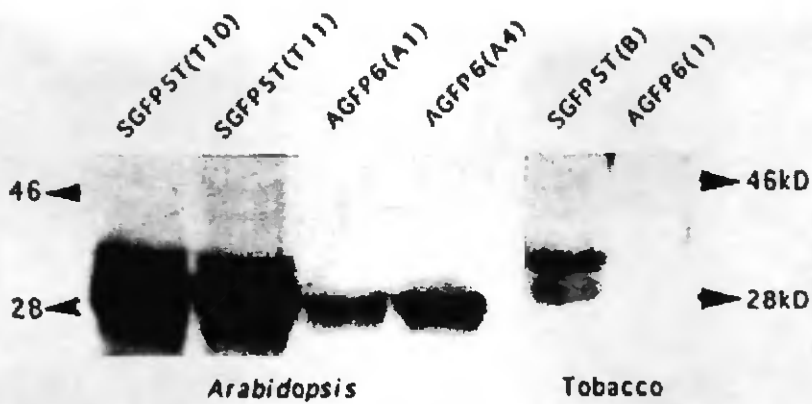


Figure 9: Western blot of proteins extracted from transgenic Arabidopsis and tobacco plants. Anti-GFP antiserum does not detect anything in the tobacco clone AGFP6(1), while in the Arabidopsis clones AGFP6(A1) and (A4) the detected protein has the same apparent size of the mature SGFP5T.

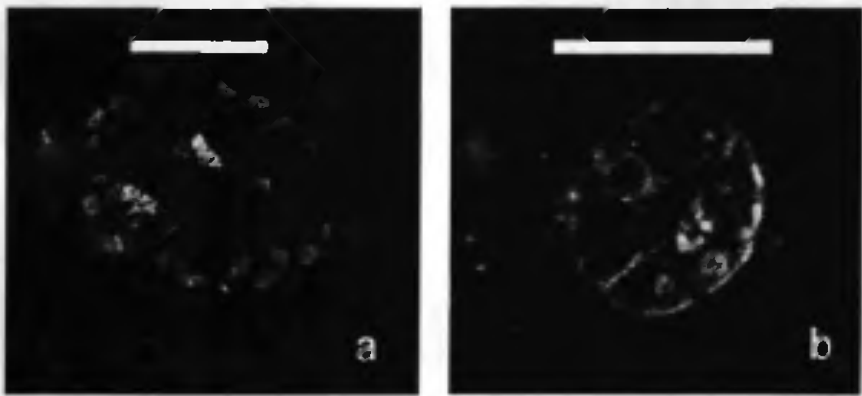


Figure 10: Stomata from tobacco **(a)** and Arabidopsis **(b)**.

Discussion

The identification of differences between two popular model plants can contribute to avoid incorrect interpretations as well as difficulties in conciliating results obtained in different laboratories on different plants. Here I present preliminary results indicating a different distribution of vacuolar soluble proteins in different plant species as well as in tissues of the same plant.

In transgenic tobacco plants AGFP6 is not detectable, while SGFP5T is accumulated only in few cells, the guard cells, some elongating root cells and rarely, the trichome's basal cell.

In transgenic *Arabidopsis* plants AGFP6 and SGFP5T are accumulated in some cells in distinct vacuolar compartments, while in other cells they are accumulated in a single central vacuole.

I propose that the tobacco and arabidopsis vacuolar complexes appear very different in GFP accumulation but differ in fact only in the proteolytic activity of the LV.

The lytic vacuole marker AGFP6 seems to be targeted generally to the vacuole with the most important volume. This marker is found in small compartment only in not fully expanded cells. In tobacco this vacuole must be so active in protein degradation that AGFP6 is too rapidly degraded to accumulate, while in contrary in arabidopsis this vacuole accumulates AGFP6 because it has a lower degradative activity. Cells in which only one vacuole is generated by the fusion of the PSV and the LV accumulate AGFP6 together with SGFP5T. This situation seems to be common to the majority of differentiated tissues but not to protoplasts. As the very degradative unique tobacco vacuole degrades both GFPs, we see SGFP5T only if the PSV remains separate from the LV.

In chapter 3 we saw that the LV seems to be responsible of re-establishing cell turgor. In miniprotoplasts this vacuole remains separate from the vacuoles containing SGFP5T at least until the cell reaches its full expansion. By analogy we can imagine that other cells in which the volume is changing also maintain separate vacuoles. Indeed in tobacco we see small PSV containing SGFP5T in elongating root cells and guard cells, cells that actively change their volume. Nevertheless not all expanding cells present this characteristic so that an absolute link cannot be established. Another possibility is that the presence of separate PSV and LV vacuoles is common to cells that recently divided and developed a new function. Again we find in this category the tobacco cells that show small vacuoles containing SGFP5T: elongating root cells, guard cells and basal cells of trichomes.

In arabidopsis we observe small vacuoles containing SGFP5T in all these cell types, as well as in many others. Guard cells represent an

exception: in fact, while SGFP5T is accumulated both in small compartments and in the large central vacuole, AGFP6 is not accumulated at all.

An argument to support the idea that the vacuolar complex in tobacco and arabidopsis is only apparently different is the presence in arabidopsis of a specific proteolytic activity not detectable in tobacco. If we consider the AGFP6 accumulation, we observed in arabidopsis the accumulation of a mature form we did not see in tobacco cells (protoplasts or plant tissues). This mature form must be due to a specific proteolytic activity in the vacuole. We postulate that in tobacco the vacuole has a much stronger proteolytic activity and degrade completely the GFP. To know if tobacco vacuoles are indeed more degradative than arabidopsis vacuoles, it will be necessary to measure their proteolytic activity (Hörtensteiner *et al.*, 1992).

The production of transgenic plants was necessary to study the vacuolar system in natural conditions and not only in artificial systems like protoplasts. There are not yet definitive results about the differences between the tobacco and arabidopsis vacuolar system. The characterisation of the two system appears now an interesting but unexpected task.

The arabidopsis transgenic plants, because of their lower proteolytic activity, represent a very good material for further investigations. We produced these transgenic plants to obtain a model plant in which the tagging of genes involved in the correct sorting of soluble proteins to the vacuoles could be easily identified. With these plants in hands we are now evaluating the best strategies for the screening of mutants.

Before using these plants to generate a mutant collection, a large quantity of work remains to be done. We have to precisely characterise the fluorescence pattern of each transformant and evaluate the practical possibilities of screening. Even if preliminary considerations suggest that the direct observation of mutants with a low magnification fluorescence microscope will allow us to recognise any alteration of GFP distribution, we have to consider the high investment (in time more than in money) of a mutagenesis and screening project.

These plants, once precisely characterised, can also give precious information about the role of vacuoles in different tissues. The distribution of large and small vacuoles (acidic and lytic or neutral and storage vacuoles) in different tissues will be the subject of additional studies.

Experimental procedures

Plant transformation

N.tabacum cultivar SR1 plants were germinated and grown in sterile conditions for 4 - 6 weeks on MS classic medium (Calbiochem) at 25°C under continuous light.

Agrobacterium tumefaciens (GV3101) was grown on solid LB containing 50mg/l Kanamycin for at least 2 days to select for the presence of the binary vector.

Pieces from 4 tobacco leaves (3-6 cm) were incubated for 10 minutes in a 50 ml Falcon tube with 30 ml liquid MS medium in which ten inoculating loops of taken directly from the plate..

Excess Agrobacterium was blotted from the leaf pieces before placing them on MSS (MS, 3% sucrose, 1mg/l 6-BAP, pH 5.8) plates. After 2 days of incubation at 25°C in the dark, plates were transferred to the light for two more days. In the case of Agrobacterium overgrowth *leaf pieces were transferred to new plates.*

After the 4 day cocultivation, leaf pieces were transferred on MSS containing the selective antibiotics (100 mg/l kanamycin, 400 mg/l cefotaxime) and incubated at 25°C under continuous light condition. Leaf pieces were transferred to fresh MSS plates with selection antibiotics every week. When shoots appeared (after 4-6 weeks), the leef pieces were transferred on MSS selective media in SIGMA boxes. 3 cm long shoots were transferred on MS media with 50mg/l kanamycin but without hormones; after substantial root growth, plants were transferred in soil (Fisher and Guiltinan, 1995).

The protocol to obtain transgenic *Arabidopsis thaliana* plants by the *in planta* transformation method, was essentially the same as described by Bechtold and Bouchez (1993).

Nucleic acid extraction and Northern blotting

Genomic DNA was isolated from tobacco and Arabidopsis by the CTAB extraction method modified accordint to Murray and Thompson (1980). Total RNA was isolated according to the instructions from LIFE-TECHNOLOGIES (GIBCO BRL) for TRIzol Reagent with few modifications that increased the purified material (see chapter 6).

RNA samples were then transferred from the gel to a positively charged nylon membrane, by capillarity, essentially as described in Sambrook's manual (1989). Total RNA was hybridised either with a radioactive probe (Figure 2) or with a probe labelled by the DIG-Chem-Link (Boehringer) (Figure 5).

Protein extraction and Western blotting

Plant tissue samples (<0.2 g) were reduced to powder in liquid nitrogen and incubated for 30 min in the extraction buffer (1% TBS and 2% SDS). Insoluble proteins were eliminated by centrifugation for 5 min at 14000 g. Soluble proteins were precipitate by 10% TCA and washed in acetone before electrophoresis and blotting following standard conditions (Laemmli, 1972). Protoplasts were harvested and resuspended in the extraction buffer (1% TBS). To extract total intracellular soluble proteins, cells were lysed by 3 consecutive cycles of freezing and thawing.

Confocal microscopy

Transgenic arabidopsis and tobacco plants were grown in sterile agar culture for variable periods of time and then mounted for microscopy in water under glass coverslips. The specimens were examined using a confocal laser-microscope Leica DMR using the Leica TCS 4D operating system. GFP was detected with the filter set for FITC while chlorophyll epifluorescence and antibody immunofluorescence were detected with the filter set for TRITC. The stored digital images were pseudocolored as red or green images, using Photoshop 4.1 (Adobe, Mountain View, CA) corresponding to the real red or green emission.

Chapter 6

Materials and Methods

The material used in this thesis is described in all necessary detail to allow confirmation of our data or continue the research. Protocols, when they introduce original solutions or represent important key factor of the research work, are reported as a step by step procedure, followed by composition of solutions.

NUCLEIC ACIDS MANIPULATIONS

STANDARD TECHNIQUES

Standard techniques for DNA manipulation were used, as described in Sambrook's laboratory manual (1989). DNA modifying enzymes were used according to the manufacturer's instructions. PCR was performed in 20 µl volumes in a DNA thermal cycler PTC-100 (MJ Research Inc.) using Taq polymerase (Life technologies AG, Basel, CH). 50-150 ng template DNA was used in reactions containing the standard buffer suggested by Life technologies with 2.5 mM $MgCl_2$, 1 µM of each primer and 0,5 units of Taq polymerase. After a template melting step (5 min at 94°C), amplification was performed in 30 cycles as follows: denaturation for 1 min at 94°C, annealing for 1 min at the primer's specific annealing temperature and extension for 1 min at 72°C. The PCR products were separated on a 1% preparative Agarose Gel and the expected bands were directly isolated from the gel by pumping out the TBE buffer contained in the gel with a 20 µl pipette. PCR products were cloned in an EcoRV digested, T-tailed pBluescript without additional purification steps. DNA inserts cut out of pBluescript were then combined in different vectors using standard digestion and ligation techniques.

GFP CONSTRUCTS

The plasmids pBIN-mGFP4 and pBIN-mGFP5-ER encoding a cytosolic and an ER-targeted GFP respectively, and adapted for expression in plants, were kindly given by D. Haseloff (Cambridge, UK) (Haseloff *et al.*, 1997). 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 pBIN-mGFP4 was isolated as a BamHI-SacI fragment, blunt ended with Klenow polymerase, cloned into the SmaI site of pGY1, producing plasmid pGFP4, which encodes a cytosolic GFP (E. Freydl, Zürich). The 3' BamHI site was destroyed by partial BamHI digestion, filling up with Klenow polymerase and religation. A NheI 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 the 3' reverse vector primer 2 (tgtag agaga gactg gtgat ttc). An NheI 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 the 5' forward vector primer 3 (tgacg cacaa tccca ctatc cttcg ca) and primer 4 (gttct **gcgct agc**ag aaagc agtag g). This allowed to construct plasmid pSGFP4, encoding a GFP fused to the signal sequence of chitinase A. Furthermore a BglII site was introduced at the end of the coding sequence of the GFP, using the 5' forward vector primer 3 and primer 5 (gtcga ctcta **gagat ct**ttg tatag ttcat c). This replaced the stop codon by Glu and Ser codons. The BglII 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 BglII site in the same reading frame, Neuhaus *et al.*, 1994) resulting in plasmid pSGFP4T. In another construction, a C-terminal STKDEL sequencecontain an ER-retention signal was introduced by PCR using the forward primer 1 and primer 6 (**cctgc** agtca **gagct c**gtcc ttggt **cgact** tgtat agttc atc). This also introduced SalI and SacI sites within the coding sequence and a PstI site after the stop codon in the resulting plasmid pSGFP4K.

Because of the problems of thermostability of GFP4 in the secretory pathway of plants, a new variant (GFP5), was produced and kindly given to us by J. Haseloff. The BamHI-PvuII fragments from pSGFP4, pSGFP4T and pSGFP4K were therefore replaced by the corresponding fragment from pBIN-mGFP5-ER containing the sequence encoding the signal peptide of *Arabidopsis* chitinase and most of the thermostable GFP5. This produced the plasmids pSGFP5, pSGFP5T and pSGFP5K which were mostly used in this work (Figure 1).

pAGFP5 plasmid was obtained by the substitution of the signal sequence of pSGFP5 with the first 431 bases (143 aa) of barley aleurain cDNA as a fragment BamHI/NheI. We introduced a NheI site at the beginning of the coding sequence for GFP5 or GFP6 with the primer NheGFP (tctgc **tagc** gcaat gagta aagga gaagaac) and at the 3' end of the sequence coding for the aleurain N-terminal fragment by directed mutagenesis with the primer NTPP2 (**ggct**a gcggc ggcgt cccgc).

Mutations F64L/S65T were introduced in all constructs by directed mutagenesis with the primer gfpS65T (ccatg gccaa cactt gtcac tactc tcact).
Plasmids were isolated by alkaline lysis in presence of SDS (Sambrook *et al.*, 1989) and purified on an ethidium bromide-CsCl density gradient.
For stable plant transformation, a binary vector (maintainable *in E. coli* under kanamycin selection) was derived from pBIN-m-gfp5-ER (Haseloff *et al.*, 1997). All GFPs were cloned into pBIN as BamHI-SacI fragments including the GFP encoding sequence and the terminator, in replacement of the fragment encoding GFP5-ER in pBIN-mGFP5-ER.

RAT ß-GLUCURONIDASE CONSTRUCTS

Plasmids pGY1 containing the rat ß-glucuronidase forms described in the annexe to chapter 2 (pRGUS; pRGUSD15; pRGUSD15+T), were produced by M. Pietrzak and J-M. Neuhaus in Basel.
Binary vectors pBIN-RGUSD15 and pBIN-RGUSD15+T were obtained by the substitution of the BamHI/SacI cassette of pBIN--mGFP5-ER containing the GFP coding sequence, with the coding sequence of the different forms of rat ß-glucuronidase.

FIGURE 1: Schematic representation of all constructs.

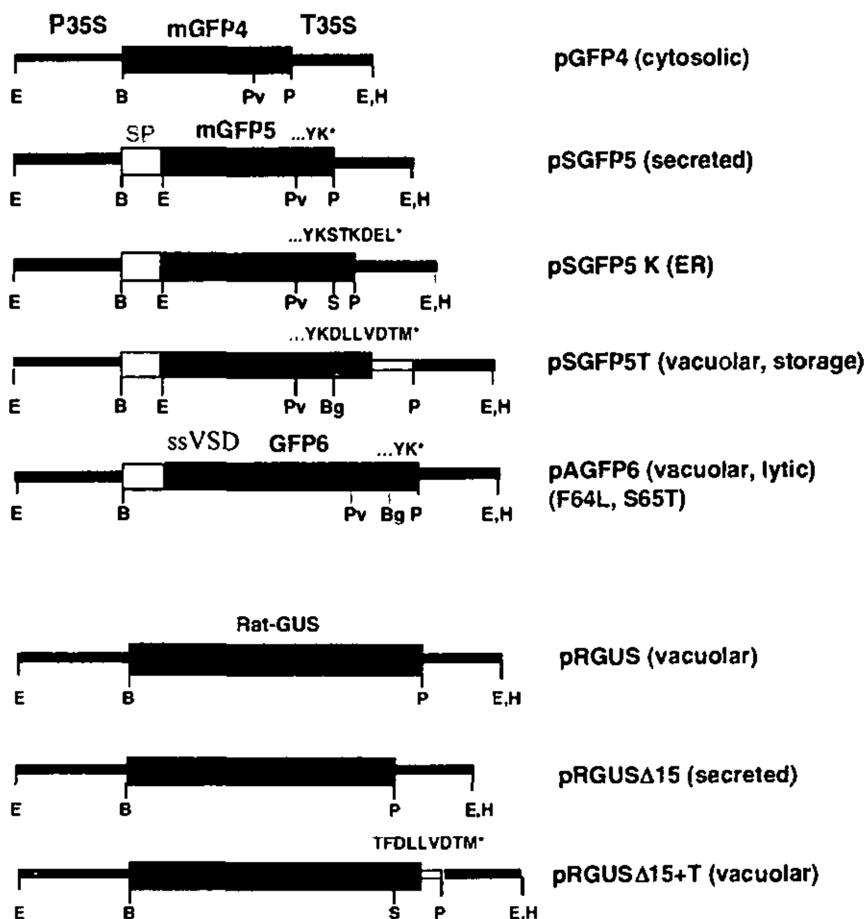


Figure 2: Comparison between GFP4 (first row) and GFP5 (second row) sequences. The third row indicate mutations introduced in GFP5 to obtain GFP6 and glycosylated GFPs GL80, GL133 and GL172.

741 GGGGTCCCCGGGCTGCGGAACCGATACCTGTTCCATGGCCAAAACTTGTC 696 GFP4
800 aattattgcactactggaaactacctgttccatggccaacacttgtc 849 GFP5

695 ACTCCTTTCTCCTACGGTGTTCAATGCTTTTCAAGATACCCAGGTCATTT 651 GFP4
850 actactttctcttatggtgttcaatgcttttcaagatacccagatcatat 899 GFP5
actctcactt . . . enhancing mutations (GFP6)
F64L/S65T

650 GAAGCGGGACGACTCCTTCAAGAGCGCCATGCCTTAGGGATACGTTCAGG 606 GFP4
900 gaaGcggcaCgacttCttcaagagCgccatgccTgaGggAtaCgtGcagg 949 GFP5
gaagaaccacacctt . . . . . glycosylation. site GL80
R80N/D82T

605 AGAGGACCATCTTCTTCGACGACGACGGGAACTACAAGAMCCGTGCTGAA 558 GFP4
950 aGagGacCatCttCttcaaGgaCgacgggaactacaagacacgtgctgaa 999 GFP5

557 GTCAAGTTTTGGGGGAGACACCCCCGTCAAAGGGATCGAGCTTAGGGGAA 508 GFP4
1000 gtcaagtttgaGggAgaCacccCgtCaaCagGatcgagCtTaaGggAa 1048 GFP5

507 TCGATTTCAGGTAGGACGGAAACATCCTCGGCCACAAGTTGGAATACAAC 458 GFP4
1049 tCgattCaaGgaGgaCggaaacatCctCggCcacaaGttggaatacaac 1098 GFP5
ggagaacggaagcatc. . . glycosylation site GL133
D133N/N135T

457 TACAACTCCCACAACGTATACATCACGGCAGACAAACAAAGAATGGAAT 409 GFP4
1099 taCaactcCcacaaCgtatacatcatggcagacaaacaaaagaatggaat 1148 GFP5

408 CAAAGCTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAAC 359 GFP4
1149 caaagttaacttcaaaattagacacaacattgaagatggaagcgttcaac 1198 GFP5
. . acatcgcaaaacgacagcg glycosylation site GL172
E172A/D173N/G174D/G175S

358 TAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTT 309 GFP4
1199 tagcagaccattatcaacaaaatactccaattggcgatggccctgtcctt 1248 GFP5

308 TTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCC 259 GFP4
1249 ttaccagacaaccattacctgtccacacaatctgccctttcgaaagatcc 1298 GFP5

258 CAACGAAAASAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTG 209 GFP4
1299 caacgaaaagagagaccacatggtccttcttgagtttgtaacagctgctg 1348 GFP5

208 GGATTACACATGGCATGGATGAACTATACAAATAACGGGGATCCTCTARA 159 GFP4
1349 ggattacacatggcatggatgaactatacaaataaGggggatcctctaga 1398 GFP5

158 GTCGACCTGCAGGCATGCCCGCTGAAATCACCAGTCTCTCTCTACAATCA 109 GFP4
1399 gtcgacCTGCAGGCATGcCCGCTGAAATCACCAGTCTCTCTCTACAA... 1443 GFP5

Figure 3: Sequences of GFP fusion proteins from EcoRI to HindIII in pGY1. Usefull restriction site are indicated by underlined DNA sequences; VSDs are indicated as underlined aminoacidic sequences.

PSGFP5

```
 EcoRI      XhoI
 GAATTCCCATGCCTCGAGGCATGGAGTCAAAGATTCAAATAGAGGACCTAACAGAACTCG
1 ---------+---------+---------+---------+---------+---------+ 60
 CCGTAAAGACTGGCGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAA
61 ---------+---------+---------+---------+---------+---------+ 120
 TCTTCGTCAACATGGTGGAGCACGACACGCTTGTCTACTCCAAAAATATCAAAGATACAG
121 ---------+---------+---------+---------+---------+---------+ 180
 TCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCC
181 ---------+---------+---------+---------+---------+---------+ 240
 TCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTG
241 ---------+---------+---------+---------+---------+---------+ 300
 GCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCG
301 ---------+---------+---------+---------+---------+---------+ 360
 ACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTC
361 ---------+---------+---------+---------+---------+---------+ 420
                               EcoRV
 CAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACG
421 ---------+---------+---------+---------+---------+---------+ 480
 CACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGG
481 ---------+---------+---------+---------+---------+---------+ 540
      KpnI/SmaI/BamHI
 AGAGGACAGGGTACCCGGGGATCCAAGGAGATATAACAATGAAGACTAATCTTTTTCTCT
541 ---------+---------+---------+---------+---------+---------+ 600
c                                   M  K  T  N  L  F  L  F -
                                    EcoRI
 TTCTCATCTTTTCACTTCTCCTATCATTATCCTCGGCCGAATTCagtaaaggagaagaac
601 ---------+---------+---------+---------+---------+---------+ 660
c     L  I  F  S  L  L  L  S  L  S  S  A  E  F  S  K  G  E  E  L -

 ttttcactggagttgtcccaattcttgttgaattagatggtgatgttaatgggcacaaat
661 ---------+---------+---------+---------+---------+---------+ 720
c     F  T  G  V  V  P  I  L  V  E  L  D  G  D  V  N  G  H  K  F -

 ttctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaattta
721 ---------+---------+---------+---------+---------+---------+ 780
c     S  V  S  G  E  G  E  G  D  A  T  Y  G  K  L  T  L  K  F  I -
                      NcoI
 tttgcactactggaaaactacctgttccatggccaacacttgtcactactttctcttatg
781 ---------+---------+---------+---------+---------+---------+ 840
c     C  T  T  G  K  L  P  V  P  W  P  T  L  V  T  T  F  S  Y  G -
                          NdeI
 gtgttcaatgcttttcaagatacccagatcatatgaaGcggcaCgacttCttcaagagCg
841 ---------+---------+---------+---------+---------+---------+ 900
c     V  Q  C  F  S  R  Y  P  D  H  M  K  R  H  D  F  F  K  S  A -

 ccatgccTgaGggAtaCgtGcaggaGagGacCatCttCttcaaGgaCgacgggaactaca
901 ---------+---------+---------+---------+---------+---------+ 960
c     M  P  E  G  Y  V  Q  E  R  T  I  F  F  K  D  D  G  N  Y  K -


 agacacgtgctgaagtcaagtttgaGggAgaCaccctCgtCaaCagGatcgagCtTaaGg
```

```
961 ---------+---------+---------+---------+---------+---------+ 1020
c      T  R  A  E  V  K  F  E  G  D  T  L  V  N  R  I  E  L  K  G -
        ClaI
      gAatCgatttCaaGgaGgaCggaaacatCctCggCcacaaGttggaatacaactaCaact
1021 ---------+---------+---------+---------+---------+---------+ 1080
c      I  D  F  K  E  D  G  N  I  L  G  H  K  L  E  Y  N  Y  N  S -
           AccI
      cCcacaaCgtatacatcatggcCgacaaGcaaaagaaCggCatcaagCCaacttcaaGA
1081 ---------+---------+---------+---------+---------+---------+ 1140
c      H  N  V  Y  I  M  A  D  K  Q  K  N  G  I  K  A  N  F  K  T -
                                    BclI
      CCCgCcacaacatCgaagaCggCGgcgtGcaactCgcTgaTcattatcaacaaaatactc
1141 ---------+---------+---------+---------+---------+---------+ 1200
c      R  H  N  I  E  D  G  G  V  Q  L  A  D  H  Y  Q  Q  N  T  P -
      caattggcgatggccctgtccttttaccagacaaccattacctgtccacacaatctgccc
1201 ---------+---------+---------+---------+---------+---------+ 1260
c      I  G  D  G  P  V  L  L  P  D  N  H  Y  L  S  T  Q  S  A  L -
                                                    PvuII
      tttcgaaagatcccaacgaaaagagagaccacatggtccttcttgagtttgtaacagctg
1261 ---------+---------+---------+---------+---------+---------+ 1320
c      S  K  D  P  N  E  K  R  D  H  M  V  L  L  E  F  V  T  A  A -

                                            XbaI  SalI
      ctgggattacacatggcatggatgaactatacaataaGgggGATCgatcctctagagtcg
1321 ---------+---------+---------+---------+---------+---------+ 1380
c      G  I  T  H  G  M  D  E  L  Y  K  *


      TTCATGATCTGTTTTGTTGTATTCCCTTGCAATGCAGGGCCTAGGGCTATGAATAAAGTT
1381 +---------+---------+---------+---------+---------+--------- 1440
      AATGTGTGAATGTGAATGTGTGATTGTGACCTGAAGGGATCACGACTATAATCGTTTATA
1441 +---------+---------+---------+---------+---------+--------- 1500
                                 PstI  SphI
      ATAAACAAAGACTTTGTCCCAAAAACCCCCCCCCCCCTGCAGGCATGCCCGCTGAAATCA
1501 +---------+---------+---------+---------+---------+--------- 1560
      CCAGTCTCTCTCTACAAATCTATCTCTCTCTATAATAATGTGTGAGTAGTTCCCAGATAA
1561 +---------+---------+---------+---------+---------+--------- 1620
      GGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTA
1621 +---------+---------+---------+---------+---------+--------- 1680
      TGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATC
1681 +---------+---------+---------+---------+---------+--------- 1740
        KpnI  SacI  EcoRI HindIII
      CAGTGGGTACCGAGCTCGAATTCAAGCTT
1741 +---------+---------+-------- 1768
```

pSGFP5T

```
                                      BglII
   ctgggattacacatggcatggatgaactatacaaAGATCTTTTAGTCGATACTATGTAAT
 1321 ---------+---------+---------+---------+---------+---------+ 1380
c      G I T H G M D E L Y K D L L V D T M *

   TTCATGATCTGTTTTGTTGTATTCCCTTGCAATGCAGGGCCTAGGGCTATGAATAAAGTT
 1381 +---------+---------+---------+---------+---------+--------- 1440
   AATGTGTGAATGTGAATGTGTGATTGTGACCTGAAGGGATCACGACTATAATCGTTTATA
 1441 +---------+---------+---------+---------+---------+--------- 1500
                                             PstI  SphI
   ATAAACAAAGACTTTGTCCCAAAAACCCCCCCCCCCCTGCAGGCATGCCCGCTGAAATCA
 1501 +---------+---------+---------+---------+---------+--------- 1560
   CCAGTCTCTCTCTACAAATCTATCTCTCTCTATAATAATGTGTGAGTAGTTCCCAGATAA
 1561 +---------+---------+---------+---------+---------+--------- 1620
   GGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTA
 1621 +---------+---------+---------+---------+---------+--------- 1680
   TGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATC
 1681 +---------+---------+---------+---------+---------+--------- 1740
     KpnI SacI EcoRI HindIII
   CACTCGGTACCCGAGCTCGAATTCAAGCTT
 1741 +---------+---------+-------- 1768
```

pSGFP5K

```
                                     SalI      SacI    PstI
   ctgggattacacatggcatggatgaactatacaaGTCGACCAAGGACGAGCTCTGACTGC
 1321 ---------+---------+---------+---------+---------+---------+ 1380
c      G I T H G M D E L Y K S T K D E L *
     SphI
   AGGCATGCCCGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATAATAATG
 1381 ---------+---------+---------+---------+---------+---------+ 1440
   TGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAG
 1441 ---------+---------+---------+---------+---------+---------+ 1500
   CATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTC
 1501 ---------+---------+---------+---------+---------+---------+ 1560
                   KpnI SacI EcoRI HindIII
   TAATTCCTAAAACCAAAATCCAGTGGGTACCGAGCTCGAATTCAAGCTT
 1561 ---------+---------+---------+---------+-------- 1609
```

AGFP6

```
            KpnI/SalI/BamHI
     AGAGGACAGGGTACCCGGGGATCCggcgaaacgaaatggcccacgcccgcgtcctcctcc
 541 ---------+---------+---------+---------+---------+---------+ 600
c                                  M  A  H  A  R  V  L  L  L -

     tggcgctcgccgtcctggccacggccgccgtcgccgtcgcctcctcctcctccttcgccg
 601 ---------+---------+---------+---------+---------+---------+ 660
c     A  L  A  V  L  A  T  A  A  V  A  V  A  S  S  S  S  F  A  D -
                                         XhoI
     actccaacccgatccggcccgtcaccgaccgcgccgcctcgacgctcgagtccgccgtcc
 661 ---------+---------+---------+---------+---------+---------+ 720
c     S  N  P  I  R  P  V  T  D  R  A  A  S  T  L  E  S  A  V  L -

     tcggcgcgctcggccgcacccgccacgccctccgcttcgcgcgcttcgccgtcaggtacg
 721 ---------+---------+---------+---------+---------+---------+ 780
c     G  A  L  G  R  T  R  H  A  L  R  F  A  R  F  A  V  R  Y  G -

     gcaagagctacgagagcgcggcggaggtgcggaggcggttccggatcttctccgagagcc
 781 ---------+---------+---------+---------+---------+---------+ 840
c     K  S  Y  E  S  A  A  E  V  R  R  R  F  R  I  F  S  E  S  L -
      XhoI
     tcgaggaggtgcgctccaccaaccggaagggcctcccctaccgcctcggcatcaaccgct
 841 ---------+---------+---------+---------+---------+---------+ 900
c     E  E  V  R  S  T  N  R  K  G  L  P  Y  R  L  G  I  N  R  F -

     tctcggacatgagctgggaggagttccaggcgacccgtctcggcgcggcgcagacctgct
 901 ---------+---------+---------+---------+---------+---------+ 960
c     S  D  M  S  W  E  E  F  Q  A  T  R  L  G  A  A  Q  T  C  S -
                                         NheI
     cggcgacgctcgccggcaaccacctcatgcgggacgccgccgcTAGCGCAatgagtaaag
 961 ---------+---------+---------+---------+---------+---------+ 1020
c     A  T  L  A  G  N  H  L  M  R  D  A  A  A  S  A  M  S  K  G -

     gagaagaactttcactggagttgtcccaattcttgttgaattagatggtgatgttaatg
1021 ---------+---------+---------+---------+---------+---------+ 1080
c     E  E  L  F  T  G  V  V  P  I  L  V  E  L  D  G  D  V  N  G -
```

DNA EXTRACTION

Genomic DNA is isolated from tobacco and *Arabidopsis* by CTAB extraction modified after Murray and Thompson (Murray and Thompson, 1980).

STEP1: Take approximately 0.15 g of young tobacco or *Arabidopsis* leaves, shock-freeze samples in liquid nitrogen and homogenise in mortar;
STEP2: add 2 ml Extraction buffer (Tris (HCl) 50mM pH 8; NaCl 0.7M; EDTA 10mM; CTAB (Cetyltriethylammoniumbromide) 1% (w/v));
STEP3: incubate at 60°C for 20 min and let cool down to R.T.;
STEP4: add 2 ml chloroform and mix well;
STEP5: centrifuge 5 min 2000g at R.T.;
STEP6: transfer aqueous phase (upper) to fresh tube, be careful not to disturb the interphase;
STEP7: add 0,1 vol. CTAB stock (CTAB 10% (w/v); 0.7M NaCl), mix gently;
STEP8: add 1 vol. chloroform and mix well;
STEP9: centrifuge 5 min 2000g at R.T.;
STEP10: transfer aqueous phase (upper) to fresh tube;
STEP11: add 2 vol. 100% ethanol and incubate 1 h at -20°C;
STEP12: centrifuge 30 min 2000g at R.T.;
STEP13: dissolve the pellet in 1 ml water or TE;
STEP14: add 2µl RNAse (1mg/ml) and incubate 30 min at 37°C;
STEP15: add 1 ml LiCl 5M and incubate 10 min at R.T.;
STEP16: centrifuge 15 min 2000g at R.T.;
STEP17: transfer liquid phase in a fresh tube and add 2 vol. 100% ethanol, incubate 1 h at -20°C;
STEP18: centrifuge 30 min 2000g at R.T.;
STEP19: resuspend pellet in TE

SOUTHERN BLOTTING

Genomic DNA samples are digested O.N. by restriction enzymes and fragments are separated by electrophoresis on a 1% agarose gel with 0.5X TBE as buffer.
DNA samples are then transferred from the gel to a cellulose-nitrate membrane (BA85; 0,45µm; Schleicher&Schuell) by capillarity, essentially as described in Sambrook's manual (1989).

DNA samples are hybridised with a probe labelled by the DIG-Chem-Link (Boehringer); labelling of the probe is described in the following paragraphs.

RNA EXTRACTION

Total RNA is isolated in accordance to instructions from LIFE - TECHNOLOGIES (GIBCO BRL) for *TRIzol* Reagent with few modifications that increase the purified material.

STEP1: Take approximately 0.15 g of young tobacco or *Arabidopsis* leaves, shock-freeze samples in liquid nitrogen and homogenise in mortar;
STEP2: transfer to a 15ml tube and add 2 ml 2x CTAB (2% CTAB; 0.7M NaCl);
STEP3: incubate tubes at 60°C for 25 min (mix every 5 min);
STEP4: cool down to R.T. and recover the condensation water by a short centrifugation;
STEP5: add 2ml CHCl3, mix;
STEP6: centrifuge 10', at 2000g;
STEP7: save upper phase in a new tube and add 2 volumes 100% EtOH;
STEP8: centrifuge 10', at 2000g;
STEP9: dry the pellet; resuspend in 200µl H_2O;
STEP10: add 1.5 ml TRIZOL (GIBCO BRL);
STEP11: incubate 5 min at R.T.;
STEP12: add 300µl CHCl3; vortex;
STEP13: centrifuge for 15 min at 12000g, at a temperature of 4°C;
STEP14: save upper phase in a new tube and add 1/2 vol. isopropanol; precipitate minimum 10 min at R.T.;
STEP15: *centrifuge 10 min at 12000g (4°C)*;
STEP16: wash the pellet in EtOH 75%;
STEP17: resuspend in treated H_2O RNAse free.

NORTHERN BLOTTING

RNA samples are mixed with two volumes of RNA sample buffer (10ml Formamide; 3.5ml Formaldehyde 37%, 2ml 5X MOPS) and incubated 5min at 65°C.
5X MOPS stock solution can be stored many months at R.T.; 1 litre is prepared dissolving 3.28 g anhydrous sodium acetate and 20.6 g MOPS in *water; the pH is adjusted to 7 with* NaOH and EDTA is added to a final concentration of 5mM.

RNA samples are separated by electrophoresis on a 1% agarose gel with 1X MOPS as buffer.
RNA samples are then transferred from the gel to a positively charged nylon membrane (Boehringer), by capillarity, essentially as described in Sambrook's manual (1989).

Total RNA is hybridised either with a radioactive probe or with a probe labelled by the DIG-Chem-Link (Boehringer).

HYBRIDIZATION WITH A RADIOACTIVE PROBE

Wet membrane in 2X SSC
Place the membrane in the tube with 10ml PREHYBRIDATION MIX:

	Final	for 20ml
20X SSC	5X	5 ml
50X Denhardt's	8X	3.2 ml
10% SDS	0.1%	0.4ml
formamide	50%	20 ml
Na_2HPO_4 pH6.5	50mM	1 ml
Na_2HPO_4 pH6.5	5'mM	1 ml

Incubate 1 hour 42°C

Denature the PROBE DNA FRAGMENT (100 ng in 12 µl) 5min at 95°C
Add 4 µl 5X OLB primers mix (Boehringer)
2 µl 10X dNTPs mix
1 µl 32P-dATP (400Ci/mmoles)
1 µl Klenow (2U/µl)
Incubate 1h 37°C
Add 80 µl STOP SOLUTION (Boehringer)
Prepare a minicolumn with Bio-Gel P4
Spin (Eppendorf pos.2) 1 min and eliminate liquid
Wash with TE; Spin
Charge with the probe and spin 1 min.
Add filtered probe to PREHYBRIDIZATION MIX
Incubate ON 42°C
Recover the probe and stock at -20°C
Wash filter twice with 50 ml 2x SSC; 0.1% SDS 20 min 42°C
Wash in 0.2XSSC; 0.1%SDS 30' 42°C
More washing can follow to reduce background.
Expose radiography films to the filter in a sealed transparent bags.

HYBRIDIZATION WITH A Dig-Chem-Link PROBE

Probe labelling by PCR (DIG Probe Synthesis KIT -BOEHRINGER M.):
In a mini-eppendorf on ice, prepare the following PCR mix:

PCR 10X buffer	5 µl
PCR DIG-Mix	5 µl
Primer GFPY66H	5 µl
Primer GFPend	5 µl
Enzyme mix	0.75 µl
DNA psGFP6T	0.5 µl
H_2O	28.75µl

Program: 10(48°C)+30(54°C)

Hybridisation (DIG Easy Hyb -Boehringer):

STEP1: Put the filter on agitation (rotation) with "DIG E H" at 50°C for pre-hybridisation (20ml).
STEP2: Prewarm at 50°C 10ml "DIG E H" (for each 100 cm^2 of filter)
STEP3: Denature PCR product (PCRp.) 10 min. 100°C
STEP4: Put on ice
STEP5: Add 2 µl PCRp. per ml to the prewarmed DIG E H solution
STEP 6: Replace the pre-hyb. solution (on the filters) by the DIG E H + PCRp.
STEP7: Incubate at 50°C on agitation for 16h
STEP8: Wash 5min in 2X SSC; 0,1% SDS at rt.
STEP9: Repeat wash
STEP10: Wash 15 min in 0.1X ; 0,1% SDS
STEP11: Repeat wash
STEP12: wash in Maleic buffer (0.1M Maleic acid, 0.15M NaCl)
STEP13: Prepare 100 ml Blocking Solution (5%milk in Maleic buffer) wash the filters 30 min with 80 ml .
STEP14: Dilute the remaining 20 ml with 80 ml Maleic Buffer (final 1%milk) and add 2µl anti-digoxigenin-AP, incubate 30 min.
STEP15: Wash rapidly in Maleic Buffer
STEP16: wash twice in Maleic Buffer + 0.3% Tween20
STEP17: Wash in Maleic Buffer 5 min.
STEP18: Incubate 10 min in AP buffer (0.1M Tris-HCl pH9.5; 0.1M NaCl)
STEP19: Put filters on a glass plate, add immune-star mix (2ml immune-star substrate +100 µl immune-star.
STEP20: Drain the liquid and expose in sealed bags.

PLANT MATERIAL

Leaf mesophyll protoplasts of *Nicotiana tabacum* cv. SRI are obtained from plants grown under *sterile* conditions to the age of about 5-7 weeks on MS (Duchefa medium including micro, macro elements and vitamins) agar medium (8% agar) supplemented with 15% saccharose, at 23°C with 16 hours of daily light. The shoot cultures are transferred to fresh medium every 4-8 weeks. Transgenic Nicotiana tabacum plants are grown in a greenhouse at 22°C, 16 hours of daily light and 70% humidity.

ISOLATION OF *N. TABACUM* PROTOPLASTS

After their isolation, the protoplasts (pps) are healthy and useful for transformation, even after 18 hours at 4°C.

STEP1: Cut the fully expanded leaves of a shoot culture under sterile conditions and transfer them to a 9 cm Petri dish. *Two leaves per dish, 5 dishes.*

STEP2: Wet the leaves thoroughly with Enzyme Solution and remove the mid-ribs.

STEP3: Cut leaves in 2 pieces and wound the upper epidermis.

STEP4: Add enzyme solution to 10ml.

STEP5: Seal with parafilm and incubate over night (14-18h) at 26°C in the dark without shaking.

STEP6: Gently agitate the dishes for 30 min.

STEP7: Take the pps suspension with a sterile pipette with cut-off tip and pour through a 100µm stainless steel mesh sieve.

STEP8: Divide into 6ml aliquots. Use K3 to adjust volume.

STEP9: Carefully overlay the suspension with 1ml of W5 solution. Don't mix.

STEP10: Spin for 10 min. at 80g (570rpm). Good pps will float at the interphase.

STEP11: Collect the pps with a sterile pipette with cut-off tip taking as little as possible of the lower phase.

STEP12: Pool the pps in 4ml aliquots.

STEP13: Gradually add 10ml W5, mix gently.

STEP14: Pellet pps (80g 5min)

STEP15: repeat STEP 13 & 14

STEP16: Resuspend pps in a total volume of 11ml W5 and store at 4°C in the dark for 2h.

STEP17: Dilute 1ml in 9ml W5; count the pps in a 0.2µl hematocytometer. Obs. pps*500000= tot. pps

STEP18: Pellet pps as in STEP14

STEP19: Resuspend pps in MMM at a concentration of $2.5^{*}10^{6}$ pps/ml.

PROTOPLAST TRANSFORMATION FOR TRANSIENT EXPRESSION

STEP1: Prepare new tubes with 5 µl plasmid DNA (10mg)

STEP2: Distribute 300ml of the pps suspension into the tubes. Use a *clipped blue tip. Mix.*

STEP3: Add 300ml PEG solution (40% PEG4000; Mannitol 0.4M; $Ca(NO_3)_2$ 0.1M pH 8); pipette slowly because of high viscosity. Shake gently. Incubate 5 min.

NB: PEG is a tensioactive that helps the DNA to adhere to the plasma membrane. This adhesion and the permeabilization of the plasma membrane, with a contribution of Mg^{2+} ions, makes the uptake of DNA by the cells easier.

STEP4: Add 2 ml K3 + dichlorobenzonitrile 2 µg/ml (DCB is a cellulose synthesis inhibitor; the stock of 4mg/ml DMSO is stored at 4°C), incubate 2h at 26°C in the dark for the pps to recover.

STEP5: Add 5 ml of W5 solution to wash away PEG. Pellet pps (70g, 5min).

STEP6: Resuspend pps in 2ml K3 supplemented with DCB to inhibit the synthesis of a new cell wall.

STEP7: Incubate over-night at 26°C in the dark.

SOLUTIONS FOR PROTOPLASTS PREPARATION AND TRANSFORMATION:

Enzyme Solution

In 100ml K3 solution dissolve:

1.2 g Cellulase Onozuka R10

0.4 g Macerozyme R10

0.3 g Sucrose

Filter sterilisation.

PEG solution

40% PEG4000

0.4M Mannitol

0.1M $Ca(NO_3)_2$

pH 8

autoclave.

K3 Macro 1 l

KNO_3	25.3 g
NH_4NO_3	2.5 g
$NaH_2PO_4 \cdot H_2O$	1.5 g
$CaCl_2 \cdot 2H_2O$	9 g
$MgSO_4 \cdot 7H_2O$	2.5 g
$(NH_4)2SO_4$	1.34 g

B5 Micro 100ml

H3BO3	0.3 g
KI	0.075g
$MnSO_4 \cdot H_2O$	1g
$ZnSO_4 \cdot 7H_2O$	0.2g
$CuSO_4 \cdot 5H_2O$	2.5mg
$Na_2MoO_4 \cdot 2H_2O$	25 mg
$CoCl_2 \cdot 6H_2O$	2.5mg

K3 100X-vit. 100Ml

Inositol	1g
Pyridoxine HCl	10mg
Thiamine HCl	100mg
Nicotinic acid	10mg

Na_2EDTA(200X): 40mM

14.92 g/l

$FeCl_3 \cdot 6H_2O$(200X): 20mM

5.4 g/l

2,4-D: 20mg in 1ml EtOH abs. Complete to 100ml with H_2O

NAA: 20mg in 1ml EtOH abs. Complete to 100ml with H_2O

BAP: 20mg in 1ml KOH 1M. Complete to 100ml with H_2O

Filter sterilisation

K3 (0.3M Sucrose) 1 l

K3-macro	100ml
(200X)Na_2EDTA	5 ml
(200X)$FeCl_3 \cdot 6H_2O$	5 ml
B5-micro	1ml
K3-vit.	10 ml
Sucrose	102.7 g
Xylose	0.25 g
pH 5.8 (KOH)	
--- Autoclave 20'- 120°C ---	
2,4-D	0.5 ml
NAA	5 ml
BAP	1 ml

W5 osmoticum 1 l

NaCl 154 mM	9 g
$CaCl_2$125mM	18.3g
KCl 5 mM	0.37 g
Glucose 5 mM	1g

MMM 100ml

Mannitol 0.5 mM	9.11g
$MgCl_2$ 15 mM	0.3g
MES 0.1%	0.1g

VACUOLE ISOLATION

STEP1: Add 3ml W5 solution to the pps, mix gently and pellet the pps as in step 14 of pps preparation.

STEP2: Resuspend the pellet in 2ml Lysis buffer (Mannitol 0.2M; Ficoll 400 10%; EDTA 20mM; DTT 2mM; HEPES 5mM pH8; BSA 150mg/ml) prewarmed at 42°C. Wait 2 min at 42°C.

STEP3: The lysate is overlaid by 1ml of 5% Ficoll 400 (1 vol. lysis buffer + 1 vol. vacuole buffer) and then on top 0.5ml of Vacuole buffer (Mannitol 0.6M; HEPES 10mM pH7.5; BSA 150mg/ml) is added.

STEP4: Centrifuge at 80g (570rpm) 10 min. The density gradient makes the vacuoles float at the upper interphase, between the vacuole buffer and 5% Ficoll.

STEP5: Collect the vacuoles taking as little as possible of the lower phase.

PROTOPLAST EVACUOLATION

N. tabacum protoplasts are evacuolated 2 hours after transformation (STEP5 for pps transformation); after evacuolation they are named miniprotoplasts (MIPS).

STEP1: Protoplasts are pelleted in the osmoticum (W5) and resuspended in 2 ml of E.Sol.1.

STEP2: Overlay pps on 8 ml E.Sol.2 in a 10 ml ultracentrifuge tube; mix partially the two phases to create a gradient.

STEP3: Centrifuge at 90'000 g at room temperature for 45 min.

STEP4: Collect all green phases (Ultracentrifugation evacuolates the PPS but the gradient does not permit to separate them from non-evacuolated PPS and vacuoplasts).

STEP5: Dilute the E.Sol.2 containing the MIPS, with 9 volumes E.Sol.1.

STEP6: Sediment MIPS and PPS 5 min at 120 g.

STEP7: Eliminate supernatant except the last 2 ml and resuspend the MIPS and PPS.

STEP8: In a 12 ml Falcon tube prepare a Percoll gradient as follow: on top of 1,5 ml 60% Percoll solution lay on 5 ml 40% solution, then 1,5 ml 20% solution, finally the 2ml of E.Sol.1 containing the MIPS and PPS mixture.

STEP9: Centrifuge 10 min at 1000 g and at R.T.

STEP10: Collect MIPS contained in the green band between 60 and 40% Percoll solution and discard normal PPS in the upper band.

STEP11: Wash MIPS with 2 volumes of W5 osmoticum.
STEP12: Resuspend MIPS in 2 ml K3 culture medium.

SOLUTIONS FOR PROTOPLAST EVACUOLATION:

Evacuolation Sol. 1 (E.Sol.1)

Mannitol	0.5 M
$CaCl_2$	1 mM
MES	10 mM

in water (pH~7)

Evacuolation Sol. 2 (E.Sol.2)

Mannitol	0.5 M
$CaCl_2$	50 mM
HEPES	20 mM

in Percoll (pH~7)

100% Percoll solution for 100 ml

Mannitol	0,5 M	9,1 g
MES	20 mM	0,1 g

in Percoll (pH~6)

Dilute with E.Sol.1 to obtain 60-20% Percoll solutions.

TOBACCO TRANSFORMATION BY AGROINFECTION.

To transform Nicotiana tabacum, sterile shoots are used. *Agrobacterium tumefaciens* strain GV3101 , transformed with our pBIN constructs (see later), is grown in liquid LB + 25 mg/l Gentamycin + 50 mg/l Kanamycin. The *Agrobacterium* culture is pelleted by centrifugation (10 min at 1500 g) and the bacteria are resuspended in MS (Duchefa) containing 15% w/v sucrose. Leaves from different plants are cut and submerged into the *Agrobacterium* suspension. Plant pieces are left 15 min in the bacterial suspension, then the excess of liquid is eliminated by blotting on sterile paper filters. Leaf pieces are plated on solid MSS (MS, 3% sucrose, 1mg/l 6-BAP, pH 5.8) and incubated 24h at 25°C in the dark for cocultivation.
The plates then are moved to the light (25°C) and incubated 4 days. In case of bacterial overgrowth, leaf pieces have to be moved to fresh medium.
Wash the leaves fragments in a solution of MS + 500mg Cefotaxime.
Move the leaf pieces to MSSCK plates (MSS + 400mg Cefotaxime; 100 mg Kanamycin/litre).
The fragments have to be transferred weekly on new MSSCK plates.
After 5 weeks regenerants normally appear.
Regenerating shoots are moved to MS + 50mg/l Kanamycin but without hormones for root formation.

Once plants have a strong root apparatus, they can be transferred to soil for complete maturation.

ARABIDOPSIS IN PLANTA INFILTRATION.

The protocol for obtaining transgenic *Arabidopsis thaliana* plants by a so-called *in planta* transformation method, is essentially the same described by Bechtold and Bouchez (1993).

PLANT MATERIAL.

Arabidopsis thaliana plants, cv. Wassilevskaja, are germinated after 2 days vernalization, in small (3/5 cm x 3/5 cm) pots with sixteen-hour day photoperiod (artificial light ideally at $105 \mu E/m^2/s$), 22°C temperature, 70% humidity and always wet sowing compost. Plants are watered by sub-irrigation with tap water. When the plant inflorescence is about 5 cm high, it is cut at the base. Within 3 weeks new inflorescences grow and plants are ready for infiltration. Flowers should not yet be at maturity.

AGROBACTERIUM TUMEFACIENS

We use the *Agrobacterium tumefaciens* strain GV3101, described to be the most efficient to infect *A. thaliana* cv. *Wassilewskaja*. This strain contains the binary vector pBIN described above (pBIN-SGFP5 /SGFP5K /AGFP6 /SGFP5T). The bacterium is transformed by triparental mating as described by Ditta *et al.* (1980) using *E. coli* HB101 pRK2013 helper strain (Ditta *et al.*, 1980; Spielman and Simpson, 1986).
One litre LB containing appropriate antibiotics (50 mg/l Kanamycin; 25 mg/l Gentamycin) is inoculated with 5 ml of an over-night culture of *Agrobacterium* (50 mg/l Rifampicin; 50mg/l Kanamycin; 25 mg/l Gentamycin). Grown at 28°C with a good aeration (rotary shaker, 200 rpm) to an OD (600nm)=0.8 (24hs), the *Agrobacterium* culture is centrifuged 15 min. at 2500g and then gently resuspended in 300-400 ml of infiltration medium (1/2MS + 0.15% TWEEN 80).

INFILTRATION.

Plants are left in the soil pots, only the aerial part is immersed in the infiltration solution. All material must be contained in a vacuum chamber. Vacuum is applied 10 minutes but it can take from 5 to 10 minutes to be complete. It is applied 3 consecutive times. Excess liquid

is removed from the plants which are then incubated in conditions of high humidity, in order to allow the plants to recover. When all pre-existing inflorescences arrive to maturity, the plants are left to dry progressively. Seeds are harvested in bulk from all infiltrated plants. Seeds are sterilised for 5 minutes in 1% sodium hypochlorite and washed in abundant sterile water. Dried or wet seeds can be plated for germination on solid selective medium (1/2MS + 50 mg/l Kanamycin + 0.8% Agar).
All observations and experiment are done on T1 (progeny of the primary transformant) or on T2 plants (progeny of T1).

PROTEINS ACCUMULATION AND EXTRACTION.

DRUGS SUBMINISTRATION TO PPS

The following inhibitors are added to the culture medium used to resuspend the protoplasts after rinsing the PEG and are present for the entire incubation time.
NH_4Cl (Merck, Darmstadt, Germany) is filter sterilised 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) is dissolved in ethanol and used at the final concentrations of 5 and 10 µM (Ehara *et al.*, 1996). Batilomycin A1 (Calbiochem, Lucerne, Switzerland) is dissolved in DMSO and used at concentrations of 2.5, 5 and 10 µM (Matsuoka *et al.*, 1997). Wortmannin (Sigma, Buchs, Switzerland) is dissolved in DMSO in a 10mM stock solution. Final concentrations of 15, 25, 35 and 45 µM are used (Nakamura *et al.*, 1993).

PROTEIN EXTRACTION

Plant tissue samples (<0.2 g) are reduced to powder in liquid nitrogen and incubated for 30 min in the extraction buffer (TBS 1% and SDS 2%). Insoluble proteins are eliminated by centrifugation 5 min at 14000 g. Soluble proteins precipitate in TCA 10% and are washed in acetone.
Protoplasts are harvested and resuspended in the extraction buffer (TBS 1%). Cells are lysed by 3 consecutive freezing-thawing cycles. To separate vacuolar content from most of microsome fraction, cells residues are separated from soluble fraction after a single cycle.

ENZYMATIC ACTIVITY OF RAT ß-GLUCURONIDASE AND MANNOSIDASE

Protoplasts are harvested, resuspended in 0.1 M Na-acetate pH 5 and lysed by 3 cycles of freezing (in liquid nitrogen) and thawing.
The soluble proteins are separated from insoluble residues by centrifugation. The same extraction buffer (0.1 M Na-acetate pH 5) is directly used to measure enzymatic activities of RGUS and α-mannosidase (the constitutive enzyme used as internal control). Measurements are done in a FL500 microplate fluorescence reader (Bio-Tek Instruments).
The reaction substrate is 4-Methyl-Umbelliferyl-ß-D-Glucuronide (BIOSYNTH, Staad, Switzerland) to test RGUS activity and 4-Methyl-Umbelliferyl-α-D-Mannoside (SIGMA, Steinheim, Germany) to test mannosidase activity. Tests in normal condition were normalised by comparing RGUS activity to the internal control (α-mannosidase).

WESTERN BLOT
The proteins are separated in polyacrylamide gels with SDS (15% separation gel; 4% stacking gel) using the protocol described by Laemmli et al. (1972). We use the minigel system "Mini-Protean II Dual Slab Gel System" from Biorad. and we use an electrophoresis buffer (25mM Tris; 192 mM Glycine; 0.1% SDS) at pH 8.3, 200 V are applied .
The samples separated on SDS-gel are directly transferred on a nitro-cellulose membrane (BA83; 0.2µm; Schleicher and Schuell), with a "semi-dry" Sartorius apparatus (Sartoblot II)
Procedure:
° Put 3 "3M " paper pieces (of gel size), wet with TRANSFER-BUFF (48mM Tris; 25mM glycine; 1.3mM SDS; 20% MeOH= 2.9g Tris, 1.46 g Glycine, 0.187 g SDS 100ml MeOH in 0.5 l) on the cathode.
° Put the gel on the paper (no bubbles!)
° Wet nitro-cellulose in water and cover the filter (sign the upper-right corner).
° Put 3 more layers of paper wetted in transfer buffer.
° Apply 2mA/cm^2 30 min.

IMMUNOLABELING

STEP1: The western blot is incubated in 100 ml milk-TBS 5% (20 mM Tris-Cl pH7.5; 500 mM NaCl; 5% w/v milk powder) to saturate the nitro-cellulose membrane with proteins for 45 min.
STEP2: A membrane of 40 cm^2 is then incubated in a plastic bag with 10 ml milk-TBS 1% (TBS + 1% w/v milk) containing a 1/5000 dilution of anti-GFP antibodies (Molecular Probes: A6455).

STEP3: Leave on agitation for 2 hours minimum or ON.

STEP4: Wash the filter with H_2O, 2x10 min with TTBS (250 µl TWEEN-20 in 500ml TBS), wash finally with H_2O.

STEP5: Close the filter in a new plastic bag with 10ml milk-TBS 1% containing a dilution 1/30000 of anti-rabbit secondary antibody conjugated to alkaline phosphatase (SIGMA: A-3687) incubate for 2 hours.

STEP6: Wash the filter with H_2O, 3x10 min with TTBS, wash finally with H_2O.

STEP7: Put the filter in a new plastic bag, drain liquid excess but without letting the blot dry. Add Substrate solution (1ml substrate + 50 µl enhancer) IMMUN-STAR chemioluminescent protein detection system Biorad.

STEP8: After 5 min incubation change plastic bag and expose (do not dry the filter!)

PULSE-CHASE on GFP transient expression.

I end II DAY:

To perform pulse-chase experiments, use a standard protoplast preparation (final concentration ~2-2.5*10^6/ml).

The equivalent of two transformations per construct are needed.

STEP1: The usual two hours after transformation, pool the two transtormation (for each construct) in one tube add one volume W5 and pellet.

STEP2: Resuspend transformed PPS in 0.5 ml K3.

PULSE:

STEP3: Add 3.5 µl PRO-MIX (70% L-[^{35}S] methionine and 30% [^{35}S] cysteine; Amersham, UK) cell labelling mix (3 Mbq/ ml)

STEP4: Incubate 1 hour 28°C

CHASE:

STEP5: Add 0.5 ml K4 (or K3) + 25µl 10 mM cold methionine and 5mM cold cysteine.

STEP6: At variuous times, transfer a 0.2 ml aliquot to a 2 ml tube, add 1ml W5, centrifuge shortly at 600 rpm and resuspend pellet in 50µl extraction buffer (PBS1X; NP40 2.5%; SDS 1%) -Freeze

For time 0 take the aliquot immediately after addition of the chase.

IMMUNO-PRECIPITATION:

III DAY:

STEP7: Boil extracts 10'
STEP8: Spin 3' 13000 g, save supernatant.
STEP9: Add 130 µl H_2O + 50 µl NP40 + 20 µl PBS 10X
STEP10: Add 1 µl anti-GFP antibodies
STEP11: Incubate over night.

IV DAY:

STEP12: Spin 2' 13000 g, save supernatant
STEP13: Add 250 µl of 1X PBS, 0.2% SDS, 2.5% NP40.
STEP14: Add 20 µl Protein A Sepharose
STEP15: Incubate 2 hours with agitation at R.T.
STEP16: Pellet resin by spinning at 1500 rpm
STEP17: Wash 4 times with 1ml wash buffer (PBS 1X+ NP40 1% + SDS 0.2%)
STEP18: Add to resine 20 µl charge buffer + 2-mercaptoethanol; Boil10'
STEP19: Charge on Gel SDS-PAGE (see above).
STEP20: Fix the gel in acetic acid 10% and isopropanol 25% -30'
STEP21: Treat 30 min in Amplify solution from Amersham
STEP22: Dry the gel on 3M paper
STEP23: Expose

ENDOGLYCOSIDASE H RESISTANCE

Start from standard immuno-precipitation samples.
Proteins adsorbed to Sepharose are eluted at 70 °C for 20 min in 100 mM TrisHCl, pH7.8, with 0.5 % SDS and 1% 2-mercaptoethanol (20µl), and then the beads are pelleted by centrifugation at 14000 g for 5 min.

STEP1: Dilute supernatant with nine volumes of 0.1 M sodium acetate, pH 5.6, supplemented with BSA (1mg/ml final conc.) and proteinase inhibitors (1µg/ml leupeptin, pepstatin A). This mixture is divided in two aliquots,
STEP2: Add 5 mU endo H (diluted 1mU/µl) to one and 5µl H_2O in the other; incubate 16 h.

STEP3: Precipite by TCA (10%) and wash in acetone before resuspending in Protein-sample-buffer. Separate by SDS-page and reveal as for a pulse-chase experiment

CELL TREATMENTS AND PREPARATION FOR MICROSCOPY

NEUTRAL RED STAINING

Neutral red (NR) (Fluka, Buchs, Switzerland) is dissolved directly in the culture medium at a concentration of 10 mg/ml. Final concentration for staining is 1 mg/ml. Cells are incubated with NR for 30 min. at room temperature. One volume of osmoticum is added, cells are centrifuged 5 min. at 80g, resuspended in 1 volume of fresh culture medium and evaluated within 30 min.

PROTOPLASTS FIXING

Protoplasts can be fixed at any stage of expression.
Cells are harvested in W5 solution by centrifuging at 80g, and resuspended gently in 1 ml FIXING SOLUTION.

FIXING SOLUTION has to be prepared freshly:

0.4 g of paraformaldehyde (FLUKA) are dissolved in 10 ml water in a boiling water bath; add to the obtained solution 2 drops of NaOH 0.1N to obtain a clear solution. Cool down the solution to R.T. and add mannitol (0.91 g to a final concentration of 0.5 M) and Hepes (from a stock at pH 5.8 to a final concentration of 50 mM) .

Cells are incubated at R.T. 1 hour with periodic gentle mixing. They can now be labelled or stored at 4°C for a few days.

PROTOPLASTS IMMUNOLABELLING

STEP1: Fixed cells are washed 3 times with one volume of BLOCKING BUFFER (1X PBS; 0.25% BSA; 0.25% Gelatine; 0.05% NP40; 0.02% azide; 0.5M Mannitol)

STEP2: Incubate *cells* in BLOCKING BUFFER containing 0.5% *Triton* X100 freshly prepared, for 5 min.

STEP3: Wash cells as in step 1.

STEP4: Incubate cells in BLOCKING BUFFER containing 1% BSA for 20min, to saturate non-specific interactions.

STEP5: Wash cells as in step 1.

STEP6: Incubate cells in BLOCKING BUFFER containing 5 ng/µl primary antibody for 1 to 2 hours; JIM84 antibodies obtained from rat hybridomas, were a kind gift of Chris Hawes, (Satiat-Jeunemaitre

and Hawes, 1992); anti α-TIP antibodieswere a kind gift of John Rogers (Johnson *et al.*, 1989).

STEP7: Wash cells as in step 1.

STEP8: Incubate cells in BLOCKING BUFFER containing a dilution 1/30000 of anti-rabbit secondary antibody conjugated to lissamine rhodamine (SIGMA: A-3687) incubate for 2 hours.

STEP9: Transfer cells in a new tube and wash as in step 1.

STEP10: Observe cells with the confocal microscope.

Chapter 7

GENERAL DISCUSSION

Once upon a time...

When this thesis work was started in 1995 the plant vacuole was still considered by many plant physiologists as a unique compartment, the largest organelle of the plant cell with very diverse functions. Plant anatomists and microscopists knew cases of coexistence of two different vacuoles in cells of certain tissues, vacuoles differing by their contents (pigments, tannins, etc.) (Michel *et al.*, 1992). The plant vacuole was known to be a lytic compartment, the equivalent of mammalian lysosomes, to be involved in the storage of organic and inorganic nutrients and metabolites, but also to be important for the generation of turgor and cell growth. Since a few years the study of different vacuolar proteins revealed that in plants the situation was more complex than in animals or yeast. The signal for sorting most soluble proteins to mammalian lysosomes is the phosphorylation of terminal mannose residues on N-linked glycans whereas proteins destined to the vacuole in yeast have an N-terminal peptidic targeting signal. In contrast, in plants, proteins sorted to the vacuole have one of at least three types of sorting signals: the C-terminal, signal-specific or physical-structure Vacuolar Sorting Determinants (ctVSD; ssVSD; psVSD; see general introduction). In 1995 the first evidence was published that at least two different mechanisms exist for the transport of soluble proteins with different VSDs to the vacuole in plants (Matsuoka *et al.*, 1995).

Accumulation of storage proteins in seeds is the most dramatic example of the necessity to sort proteins differently in different cell types, but the operating hypothesis at that time was that protein storage vacuoles derived directly from the same vacuole that has acidic pH and hydrolytic functions. The evidence for the presence of two completely distinct vacuolar compartments, with different membrane composition and different functions, was presented in 1996 (Paris *et al.*, 1996) but was limited to the undifferentiated cells of root tips from germinating pea seedlings. In 1998 results described in this thesis (Chapter 2), were published as demonstration of the presence of two distinct vacuoles with different pH in tobacco mesophyll cells (Di Sansebastiano *et al.*, 1998). At the end of the same year a third vacuole was clearly identified (Jauh *et al.*, 1998). It is easy to predict new

surprising developments of our comprehension of the plant secretory pathway.

What could we do?

To investigate the sorting of proteins to the vacuole and the mystery of the multiple functions of this compartment, we developed reporters with characteristics similar to the soluble proteins characteristic of functionally different vacuoles. We started using a modified rat ß-glucuronidase (RGUS) to which we fused a ctVSD (Annexe to chapter 2). RGUS was useful to check if the system we planned to use reflected the situation *in vivo*. We transiently transformed tobacco protoplasts with the reporter protein and concluded that this system allowed the measurement of vacuolar targeting efficiency. It was then possible to interfere with the sorting of the reporter protein by adding a number of chemical agents, deriving informations about the sorting mechanisms. Unfortunately RGUS did not give the possibility to discriminate between different kind of vacuoles. All vacuoles were isolated as a unique subcellular fraction with mixed characteristics, a technical limit found until now in all investigations on the plant vacuoles, except in microscopy.

As emphasised by many authors, the green fluorescent protein (GFP) used for the first time in 1994 (Chalfie *et al.*, 1994) brought light to the labyrinth of membranes in the secretory pathway. As other research groups we used GFP as reporter to study the system of our interest. We produced GFP fusion proteins containing the vacuolar sorting determinants of different vacuolar proteins: a first vacuolar GFP was made with a ctVSD from the tobacco chitinase A (SGFP5T; chapter 2 and 3) and a second vacuolar GFP with a ssVSD from barley aleurain (AGFP6; chapter 3). We obtained unexpected and important results.

A small contribution to a complex investigation.

The first vacuolar GFP we tested, SGFP5T, was targeted to vacuoles with a neutral pH. This lead us to the conclusion that the large central vacuole of tobacco protoplasts 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 neutral red (NR, a dye that is trapped at low pH), may be similar to some barley root tip cells that were found to accumulate barley lectin (a ctVSD-containing protein) in a functionally distinct compartment from aleurain, a ssVSD-containing protease.

We obtained a specific labelling of the storage compartment in living cells while the lytic compartment could be counter-stained by NR. Whether these two distinct compartments coexist in all cell types isolated from tobacco leaves is difficult to say, but we showed that functionally distinct vacuoles can be present in a large variety of cell types. The fact, that not all plant cells do contain two functionally distinct vacuoles explains why proteins targeted by two different VSD types were found to accumulate in the same vacuole in tobacco (Schroeder *et al.*, 1993).
Our results with SGFP5T strongly support the existence of two vacuolar compartments. These distinct vacuoles correspond to the destinations for two different VSD-mediated pathways, the ctVSD sending SGFP5T to a non-acidic storage vacuole in a wortmannin-sensitive way.
To complete the study about the two sorting pathway, we compared the SGFP5T to AGFP6, obtained by the fusion of GFP with the ssVSD of aleurain. AGFP6 contains the mutation S65T to enhance its fluorescence. It is in fact targeted to the lytic vacuole as expected, but it does not accumulate in large quantities as SGFP5T but is rapidly degraded (see chapter 3). Confocal laser scanning microscopy was used to put on evidence the different steps of the sorting systems of the two GFPs. This revealed an interesting difference between the patterns of GFP accumulation for the two constructs. The pattern of SGFP5T accumulation led us to conclude that this GFP fusion protein transited through the ER and was accumulated either in small vacuoles or in the large central vacuole. In contrary AGFP6 spent such a short time in the ER that we only could see it when cells were incubated at low temperature. It transited through small compartments not described until now, which seem to be a sort of prevacuolar compartment. Sometime the AGFP6 then reached the large central vacuole but it did that in two protoplast subpopulations in proportions different from those observed with SGFP5T. If we consider only the more homogeneous subpopulation of chloroplast-rich protoplast, representing cells from the palisade mesophyll, we can see a clear correspondence in the percentage of cells in which AGFP6 occupied the large vacuole and cells in which SGFP5T was limited to small vacuoles and ER.
The most important argument for a difference between AGFP6 and SGFP5T vacuoles was neutral red (NR) accumulation. Contrary to the situation observed for SGFP5T, colocalisation of AGFP6 and NR was found in the majority of cells with a fluorescent vacuole, indicating the accumulation of AGFP6 in an acidic vacuole. We assume that the AGFP6 is targeted to the lytic vacuole because of three considerations: first the ssVSD derives from a protease targeted to the lytic vacuole, second

AGFP6 undergoes a rapid degradation, third low pH is generally associated to the lytic compartment.
Having shown that SGFP5T and AGFP6 are transported through different pathways to different vacuoles, we had the tools to investigate *in vivo* the vacuole biogenesis. Working on protoplasts we faced a complex situation in which these dedifferentiating cells underwent a series of unclear modifications. The compartments labelled by GFPs are in part pre-existing, in part generated *ex novo*. Confident to have the possibility to clearly label different vacuoles, we looked for a system in which it was possible to investigate whether these separate vacuoles had a different genesis or derived from a common precursor. We turned our attention to the possibility of modifying the situation in the protoplasts we had used so far. Following a suggestion from E. Martinoia we reset the secretory system by evacuolation of the protoplasts.
We used the GFP reporters to monitor vacuole regeneration in evacuolated protoplasts. We observed that evacuolated protoplasts regenerate several small vacuolar compartments at the same time, some of which contained SGFP5T, while others contained AGFP6. One of the vacuoles became the most important vacuolar compartment of the cell after 36 hours. AGFP6 was accumulated in this main compartment with few exceptions and its fluorescence was very bright. SGFP5T remained limited to small vacuoles. While its quantity increased, SGFP5T was not observed in the newly formed large central vacuole earlier than 48 hours after evacuolation, when some large green vacuoles began to appear. It seems that the vacuole that re-established the normal cell volume, was generally the lytic vacuole which accumulated AGFP6 but we could not exclude that in some cases the same kind of vacuole did not contain either AGFP6 or SGFP5T. Vacuoles containing SGFP5T are produced by the cell at the same time as the lytic vacuole but are limited in volume and do not seem able to participate in the control of the cell volume.
Our considerations have been driven by the following observations: AGFP6 and SGFP5T are targeted to different vacuoles but they can be eventually found in some conditions in the same vacuole. This corresponds to the finding that barley lectin and sporamin were colocalised in the central vacuole of all tissues examined in transgenic tobacco (Schroeder *et al.*, 1993).
Our model is that cells can fuse their lytic and storage vacuole but this doesn't happen automatically in all cell types. The moment, in which a cell fuses storage and lytic vacuoles to a single central vacuole, may depend on the cell type and its physiological state. In normal protoplasts, soon after their preparation, the majority of cells are

ready to promote the fusion of newly formed storage vacuoles to the pre-existing vacuole. In miniprotoplasts, where a pre-existing vacuole is missing, the main vacuolar compartment is regenerated well separated from the storage compartment. In few cases the new vacuole does not contain AGFP6 either and may represent a third class of vacuoles.

We performed some preliminary experiments to investigate • the tonoplast constitution of the observed compartments using antibodies against TIPs. So far we confirmed that the regenerating vacuoles containing SGFP5T also contain α-TIP, while this epitope was (as expected) excluded from the tonoplast of regenerating vacuoles containing AGFP6. We believe that the SGFP5T vacuole is a storage vacuole (PSV), and the AGFP6 vacuole is a lytic vacuole (LV), but it is not yet possible to exclude that in protoplasts from the leaf blade, these definitions do not correspond exactly to the functions of the two vacuoles. The PSV of these cells could share some functions with the LV while remaining distinct.

An invitation to revise current beliefs.

The result presented in this thesis showed a higher complexity of the vacuolar system than expected and contributed to the distinction of the two sorting machineries analysed.

We discovered for example a differential effect of Brefeldin A on the two sorting machineries targeting SGFP5T and AGFP6 to the vacuoles. We found indications that suggest a revision of our understanding of Golgi function in sorting of vacuolar proteins . In presence of BFA doses proven to affect the Golgi structure, we observed no alterations in AGFP6 distribution, nor changes in its maturation.

To explain this observation of normal patterns for AGFP6 even in BFA treated cells, we think that the vesicles are formed on the ER, where the Golgi is reabsorbed. In our reporter protein we don't observe alteration of the maturation process because there are no visible maturation steps occurring in the Golgi. A protein with sites for cleavage or glycosylation that could be modified, could show an alteration of these steps while still suffering no mistargeting, providing the molecular tool to answer the question whether or not this protein transits through the Golgi.

In the case of barley aleurain, it was shown that the proteolytic processing was inhibited by BFA in barley aleurone cells (Holwerda and Rogers, 1990) and in tobacco protoplasts (Holwerda *et al.*, 1992). This processing step was supposed to happen in a post-Golgi location but no clear information indicate that this step is necessary for a correct targeting. The relations between Golgi structure and vesiculation

demand further investigation and our results are a starting point for further experiments in this direction.

What's coming?

Nevertheless the final aim of our work was the understanding of the vacuolar complex in "normal" plant cells. As protoplasts or other cultured cells do not represent the physiological situation of cells in differentiated tissues, it was necessary to continue this investigation on whole plant tissues. For this purpose we produced stably transformed plants both from tobacco and *Arabidopsis*. These two plants are considered as model plants and are used around the world to investigate the general physiology of plants. We discovered that these two plants have some surprising differences between their vacuolar systems.

Plant tissues have very different functions and it was not surprising that different cell types exhibit a different vacuolar pattern (see chapter 5). On the other hand it was surprising that in tobacco it was not possible to recognise the same vacuole distribution as in *Arabidopsis*.

In *Arabidopsis* AGFP6 was accumulated in large vacuoles of the epidermis, trichomes, root hairs and mesophyll cells. Young roots, meristems and the other tissues showed GFP, if at all, only in small vacuoles. On the other side SGFP5T was accumulated in large vacuoles in the guard cells, trichomes, mesophyll cells but not in the epidermis or in the root hair; SGFP5T was often visible in small compartments in the majority of tissues. Surprisingly in tobacco AGFP6 was not visible at all and SGFP5T was limited to small vacuoles in a few types of cells (see chapter 5). It is possible that in most cell types both GFPs end in a common central vacuole and that this vacuole has a different lytic activity in the two plant species. *I* think that in tobacco there is such a strong proteolytic activity that we cannot observe any GFP accumulation in the lytic compartment. We can visualise GFP in tobacco only if it is accumulated in well separated storage compartments (SGFP5T). In *Arabidopsis* the common vacuole does not have such a dramatic degradative effect on GFPs. In *Arabidopsis* we find that AGFP6 is matured at a site close to the maturation site of aleurain, while we never observed this maturation of AGFP6 in tobacco (chapter 5). This means that in *Arabidopsis* the GFP arrives in a protease-containing compartment but is not degraded. It would be important to confirm that tobacco vacuoles are more degradative than *Arabidopsis* vacuoles. The production of transgenic plants offers the possibility to investigate the adaptations of the secretory systems to the cell function in a specific tissue or the evolutionary adaptation of different plants to their

environment. But to fully understand this complex machinery, it will be necessary to identify the proteins involved in these complex pathways. Because other molecular approaches have failed, transgenic plants again offer a new solution. By producing transgenic plants expressing very informative reporter proteins like GFP, we succeeded to obtain model plants in which mutagenesis and the search for mutants could help to clone genes involved in this pathway. The method is known as gene-tagging and has been only briefly described since it represents the natural future development of the present work.

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