The study of SNAREs specificity in vivo.

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Abstract

SNARE proteins are the principal actors of the concluding step of membrane traffic, the fusion of a vesicle with its acceptor membrane. The specificity of their interactions has been studied mostly in vitro and is now waiting for innovative approaches to elucidate the highly complex in vivo situations with which the multicellular organisms have satisfied the need for polarized and regulated secretion or vacuolar/lysosomal/endosomal trafficking. Plants, because of their high number of SNARE genes, their complex vacuolar system and peculiarity of established models for studying polarised and regulated exocytosis (pollen tube, root hair), represent a particularly challenging system for understanding the fine details of SNARE functions. The specificity of SNARE interactions resides in their structure but is certainly modulated by the cellular environment and a number of accessory factors; consequently, only in vivo experiments will reveal the full complexity of SNARE regulated processes. This review will highlight the major areas of interest and the approaches to study plant SNAREs specificity in vivo.

Overview on SNAREs

The very complex endomembrane system of eukaryotes probably originated initially from invaginations of the plasma membrane during evolution of a phagotrophic lifestyle, differentiating various spatially and functionally separated compartments (Roger, 1999; Cavalier-Smith, 2002). Material exchange is mediated by cargo-loaded vesicles that bud from the donor and fuse with the acceptor compartment. A key feature of vesicular trafficking is the strict specificity in the fusion reaction, such that vesicles will deliver their cargo exclusively to the target organelle. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins are the principal specificity determinants in the concluding step of membrane traffic, the fusion of a vesicle with its acceptor membrane. SNAREs are a family of small cytoplasmic orientated membrane-associated proteins with a relatively simple architecture. Their characteristic is the so-called SNARE motif, an extended segment arranged in heptad repeats. In most SNAREs, a short linker ends with a single C-terminal transmembrane domain.

They can be described as tail-anchored (TA) proteins, which are inserted post-translationally into the membrane via their C-terminal hydrophobic domain (Borgese et al.,
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This targeting process distinguishes them from classical type II membrane proteins, which are delivered to the ER by the SRP-dependent co-translational pathway. Usually very few polar residues are present in TA proteins downstream of the C-terminal hydrophobic domain and it is difficult to verify whether they span the bilayer. However their exact topology remains controversial even if it has been demonstrated that some TA proteins can translocate their COOH terminus across the bilayer (Kutay et al., 1995; Pedrazzini et al., 2000; Di Sansebastiano et al., 2006). It seems that TA proteins are firstly inserted into the ER membrane and then delivered to their final destination by vesicular transport (Jantti et al., 1994; Pedrazzini et al., 1996; Di Sansebastiano et al., 2006). Sorting signals for SNAREs are poorly characterized. The length of the TMD (Brandizzi et al., 2002a) does not seem, in SNAREs, to be a determinant for localization but in some cases di-leucine motifs (Kasai and Akagawa, 2001), the longin motif or the SNARE motif itself (Hasegawa et al., 2004) have been found relevant for targeting.

Proteins of the SNARE family function as molecular machines, self-assembling into a cluster of elongated and parallel four-helix bundle during fusion (Hong, 2005). In the interior of the bundle, the four helices are connected by 16 interaction layers with the central interaction layer consisting of three glutamine (Q) residues and one arginine (R) residue, contributed by each particular SNARE molecule. The presence of these residues led to the classification of SNAREs into Q- and R-SNAREs (Fasshauer et al., 1998). The Q-SNAREs may be further classified into three major subfamilies (Qa, Qb and Qc), based on the amino acid sequence homologies of the SNARE domain. The functional SNARE complex brings together these four kinds of helices in accord with the structural “QabcR-rule” (Bock et al., 2001).

In vitro, extensive biochemistry and protein biophysics have revealed much about the mechanism underlying SNARE function. Observed fusion of liposomes carrying synaptic SNARE proteins demonstrated that the SNARE complex formation itself is fully sufficient to drive membrane fusion (Weber et al., 1998). More recently, a second assay has been developed in which the normally cytosol-facing SNARE proteins are ectopically expressed on the surface of cells to drive whole-cell fusion (Hu et al., 2003).

SNARE complex function (Lipka et al., 2007) has been well documented by both in vivo and in vitro systems in many eukaryotes. The specificity of SNARE interactions has been also studied in vitro and in vivo but is now waiting for innovative solution to elucidate
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some of the most complex situations, including polarized and regulated secretion or vacuolar/lysosomal/endosomal trafficking. It is unclear how the morphological diversity of eukaryotes is reflected in their SNARE complement. For example the highest number of SNAREs was discovered in green plants (Sanderfoot, 2007) but it is not yet known whether the additional SNAREs mediate novel trafficking steps.

An example can be the endosomes-TGN (Trans Golgi Network) relation in plants. It was recently hypothesised that the TGN may function as an endocytic compartment in plants as in animals (Dettmer et al., 2006). Because of direct observation of TGN proteins (Rehman et al., 2008a) we support the opinion that TGN-EE (Early Endosome) compartments are closely related but not necessarily identical as demonstrated also by other researchers observing the effects of BFA on endomembrane system (Wee et al., 1998; Geldner et al., 2001; Grebe et al., 2003). The TGN and the EE are certainly strictly related and they may coincide in some cells but we consider it premature, at this stage, to generalize as done by Lam and co-workers (Lam et al., 2007b) in predicting that the PRC (Partially Coated Reticulum) PRC (Pericentriolar Recycling Compartment)/TGN/EE are in fact the same compartment.

The most complex endomembrane system.

Early land plants arose from a group of aquatic green algae related to Chara, themselves related to unicellular green algae similar to Chlamydomonas. Flowering plants are the most recent of this long lineage and their unicellular ancestors are completely independent from animal evolution (Sanderfoot, 2007; Dacks et al., 2008). These plants represent a challenging material for future investigations, because of their higher number of SNARE genes, almost double than mammals and triple than Saccharomyces or Drosophila (Pratelli et al., 2004), their complex vacuolar system and the lack of easy-to-study model-cell for polarized and regulated exocytosis (Figure 1).

The number of SNARE-encoding genes increases in the vascular plants compared to the unicellular plants. In addition to an increase in the actual number of genes, new gene families and subfamilies appear among the SNARE genes with products that are associated with secretion and with the endosomal system. The increase in gene number for the endosomal SNAREs might be associated with the development of the typical extended vacuolar system of the plant cell (Figure 1).
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Angiosperms have between 62 and 76 SNAREs, only a few more than mosses (63 in *P. patens*) but twice the number present in unicellular green plants (30 to 35). Often, SNAREs that are single genes in unicellular organisms are present as small gene families, or multiple novel gene families that may have gained highly specialized functions.
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The increase in gene number may be associated with the rise of multicellularity that would require novel trafficking functions such as polarized secretion (Sanderfoot, 2007) and would result in SNARE specialization in novel processes (Lukowitz et al., 1996; Collins et al., 2003; Leucci et al., 2007b).

As described above, only in vivo experiments may reveal the full complexity of SNARE dependent processes.

This review will highlight the studies about SNARE specificity in exocytosis and in vacuole organization, demonstrated by in vivo approaches: genetic knock-outs, introduction of dominant-negative inhibitory fragments, and overexpression of mutant/modified SNARE constructs.

**Early steps of the secretory pathway.**

SNAREs that operate between the ER and Golgi apparatus, or within the Golgi complex, are similar in yeast (S. cerevisiae), mammals and green plants (Sanderfoot, 2007).

At the TGN level, plants again have SNAREs similar to mammalian and fungal proteins. The plant Qa-SYP4 family, localized to the TGN (Bassham et al., 2000), is similar to the mammalian syntaxin 16 and the yeast Tlg2p. Its members are topologically localized to distinct domains of this compartment, as shown for SYP41 and SYP42 (Bassham et al., 2000). Qa-SYP41 in particular was shown to distinguish between two members of the Qb-VTI1 gene family products, indicating some specialization among multiple copies of these SNAREs in angiosperms (Sanderfoot et al., 2001). In addition, the knock-out mutants for SYP41 and SYP42 were found to be male gametophytic lethal, further illustrating the specialization of the members of the SYP4 family.

**SNARE specificity in exocytosis.**

Many distinct types of Qa-SNAREs reside on the plasma membrane and may have diverse and specialized functions in delivery of vesicles to its various domains (Hong, 2005). The single-copy chlorophyte gene of this kind, SYP1, splits in embryophytes into two large groups: SYP12 and SYP13. The SYP12s were found on the PM and are likely involved in various aspects of secretion (Geelen et al., 2002; Di Sansebastiano et al., 2006; Leucci et al., 2007b; Tyrrell et al., 2007). The initial studies of knock-out mutants in SYP12 gene
family revealed that they might have overlapping functions (Assaad et al., 2004) but further studies of plant-pathogen interaction pointed out that the molecular functions by which SYP121 affects penetration resistance and negatively regulates other defences are different, as only the latter functions are shared with SYP122 (Zhang et al., 2007). In addition to the knock-out approach, the level of specialization and specificity in SNARE gene families can be studied by expression of SNARE protein fragments that interfere with the normal SNARE function in stable or transiently transformed plant cells. Using this approach, a cytosolic fragment of SYP121 (SP2) was found to have severe effects on growth, tissue development and secretion (Geelen et al., 2002). Consequently, transport to the PM was affected by the dominant-negative fragments of SYP121 and 122 but not those of SYP111 or SYP21 (Tyrrell et al., 2007).

The SYP132 protein was localized to the PM when overexpressed in protoplasts (Uemura et al., 2004) and its Medicago truncatula homologue may be involved in symbiogenesis (Catalano et al., 2007). Recently Kalde and co-workers (Kalde et al., 2007) evidenced that the NbSYP132-dependent secretion pathway was independent from SYP121 in the context of pathogen response. While SYP121 is dispensable for the resistance against bacteria, SYP132 contributes significantly, likely through secretion of defense-related cargo. Interacting regulatory factors may be involved in the functional diversification of SNARE complexes. In this direction we addressed our experiments using a new experimental approach that combines “in vivo” characteristics and easy measurement of an enzymatic reporter secretion (Di Sansebastiano et al., 2007b). By this method the involvement of a Rab11a homologue can be tested on secretion dependent on SYP121 or SYP122. Our data showed that SYP122 and Rab11a are involved in the same transport pathway, opening the path to further comparison of multiple proteins (Rehman et al., 2008a). Compared to secGFP quantitative analysis (Geelen et al., 2002; Leucci et al., 2007b) this method has the advantage of a simpler and more direct quantification, but its application is restricted to dedifferentiating protoplasts. However, the use of dominant negative mutants to disrupt membrane traffic in vivo, appears as a valuable addition to common approaches based on mutagenesis and complementation screens that provided characterizable developmental (Lukowitz et al., 1996; Heese et al., 2001) or cellular phenotypes (Sato et al., 1997; Zhu et al., 2002; Yano et al., 2003).
SNARE specificity in the late endosome and vacuole

 Trafficking to the vacuole in the late secretory pathway is essential (Rojo et al., 2001) and extremely complex, possibly with a high degree of cell type dependent variability. Many SNAREs are involved in membrane trafficking to the tonoplast including Qa-SNAREs SYP21 and 22, Qb-SNAREs VTI11 and 12, and Qc-SNAREs SYP51 and 52. Referring to various co-localization and co-immunoprecipitation experiments (Zheng et al., 1999; Sanderfoot et al., 2001; Surpin et al., 2003; Yano et al., 2003); it seems that both SYP21 and 22 interact with VTI11 and SYP51 at the prevacuolar compartment and/or the tonoplast, and the VTI12, SYP41/SYP42 and SYP61 SNAREs form a complex at the TGN. The VTI proteins are partially redundant in function, their corresponding null mutants were not lethal like for other SNAREs required in vacuolar traffic (SYP21/22/41/42) (Sanderfoot et al., 2001) but the phenotype was not easily interpreted. The vti11 knock-out has defects in shoot gravitropism (Kato et al., 2002), whereas the vti12 mutant displays characteristics of the autophagy mutants (Surpin et al., 2003). More recently it was possible to clarify the phenotype of the vti mutants using VAC2 as a new cargo reporter (Sanmartin et al., 2007). VAC2 is a vacuolar reporter derived from CLV3, a known regulator of the shoot stem cell proliferation, fused to a vacuolar sorting determinant. The analysis revealed that VTI11 was associated to clathrin-dependent transport to the lytic vacuole whereas VTI12 participated in trafficking of vacular storage proteins.

 Detailed functional studies on closely related SNAREs (Sanmartin et al., 2007; Rehman et al., 2008a) may help to find the residues that define the functional differences. For example a mutated allele of VTI12, zip1, was recently found to suppress the gravitropic defects of vti11 mutants with a single amino acid substitution in a strictly conserved domain (Niihama et al., 2005), suggesting that a single amino acid substitution may be responsible for a remarkable change in specificity.

 It is known that SYP21 is essential for cell development since the gene knock-out was lethal to the male gametophyte (Sanderfoot et al., 2001). Because of this extreme phenotype, it was not possible to reveal the cellular function by this method. An alternative is certainly the use of inducible knock-out but a second, more immediate alternative is a transient over-expression of mutant (Tyrrell et al., 2007) and native (Foresti et al., 2006) forms of the protein. Both assays proved to be valuable and greatly complementing each other. Tyrrell an co-workers (Tyrrell et al., 2007) found that the soluble dominant-negative
variant of SYP21 in Arabidopsis blocks traffic of TIP1;1-YFP chimera to the tonoplast, even if Foresti and co-workers (Foresti et al., 2006) were not able to find a similar effect on their, differently selected, markers in tobacco. On the contrary, the SYP21 over-expression specifically interfered with vacuolar cargo delivery without affecting other pathways such as exocytosis (Foresti et al., 2006), while the SP2 fragment of SYP21 had no measurable effect (Tyrrell et al., 2007). The over-expression experiments also suggested that the over-expressed SNAREs or their fragments might form fusogenic or non-fusogenic complexes, with various effects on membrane trafficking. A previous work from Varlamov and coworkers (Varlamov et al., 2004) postulated that even non-fusogenic SNARE complexes (i-SNAREs) could have physiological functions.

As a conclusion we must say that the mechanisms and the actors of the last sorting steps from PVC and/or Golgi to the vacuoles and between vacuole-like compartments are poorly understood. The regeneration of vacuoles in mini-protoplasts evidenced how different vacuolar compartments co-exist for a period of time variable in different cell kinds, suggesting a differential regulation better than a simple physical process driving tonoplast membrane fusion (Di Sansebastiano et al., 2001a). One of the few studies about SNARE-dependent fusion between vacuole-like compartments, involved a member of VAMP71 family of tonoplast-localized SNAREs (Carter et al., 2004). This study described the fusion of endosomes containing reactive oxygen species and the vacuole, depending on the VAMP711 expression. In fact, this fusion was blocked in the absence of VAMP711 (Leshem et al., 2006). Coherently, knock-out mutants have increased salt tolerance since they reduce production of reactive oxygen species stimulated by high-salt conditions (Leshem et al., 2006). Moreover we cannot forget that a Golgi independent pathway may exist (Park et al., 2004; Oufattole et al., 2005) and no SNARE candidates have been investigated on this regard.
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**Convergence of endocytic and vacuolar traffic, can SNAREs help?**

The possible convergence of endocytic and vacuolar pathways have been recently proposed. Evidence for a merging of secretory and endocytic protein sorting came from the localization of VSRs in relation to FM4-64 uptake in BY-2 cells (Tse et al., 2004). It appears that EE and TGN membranes share most characteristics (Dettmer et al., 2006; Lam et al., 2007b) and may represent the convergence point. If we recognize the TGN as the EE, it remains to be determined whether traffic from the Golgi apparatus to the PVC (Pre Vacuolar Compartment)/LE (Late Endosome) goes via the EE. Considering the separation of RabA2/A3- and VHA-a1-marked compartments, especially at cytokinesis, it might be prudent not to consider the TGN and the EE as a single compartment (Chow et al., 2008). LE in plants have a multivesiculate morphology and are enriched in PI(3)P (phosphatidylinositol 3-phosphate), they contain SYP21 syntaxins and polypeptides of the retromer and endosomal sorting complexes required for transport (ESCRT), as well as Rab5-type GTPases (ARA6, ARA7, RHA1) at their surface (Jaillais et al., 2008; Robinson et al., 2008). They were proposed the name of sorting endosome but this is not yet fully accepted (Robinson et al., 2008).

SNAREs certainly drive trafficking to these various compartments and may serve to univocally define them. Therefore the complexity of endosomal compartmentalization in plants may be elucidated through the full characterization of these proteins.

**SNAREs unique to plants.**

Several new Qb- and Qc-SNARE types, not found in animal or fungal genomes, were identified in Arabidopsis (Sanderfoot et al., 2000). Homologues of SYP7 and NPSN are present in simple eukaryotes like amoebae but not in animals or fungi, and as a consequence they have not received proper attention. In fact these proteins are usually found in eukaryotes that lack the Qb + Qc-SNAP25-type SNARE involved in secretion and exocytosis (Hong, 2005; Sanderfoot, 2007). In Arabidopsis where SNARE of the SNAP25-type play essential roles in exocytosis (Kargul et al., 2001; Collins et al., 2003) and cytokinesis (Heese et al., 2001), it has been shown that Qb-NSPN11 and Qc-SYP71 interact with the cell-plate-specific Qa-KNOLLE, and have a role in the secretion-related process of cytokinesis (Zheng et al., 2002; Sanderfoot, 2007). Moreover, these proteins are found on
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various organelles in the late secretory system of non-dividing cells (Zheng et al., 2002; Marmagne et al., 2004; Morel et al., 2006).

Qc-SYP7 fused to a fluorescent tag was localized on the ER (Uemura et al., 2004), but proteomic studies found them on different membranes (Marmagne et al., 2004; Mongrand et al., 2004; Morel et al., 2006). Whether these conflicting results are simply an artifact of over-expression or a reflection of specialized roles is not known but they highlight the importance of confirming results in conditions closest to the in vivo situation.

**Calcium mediated membrane fusion: SNAREs and synaptotagmins**

As reported above, SNAREs may be sufficient to drive membrane fusion but do not operate alone. Relative to regulated exocytosis, SNARE fusion is slow in vitro. In part, this reflects a slow activation step of the t-SNARE complex, since regulatory regions at the amino terminus and the membrane interface must undergo conformational changes to allow assembly with the v-SNARE (Melia et al., 2002).

SNARE fusion is inherently calcium-insensitive but the crucial role for additional factors in calcium-mediated fusion is evident (Melia, 2007). The basic fusion machinery is common to both regulated and constitutive form of vesicle fusion. Thus an important feature of regulated exocytosis is that docked vesicles, which have already engaged the SNARE fusion machinery, do not fuse spontaneously at any appreciable rate. A natural consequence of selectively arresting exocytosis at the level of SNARE assembly is that release can be tightly coupled to the calcium influx, such that otherwise rate-limiting steps upstream of SNARE assembly will already be complete. Calcium influx is detected by proteins called synaptotagmins. Mutations of synaptotagmin reduce or eliminate the apparent cooperativity of calcium in exocytosis, alter the vesicle release probability, and shift the calcium sensitivity (Fernandez-Chacon et al., 2001). Upon addition of calcium, synaptotagmin I enhances SNARE-mediated liposome fusion possibly influencing SNARE assembly and/or lipid-specific perturbation (Bhalla et al., 2006). What constitute the clamp that arrests vesicles in the synchronous release pool, and how this clamp responds to calcium binding at synaptotagmin have until recently been unknown (Melia, 2007). In the animal system of squid presynaptic terminals, weak interactions between SNAREs and other proteins called complexins have been evidenced in pull-down experiments (Tokumaru et al., 2001) but are not observed clearly by other means (Pabst et al., 2002;
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Bowen et al., 2005) suggesting the idea that target of complexin is the ternary SNARE complex (Melia, 2007). Anyhow the importance of complexin-SNARE interaction for exocytosis is demonstrated in vivo by over-expression of complexin II and a dominant negative mutant in chromaffin cells (Archer et al., 2002). The complexin-clamped SNARE pin assembly appear calcium-insensitive and it is the presence of synaptotagmins that induces a calcium-dependent increase of fusion rate several orders of magnitude faster than in complexin-free control samples (Giraudo et al., 2006; Schaub et al., 2006).

In plants, the cytosolic free calcium transients participate in a wide range of physiological responses, including pollen tube growth (Holdaway-Clarke et al., 2003), plant defense (Klusener et al., 2002) and stomatal closure (Garcia-Mata and Lamattina, 2003). Recently, SNARE proteins were identified to mediate directly the alterations in calcium channel gating (Serrano and Rodriguez-Navarro, 2001; Sokolovski et al., 2005). In these experiments, a dominant-negative fragment of NtSYP121 prevented stomatal closure and selectively suppressed evoked calcium transients, indicating the cytosolic-free calcium homeostasis as a target for SNARE function in vivo.

SNAREs and vacuolar pH regulation.

pH homeostasis is essential for a number of vital cellular processes. For example, protein degradation is triggered by the pH of the compartment where proteolytic enzymes are stored to avoid their activity in other parts of the cell. Also the release of ligands from receptors, and the proteolytic processing of precursor proteins into mature polypeptides is dependent on the pH of the surrounding medium. The generation of pH gradients across cellular membranes provides the driving force for the sequestration of secondary metabolites (Debeaujon et al., 2001) and for the transport of different type of ions by proton exchangers (Serrano and Rodriguez-Navarro, 2001).

The acidification of endomembrane compartments takes place in different organelles, including the Golgi, PVCs and vacuoles. Lysosomes in animals and vacuoles in plants are the most acidic cellular compartment, with pHs between 4.6 and 5.3 measured in mammalian cells and around 5 in plant cells, but can be as low as 2 in citric species such as lemon (Muller and Taiz, 2002). Regulation of pH in acidified compartments is generated and maintained by the balance between proton pump, counter ion conductance, and proton efflux. In plant cells, three types of endomembrane pumps are known, which pump proton
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across membranes. One of these is the vacuolar-type H⁺-ATPase (v-ATPase), a complex consisting of at least 26 proteins that uses ATP as an energy source. The H⁺-pyrophosphatase (PPase) is a single sub-unit protein and uses pyrophosphate as energy source. The third one is the P₃A₅-ATPase PH5, which was recently discovered by Verweij and coworkers in petunia. This protein, belonging to the same family of plant proton pumps located on the plasma membrane, is instead located on the tonoplast membrane and acidifies the lumen of the vacuole in specialized cell types that accumulate anthocyanins (as shown for petunia, rose, carnation and grape; (Provenzano, 2011), or proanthocyanins (as shown for Arabidopsis (Baxter et al., 2005).

The interaction of pumping proteins located on different endocellular membranes with SNAREs is now being discovered and different studies show that this interaction can control the activity of the pump itself (thereby influencing the final pH of the lumen of the compartment; (Grefen et al., 2010) or is required for membrane fusion (this thesis Chapter IV). We show here that some of the interactors of SNAREs can be pumps involved in the acidification of the lumen of endomembrane compartments.
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