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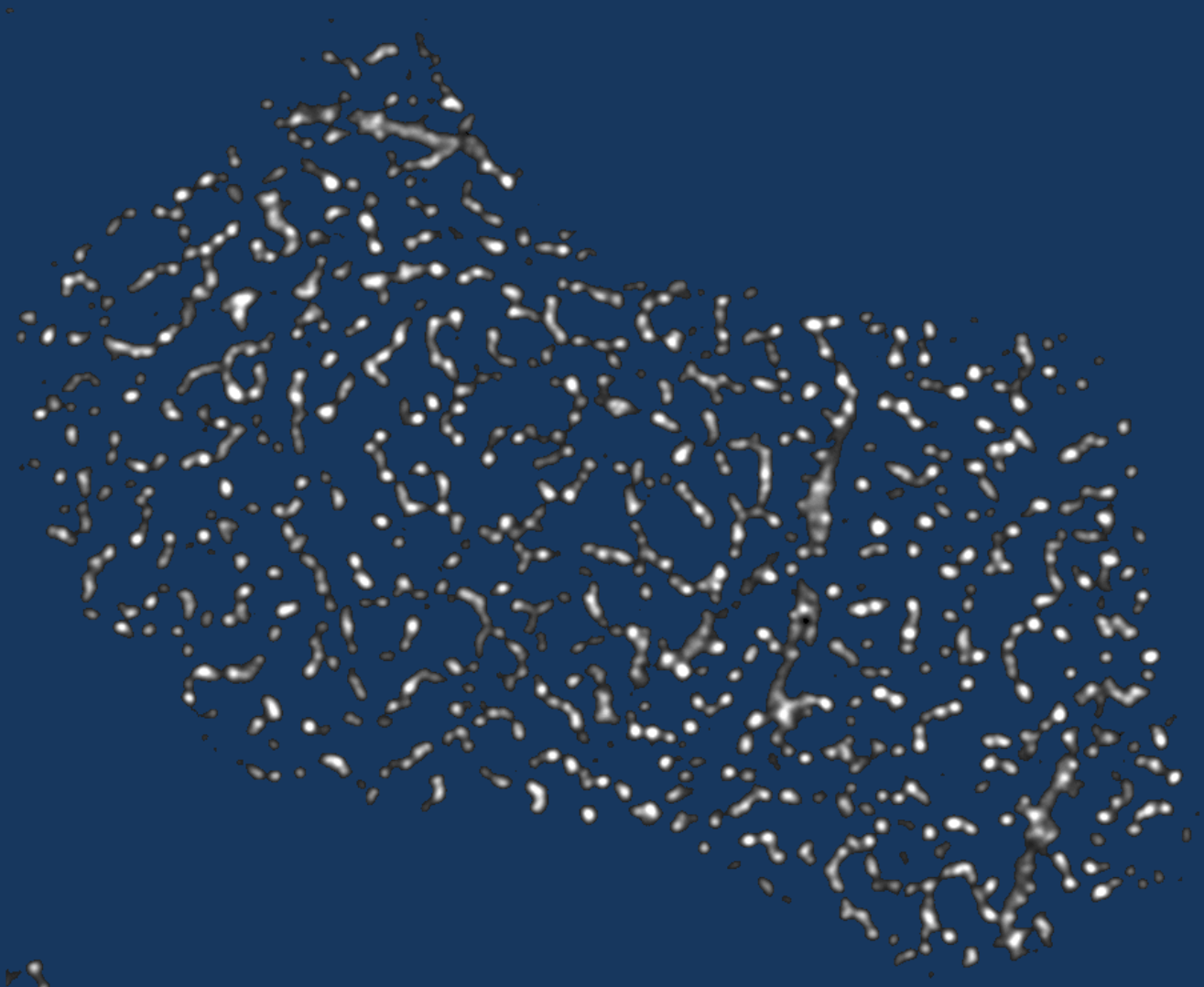
Tesis Doctoral

DECIPHERING THE ROLE OF PLANT SYNAPTOTAGMINS AT ER-PM CONTACT SITES IN THE TOLERANCE TO MULTIPLE STRESSES



Jesica Pérez Sancho

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Directores: Miguel Botella Mesa y Abel Rosado Rey

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Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga.

AUTOR: Jesica Pérez Sancho

http://orcid.org/0000-0002-6201-3240

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Deciphering the role of plant synaptotagmins at ER-PM contact sites in the tolerance to multiple stresses.

Memoria presentada para optar al grado de doctor por la Universidad de Málaga.

Firmado: Jesica Pérez Sancho.

Miguel Ángel Botella, Catedrático del departamento de Biología Molecular y Bioquímica de la Universidad de Málaga, y Abel Rosado Rey, *Assistant Proffesor* en la Universidad de British Columbia (Canadá), CERTIFICAN:

Que Jesica Pérez Sancho, Licenciada en Química por la Universidad de Málaga, ha realizado bajo nuestra dirección el trabajo de investigación correspondiente a la Tesis Doctoral titulada *Deciphering the role of plant synaptotagmins at ER-PM contact sites in the tolerance to multiple stresses* y estimamos que puede ser presentado para su evaluación ante el tribunal encargado de juzgarlo.

Por tanto, AUTORIZAMOS la presentación de esta Tesis Doctoral en la Universidad de Málaga.

Firmado: Miguel Ángel Botella

Abel Rosado Rey

Negar la ciencia es mucho más fácil que aprenderla.

Disfruta de la vida y ayuda a otros a vivir una vida digna de ser disfrutada.

Mario Bunge.

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INDEX

ABBREVIATIONS

Ca^{2+}: Calcium ion.
CACC: Ca^{2+} activated chloride channels.
cER: Cortical ER.
DAG: diacylglycerol.
DHPR: Dihydropyridine receptors.
DRM: Detergent resistant membranes.
E-Syt: Exrended Synaptotagmin.
E-SYT: General term to refer to E-Syts, SYTs and Tcbs.
ER: Endoplasmic reticulum.
FFAT: Two phenylalanines in an acidic track.
GFP: Green fluorescent protein.
IP3R: Inositol-1,4,5-triphosphate receptor.
IPTD: IP-transfer domains.
IPTP: IP-transfer proteins.
JP: Junctophilin.
LTD: Lipid transfer domain.
LTP: Lipid transfer protein.
MCS: Membrane contact sites.
MORN: Membrane occupation and recognition nexus.
MSD: Major sperm protein domain.
MSF: Membrane skeleton fence.
ORD: OSBP-related domain.
ORP: OSBP-related proteins.
PAM: PM associated membrane of the ER.
PB: Polybasic.
PI: Phosphatidylinositol.
PI4P: Phosphatidylinositol-4-phosphate.
PLC: Phospholipase C.
PM: Plasma membrane.
PS: Phosphatidylserine.
RyR: Ryanodine receptors.
SERCA: Sarco/endoplasmic reticulum Ca2+-ATPase.
SMP: Synaptotagmin-like mitochondrial lipid-binding protein.
SOCE: Store-operated Ca^{2+} entry.
SR: Sarcoplasmic reticulum.
STIM: Stromal interacting molecule.
SYT: Synaptotagmin.
Tcb: Tricalbins.
TKO: Triple knock out.
TM: Transmembrane.
VAP: VAMP-associated proteins.
WT: Wild type.

INTRODUCTION

INTRODUCTION

Cells from all organisms are subjected to stress, that is, to adverse environmental conditions that limit their development and proper function[1–3]. The plasma membrane (PM) is a biological barrier that separates the interior of the cell from the environment and, thus, its integrity is essential for cell viability[4]. At the same time, the PM serves as a communication platform between the interior and the exterior of cells, since they need to continuously interact with their environment, perceive external stimuli and transduce them into adequate physiological responses[1,5–7].

Cell membranes are not limited to the PM, especially in eukaryotes, where intracellular compartmentalization is a defining feature. Membrane-surrounded organelles such as Golgi apparatus, mitochondria, nucleus, etc., allow the segregation and regulation of specialized biochemical reactions by creating distinct microenvironments within the cell and avoid the dissemination of reaction products. On the other hand, intracellular compartmentalization also imposes a physical barrier to the free diffusion of metabolites and information throughout the cell. Therefore, cells must find a way of communication and transfer of compounds between compartments. Traditionally, vesicle trafficking was believed to be the main and almost unique mechanism for inter-organelle communication[8]. However, this communication pathway is not expected to be present in organelles isolated from the secretory pathway, such as mitochondria and plastids[9,10]. In the last years, it has become clear that the direct contact of organelles at membranous structures called membrane contact sites (MCS) participates in the coordination of segregated biochemical activities[9,11–14]. MCS are conserved microdomains where the membranes from two different organelles come to close proximity, usually less than 40 nm[15–17], favouring the direct non-vesicular trafficking of molecules between the juxtaposed organelles[11,18].

The endoplasmic reticulum (ER) is a highly dynamic organelle, with the largest surface area in the cell[19], that spreads throughout the cytoplasm of eukaryotic cells as one continuous membrane system. It is involved in many essential cellular functions, such as protein synthesis and processing, lipid synthesis and Calcium (Ca^{2+}) homeostasis. It is now clear that to properly function, the ER needs to create contacts with almost any other organelle in the cell and with the PM[20–24]. In this context, we have characterized the *Arabidopsis thaliana* Synaptotagmin1 (SYT1) as an ER-PM tethering protein essential for the plant to cope with different stresses. MCS between organelles others than ER have also been described[10,25,26].

In this thesis, we characterize the Synaptotagmins 1 and 3 of Arabidopsis as ER-PM contact site tethers involved in the tolerance to diverse stresses.

PLASMA MEMBRANE ORGANIZATION

The Fluid Mosaic Model, proposed by Singer and Nicolson in the 70´s, described the biological membranes as a 2D oriented solution of globular, amphipathic, integral proteins in a homogeneous phospholipidic bilayer solvent[27]. In this model, hydrophobic and hydrophilic interactions between non-polar and polar groups in lipids, proteins, and polysaccharides with the polar-aqueous environment are the main driving forces. This model was a significant advance in our understanding of cell membranes, but nowadays it is clear that this is a very simplistic view, at least for the PM, which has a lateral segregation of lipids and proteins into membrane compartments or microdomains, yielding a highly heterogeneous landscape[28–33].

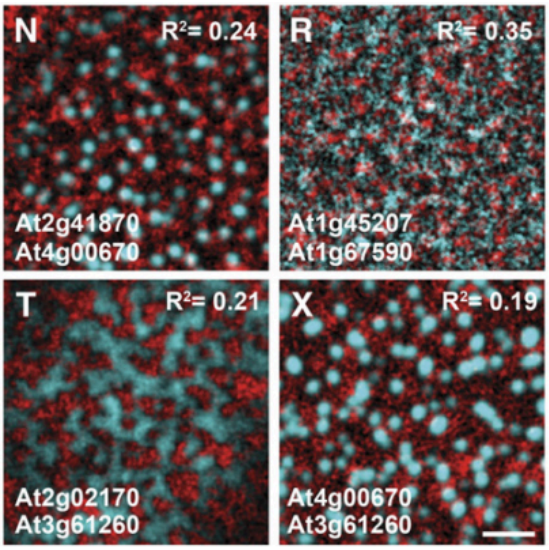


Figure 1: Different proteins localize to different PM compartments in plant cells. Modified from Jarsch et al, 2014 ([32]).

Direct visualization of stable and inducible membrane microdomains is now possible due to relatively new visualization techniques, such us (high-speed-)single particle tracking (SPT), fluorescence correlation spectroscopy (FCS), Förster resonance energy transfer (FRET)-fluorescence lifetime imaging microscopy (FLIM), fluorescence recovery after photobleaching (FRAP), and super-resolution fluorescence [5,34–39]. Although PM compartmentalization has long been proposed in animals and yeast, plants were fundamental in the development of this field, as some of the first direct visualization of PM compartments were done in plants ([32,33]), providing convincing evidence for (1) their existence and high hetereogenity (Figure 1), and (2) the elucidation of their biological roles. PM are compartmentalized in mesoscale microdomains (40-300 nm) or "membrane compartments" by its interaction with the membrane skeleton fence (MSF, mainly actin filaments in animals and yeast, and cortical microtubules in plants[33]), and with MSF-interacting transmembrane proteins ("pickets"). All these components together create a permeable mesh-like barrier that impedes the free diffusion of proteins and lipids between compartments, although they can freely diffuse within one membrane compartment[28,40,41]. Accordingly, several publications (summarised in [41]) show that the macroscopic diffusion coefficients of both lipids and proteins in the PM are 5-50 times slower than those in artificial membranes or liposomes, which can be reached after the removal of the MSF by cytoskeleton depolymerizing drugs or in blebbed

membranes[40,42,43]. The extracellular matrix and the cell wall have also been proposed as active mesh-modulators[44–46].

Compartmentalization is also achieved by self-organization of lipid and proteins in nanoscale microdomains (usually <100 nm)[39,41,47] or "lipid rafts", where sphingolipids, sterols and glycosylphosphatidylinositol (GPI)-anchored proteins are predominant[34,48,49]. Sorting has been proposed to be mediated by the particular self-associative properties of sphingolipids and sterols[34,50,51], although protein-protein and protein-lipid interactions can also participate[41,49,52], e.g. activating pre-existing sphingolipids/sterols small associations to bigger clusters in specific conditions. The different compartmentalization levels in the plasma membrane are interconnected. For example, a single protein or a single lipid hops easier from one compartment to another than a multiprotein complex or a lipid raft[28,53,54]. This phenomenon is called "oligomerization induced trapping" and is dependent on the MSF[41]. In its absence, multimerization alone also slows down the diffusion of the implicated proteins, but in much less extent[55,56].

The PM compartmentalization and subsequent slow diffusion of proteins and lipids have important consequences for cells development and survival. Evolutionarily and/or functionally related proteins colocalize more in the same membrane compartment than unrelated proteins[31,32] and there are many examples of stimulus-induced compartmentalization and decompartmentalization[36,38,53,57]. Thus, membrane microdomains may have multiple roles, such as facilitating complex formation, immobilizing protein receptors in the regions where the ligand was first perceived, separating incompatible processes, sustaining PM polarity, etc.

The formation of MCS between the PM and other membranous intracellular organelles also creates a compartmentalization in both membranes. Proteomic analyses in our lab of the interacting partners of the MCS resident protein SYT1-GFP (data not shown) revealed an enrichment in proteins involved in tethering and some others having specific functions at MCS. Interactions with actin and tubulin were also detected (data not shown and[58]), pointing to the possibility of a link between the MSF compartmentalization and MCS establishment (that we will not explore in this thesis).

ER SHAPING AND DYNAMICS

The ER has a major role in the synthesis, modification and quality control of the majority of the cell´s building blocks, that are then delivered to their final destinations primarily through the Golgi apparatus[8,59]. The ER is also an important place for Ca^{2+} storage within the cell[60], the main membrane supplier for other organelles biosynthesis (such as oil

bodies[61] and peroxisomes[62,63]), marks the position for some organelles' division[64,65], and controls organelle streaming during cell expansion[66]. In addition, plant ER have unique functions, such as the storage of hormone receptors and hydrolytic enzymes[67], the collaborative lipid biosynthesis with the chloroplasts[68], or the intercellular communication through the plasmodesmal desmotubule[69,70].

The ER is a very dynamic organelle in constant remodelling by homotypic membrane fusion[71,72]. It can be structurally classified into the ER that surrounds the nucleus and the peripheral ER, which in turn consists of flat cisternae of fixed thickness (also called sheets) and an interconnected polygonal network of tubules[71,73,74]. The alternative morphologies of the peripheral ER are proposed to preferentially accommodate the proteins needed for distinct ER functions[75,76]. The peripheral ER can be subsequently subdivided in internal ER, the ER that traverses the cytoplasm, and cortical ER (cER), the ER that underlies the PM, which is specially abundant in plant cells. Another particularity of plants is that they can subcompartmentalize and create many ER-derived structures in specific conditions. Protein bodies and precursors accumulating (PAC) vesicles accumulates storage proteins and their precursors, respectively, in developing seeds[67]. KDEL vesicles (KVs) and ricinosomes accumulate papain type proteases in germinating seeds, presumably for the degradation of excess storage proteins during early development[77]. ER bodies are plant specific ER microdomains continuous to the whole ER network that accumulate ß-glucosidases in response to internal and external stresses[78].

As mentioned, the ER is highly dynamic and tubules and sheets are not permanently defined, but they move, rearrange and interconvert. Different proteins are involved in ER shaping and dynamics. Reticulons (Rtn) and DP1(REEP)/Yop1p are conserved integral ER proteins that specifically partition into highly curved regions of the ER, namely ER tubules and the edges of the ER sheets[79–81], and are required and sufficient to generate membrane tubules[82]. These proteins harbour the reticulon homology domain (RHD) which consist on two unusually long hydrophobic segments (30–35 residues) joined by a soluble linker[79] that inserts as a double hairpin in the cytosolic leaflet of the ER membrane, promoting membrane curvature[83,84]. Atlastin (ATL) are membrane bound GTPases from metazoan that specifically localize to tubular ER, where they interact with Rtn and DP1/Yop1p and promote the homotypic fusion of ER tubules[85,86]. Yeast and Arabidopsis possess functional orthologs of Atlastin, named Sey1p and RHD3[86] respectively. The Arabidopsis RHD3 familiy also contains two, much less abundant, RHD3-like proteins (RL1 and RL2), all of them being able to promote membrane fusion both *in vitro* and *in vivo*[87,88]. On the other hand, Climp63 exclusively partition to sheets in mammalian cells, where it works as a luminal spacer and promotes the proliferation of sheets over tubules when overexpressed[80]. Nevertheless, sheets of altered intraluminal space are retained in *Climp63* mutants[80] and homologs for this protein have

not been found in plants, pointing to the existence of alternative sheet promoters. Kinectin and p180 are sheet enriched proteins in mammals that have been proposed to help in flattening the sheets[72,80].

In addition, the ER establishes MCS with almost all membrane-bound organelles in the cell, including nucleus, mitochondria, plastids, Golgi apparatus, vacuole, endosomes, peroxisomes, and lipid droplets, and with the cytoskeleton and the PM[20,24,89,90]. Such an extensive connectivity is determinant for its morphology, but, at the same time, the ER morphology determines its ability to establish MCS[72,91]. Moreover, the MCS localized SYT1 interacts with ER shaping proteins (proteomic data not shown and [92]), suggesting a main role of MCS in the determination of ER morphology.

MEMBRANE CONTACT SITES

MCS are transient or durable membranous structures where the membranes from two different organelles are very close, in the order of several nanometres, to each other [9,11,12,14]. Two distinct membranes within the same organelle can also form microdomains of great proximity, as occurs in the formation of cristae junctions in mitochondria[93,94], but this type of contacts are usually not considered MCS. Protein tethers are in charge of bringing and maintaining the two membranes close together while preventing their fusion [95,96] (although membrane hemifusion can sometimes occur [97]). Although many MCS resident proteins have been identified[9,12,14,95], we should keep in mind that not all the proteins that are located in MCS are responsible for the tethering, but may accomplish other functions in such specific locations within the cell[95], such as non-vesicular lipid transport, Ca^{2+} sensing and/or signalling, marking the position for organelle division, etc. Most of our knowledge on MCS comes from yeast and animal fields, where their functional characterization is quite developed and many of the tethering complexes between apposed membranes have already been identified and biochemically characterized[12,14,22]. Although MCS have also been visualized in plants for a long time, our understanding on their structural organization and functional behaviour has only recently started due to the identification of several plant MCS resident proteins[5,58,98,99] and the study of plant-specific MCS, such as those involving chloroplasts, and plasmodesmata (Figure 2). An updated review on plant MCS can be found in chapter 1, *Stitching organalles: Organization and Function of Specialized Membrane Contact Sites in Plants* [14].

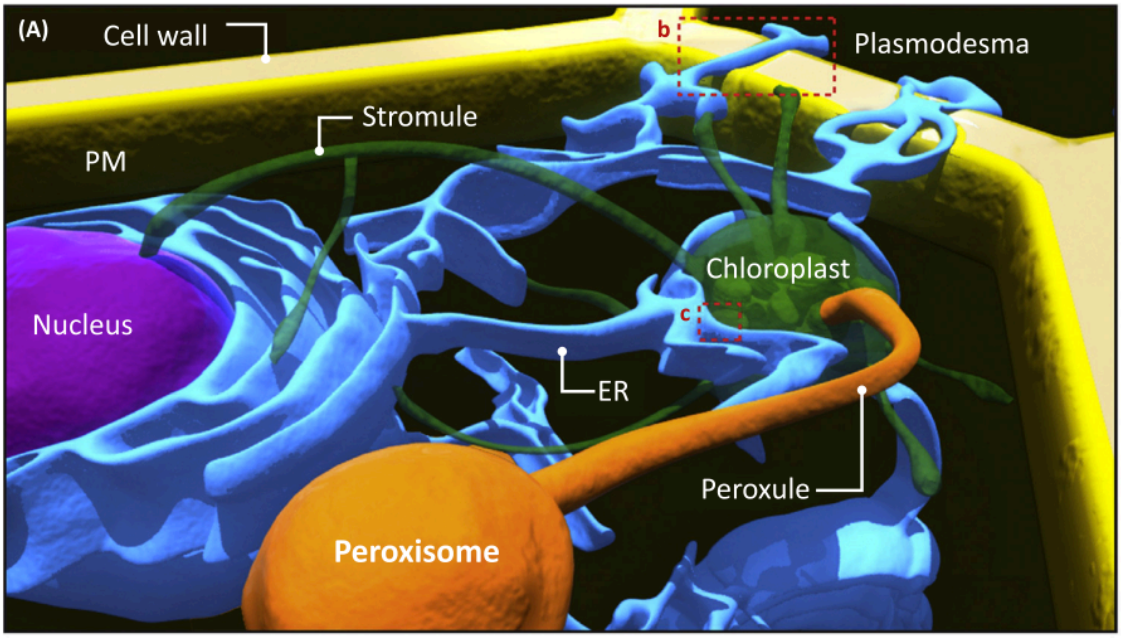


Figure 2: MCS in a plant cell. From Pérez-Sancho et al, 2016 ([14]).

ER-PM MEMBRANE CONTACT SITES

Close contacts between the ER and the PM were first observed by TEM in striated muscle cells[100] and neurons[101], and for long time they were considered exclusive of electrically excitable cells. Yet, nowadays ER-PM contact sites have been observed in numerous cell types in different organisms, including plants[14,102,103], and confirmed through the cosedimentation of a fraction of the cER (referred as PAM, for PM associated membrane of the ER) in PM purifications[104,105]. Many ER-PM contact sites resident proteins have been characterized, with an important enrichment in Ca^{2+} and lipid homeostasis related proteins[12,23,106,107] (see next sections).

In yeast, most of the peripheral ER underlies the PM[104,108] at an average distance of 33 nm (ranging from 15 to 60 nm), with very little ER distributed throughout the cytoplasm[15,109]. Similarly, the central vacuole occupies most of the volume of mature plant cells and relegates other organelles, including the ER, to a thin cortical region between the tonoplast and the PM[14,102]. However, not all the cER represent contact sites between the ER and the PM. Thus, ER-PM contact sites can be considered as subdomains of the cER adjacent and parallel to the PM at a distance smaller than 30 nm[14,109–113]. Under this criterion, up to 10 % of the PM is in contact with the cER in non-stimulated cells of different organisms[15,110,114].

CALCIUM SIGNALLING AT ER-PM CONTACT SITES.

ER is the most important intracellular Ca^{2+} store in metazoans and different stimuli in the PM trigger its release into the cytosol to function as a second messenger[115,116]. A fine regulation is needed in order to keep Ca^{2+} at nanomolar levels in the cytosol while it is in the high micromolar range in the ER[115]. MCS function as Ca^{2+} sensors that are induced when cytosolic Ca^{2+} increases[117,118] and are determinant for this highly regulated Ca^{2+} homeostasis[23]. ER is also expected to be an important Ca^{2+} store in plants, although the vacuole is the main reservoir[119].

The PM of striated muscle cells presents deep invaginations, called transverse (T)-tubules, that establish extensive contact sites (at a maximal distance of 15 nm[120]) with the sarcoplasmic reticulum (SR, ER in muscle cells) in special arrangements known as diads and triads, that are essential for the coupling of excitation and contraction[11,121]. Muscle contraction is triggered by PM depolarization followed by a massive and coordinated flux of Ca^{2+} into the cytosol. Dihydropyridine receptors (DHPRs) are voltage-activated Ca^{2+} channels in the T-tubule that open after PM depolarization and allow a small and slow influx of extracellular Ca^{2+} into the cytosol[122]. The tight contact of SR and T-tubules at diads and triads leads to the coupled gating of Ryanodine receptors (RyRs) Ca^{2+} channels in the SR [123–125], promoting the massive release of SR stored Ca^{2+} [122]. Although DHPR and RyR interact at diads and triads, this interaction is not involved in the ER-PM tethering[126,127].

Independently of the discharging mechanism, the luminal Ca^{2+} needs to rapidly recover resting levels in order to support subsequent signaling cascades. The store-operated Ca^{2+} entry (SOCE) is in charge of this function in both excitable and non-excitable cells[23,112,128], although it works faster in the excitable ones[129]. STIMs (stromal interacting molecule) are ER resident proteins that sense the concentration of Ca^{2+} in the ER lumen[130,131]. At high Ca^{2+}, inactive STIM is bound to Ca^{2+} and locates as monomers/dimers in the ER[132]. Ca^{2+} dissociation triggers further oligomerization[106], which in turn promotes the interaction of STIM oligomers with PM phospholipids and its relocation to ER-PM contact sites[133–135]. Then, STIM oligomers interact with and recruit the PM Ca^{2+} channel component Orai1[136,137], opening the channel and triggering Ca^{2+} uptake from outside of the cell into the cytosol[137,138] (Figure 3). Subsequently,

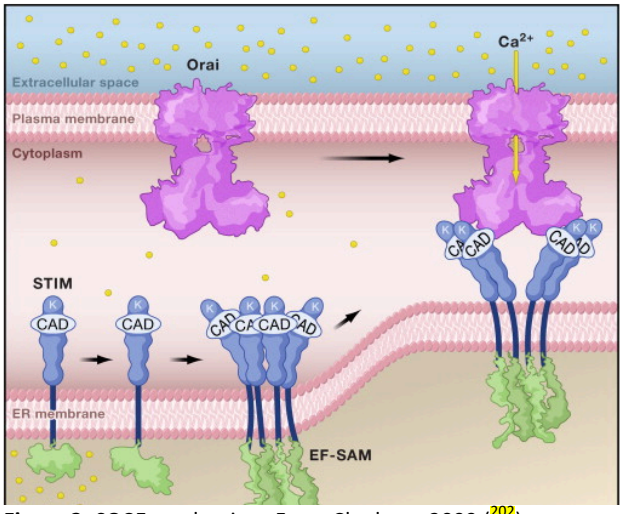


Figure 3: SOCE mechanism. From Clapham, 2009 ([202]).

the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps draw Ca^{2+} from the cytosol into the ER lumen. SERCA also accumulates at ER-PM contact sites under low luminal Ca^{2+}[139,140], favoring the rapid refilling of the ER without important increase of the cytosolic Ca^{2+} concentration[141,142].

The extended-synaptotagmin 1 (E-Syt1) can also sense cellular Ca^{2+} in mammalian cells and relocate from a wide distribution in the ER to ER-PM contact sites when cytosolic Ca^{2+} is high[113,117,118,143,144] (with a half maximal effect (EC_{50}) at 1,8 µM of free cytosolic Ca^{2+}[143]). This E-Syt1 relocation is coupled to a high increase on the ER closely apposed to the PM and a reduction in the average distance between both membranes at E-Syt1 tagged ER-PM contact sites (from 21,8 nm at resting Ca^{2+} levels to 14,8 nm when cytosolic Ca^{2+} is high[113]). Accordingly, cells lacking E-Syt1 and its homologs E-Syt2 and E-Syt3 have less ER-PM contact sites (about 0,5 % of the PM perimeter compared to 2% in WT cells[118]) and the remaining ones are not any longer sensitive to Ca^{2+} [118,143,145]. The mechanism for Ca^{2+} triggered and E-Syts mediated ER-PM tethering will be discussed in a following section, but an important aspect is that it seems to be independent of SOCE, i.e., (1) SOCE is functional in cells lacking the three E-Syts[113,118,143] and (2) SOCE activation when the external medium is depleted of Ca^{2+} does not trigger E-Syt1 relocation to ER-PM contact sites[143], indicating that release of Ca^{2+} stores in the ER is not enough to relocate E-Syt1. Contrarily, SOCE activation when the medium is rich in Ca^{2+}, which causes a massive uptake into the cytosol, does trigger E-Syt1 relocation; as so do other approaches to increase Ca^{2+} in the cytosol[143], indicating that the increase in cytosolic Ca^{2+}, and not the activation of SOCE itself, is responsible for the formation of E-Syt1 tethered ER-PM contact sites.

NON-VESICULAR PHOSPHOLIPID TRANSPORT AT ER-PM CONTACT SITES.

The ER is the main organelle for phospholipid biosynthesis[68,146], but many important reactions in lipid metabolism occur at other organelles[146,147]. This is especially relevant in plants, where phospholipids are collaboratively synthetized between the ER and the plastids[68]. Therefore, different mechanisms, including vesicular and non-vesicular lipid transport, are needed to transfer phospholipids between organelles during their synthesis and to deliver them to their final destination within the cell[148,149]. As phospholipids are the main constituents of both, cellular membranes and transport vesicles, vesicular transport is pivotal in the bulk movement of phospholipids. Yet, some processes, such as the production of phosphatidylinositol (PI) related second messengers[150], necessitate a fine and fast control of phospholipids that cannot be achieved by the classical vesicular trafficking routes. In addition, lipid composition is a defining feature for each cellular membrane, but very few examples of selective vesicular transport of phospholipids have been demonstrated[151]. Thus, non-vesicular

lipid transport is the mechanism proposed to function in those cases. Non-vesicular lipid transport is facilitated by lipid transfer proteins (LTPs), which can harbour lipids in a hydrophobic pocket in their lipid transfer domain (LTD)[148,152] and shuttle them between membranes through an aqueous phase[148]. The short distance between membranes at MCS help LTPs to greatly increase their transport efficiency, as they have less cytosol to transvers. Accordingly, many LTPs locate to different MCS, although I will only focus on phospholipid transfer at ER-PM contact sites.

OSBP-related proteins (ORPs) (Osh in yeast) is a large and conserved family of LTPs[153–155]. Yeast Osh6 and Osh7 as well as mammalian ORP5, ORP8 and ORP10 transfer phosphatidylserine (PS) against a concentration gradient from the ER to the PM at ER-PM contact sites[156]. Other ORPs also locate to ER-PM contact sites, although the specific lipids that they traffic are not unequivocally deciphered[157,158]. In addition to the OSBP-related domain (ORD), the LTD that define the family, many ORPs also contain FFAT motifs, that are recognized by VAMP-associated proteins (VAPs) in the ER[159], and PH domains, that mediate the link to the PM by binding to phosphatidylinositol-4-phosphate (PI4P)[155,158,160,161]. Indeed, PS transport need PI4P countertransport[160–163]. Recently, the mechanism governing PS/PI4P countertransport at ER-PM contact sites by ORPs have been elucidated: ORPs extract PS from the ER membrane (poor in PI4P), exchange PS with PI4P at the PM (relatively rich in PI4P), and transport PI4P back to the ER (Figure 4). Then, the ER phosphatase Sac1 hydrolizes the phosphate group, keeping PI4P levels in the ER low and providing the energy to sustaining a cyclic PS/PI4P countertransport against PS gradient[160,161].

Although ORPs are highly represented in plants (12 genes in Arabidopsis)[14,153,154], they are very poorly characterized in these organisms: PiORP1 of *P. inflate* is a pollen specific ORP that locates to PM foci (very likely ER-PM contact sites) through its PH domain[153]; ORP3a of Arabidopsis binds sterols and locates to ER-Golgi contact sites through the interaction with a VAP27-3[164] (similar to other ORPs from yeast and animals[155]). Moreover, when yeast cells expressing ORP3a, or 5 other Arabidopsis ORPs, are infected with tomato bushy stunt virus (a plant RNA virus able to infect yeast cells), all ORPs, together with the yeast VAP Scs2, are recruited by the viral p33 protein to the sites of viral replication, likely to induce membrane proliferation[165].

The output for ligand binding to many cell surface receptors is the activation of phospholipase C (PLC) enzymes. PLC hydrolyzes phosphatidylinositol-4,5-diphosphate (PI(4,5)P2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). IP3 is soluble and triggers the release of ER Ca^{2+} when it is perceived by IP3R Ca^{2+} channels in the ER membrane. DAG is instead retained at the PM, where it is then converted to phosphatidic acid (PA)[68,150]. Interestingly, the levels of PI(4,5)P2 at the PM remain relatively stable during the process, even at high PLC activity[166], an aspect that is

essential to maintain the cell´s ability to respond to future stimulation of cell surface receptors. In this context, sustained light phototransduction in *Drosophila*'s eye, that involves PI(4,5)P2 hydrolysis, rely on lipid shuttling by retinal degeneration B alfa (RGDBα) at ER-PM contact sites[167,168]. Similarly, the human RGDBα (also known as Nir2), accumulates at ER-PM contact sites after PLC activation and promotes PI(4,5)P2 replenishment in the PM[117,169]. RDGBα are cytosolic multidomain IP-transfer proteins (IPTPs) harboring a FFAT motif[148], which mediates the link to the ER through the interaction with VAPs[117,168,169], and the defining IP-transfer domain (IPTD), which works as PI/PA exchanger in a process similar to PS/PI4P countertransport at ER-PM contact sites (Figure 4): PA produced by PLC in the PM is transported to the ER, converted to PI and transferred back to the PM, where it is subsequently transformed into PI4P and PI(4,5)P2[168,169]. Interestingly, Nir2 recruitment to ER-PM contact sites is facilitated by a decrease in the gap distance between membranes induced by Extended-Synaptotagmin 1 (E-Syt1) perception of cytosolic Ca^{2+} increases[117].

E-Syts do not only work as Ca^{2+} sensible ER-PM tethers (previously described), but are also directly implicated in non-vesicular phospholipid trafficking between membranes[145]. E-Syts contain the conserved Synaptotagmin-like mitochondrial lipid-binding protein (SMP) domain[170,171], which is able to bind both, artificial liposomes and cellular membranes[111]. The crystal structure of a fragment of E-Syt2, that includes the SMP, the C2A and the C2B domains, showed that the SMP dimerizes in a tubular structure of 90 Å length that can harbor lipids in the internal hydrophobic tunnel[172]. Further analysis revealed that the structure can contain two phospholipid molecules at the same time, with the hydrophobic tails hidden inside the hydrophobic tunnel and the polar heads facing the polar solvent, with no preference for any specific phospholipid species[145,172]. Recently, the structure of the SMP domains of Mdm12 and Mmm1, proteins that locate at the ER-Mitochondria contact sites, has also been resolved. Similarly to E-Syt2 SMP domains, they can heteromerize forming tubular structures with lipid binding ability[173], pointing to a general role of SMP domains in lipid trafficking at MCS contact sites. Actually, E-Syt1 is able to transfer phospholipids in vitro between ER-like and PM-like liposomes, but lipid transfer is abolished in mutants lacking the SMP domain or impairing the tunnel formation[145]. However, PM purified fractions from triple knock out cells for the three E-Syts (TKO) showed similar phospholipid composition than wild type PM in steady-state conditions[145], indicating that E-Syts do not control steady-state PM phospholipids. An interesting observation is that the lipid transfer velocity in vitro depends on the concentration of Ca^{2+} in the media[145] that, together with the observation that cytosolic Ca^{2+} promotes E-Syt1 relocation to ER-PM contact sites, link the role of E-Syt1 as a Ca^{2+} mediated ER-PM tether with its non-vesicular lipid traffic activity. Interestingly, after activation of PLC, TKO cells displayed a more sustained accumulation of DAG at the PM compared to a very transient increase in wild type

cells[145], indicating that E-Syts could help to recover basal levels of membrane lipids after acute perturbations related to Ca^{2+} signalling.

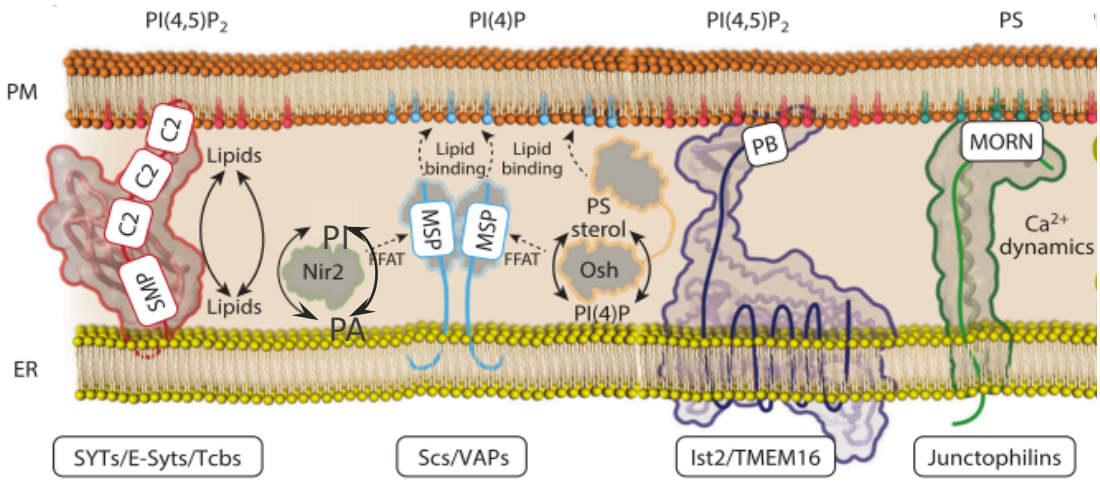


Figure 4: ER-PM tethering components and their involvement in non-vesicular lipid transport. Modified from Tilsner et al, 2016 ([70]).

ER-PM TETHERS

Among the proteins that locate to ER-PM contact sites, at least four protein families have been reported as ER-PM tethers[22,95], having the structural capacity of binding the two membranes simultaneously and promoting the contact site formation (or contact site mis-formation in its absence) (Figure 4). Only two of them (VAPs and E-SYTs) are characterized to some extent in plants.

- **Junctophilins** are a conserved family of proteins in animals (comprised of 1 protein in invertebrates and 4 proteins in vertebrates[174]) that link the connection between the ER and the PM in diads and triads in muscle cells (JP1 and JP2)[175–177], and in the subsurface cisternae of PM in neurons (JP3 and JP4)[177,178]. Junctophilins are anchored to the ER by a C-terminal TM domain and are able to bind phospholipids through multiple N-terminal membrane occupation and recognition nexus (MORN) motifs[179], that are specially highly conserved[174]. Cells lacking junctophilins present abnormal ER-PM contact sites[176] and also show altered Ca^{2+} homeostasis[176,180], while junctophilins' overexpression induce ER-PM contact sites formation[175].

- **TMEM16** (also known as Anoctamins[181]) is a protein family in eukaryotes anchored to the ER by multiple, usually 8, TMD[182]. The mammalian TMEM16A and TMEM16B are ER resident Ca^{2+} activated chloride channels (CACC)[183,184]. There is only one TMEM16 homolog in yeast (Ist2), which has been characterized as an ER-PM tether,

although there are some contradictory results[21,109]. Wolf et al. reported a 40 % decrease in the PM closely apposed (< 30 nm) to the ER in a single *Ist2* mutant compared to wild type yeast cells[109], while Manford et al. only observed an effect of *IST2* mutation in ER-PM association (in a distance within 65 nm) in a mutant background lacking other tethering proteins, but not in the I*st2* single mutant[21]. Probably, this discrepancy is due to the different criteria used to define ER-PM contact sites, as Wolf and colleagues did not observe such diminution when they considered the ER 100 or 200 nm away from the PM; highlighting that not all the cER represents ER-PM contact sites. Additionally, Ist2 overexpression highly promotes ER-PM contact sites formation[109].
Binding to PM in Ist2 is mediated by a cytosolic C-terminal polybasic (PB) domain (the cortical sorting signal, CSS) that binds phospholipids, preferentially PI(4,5)P_2[185]. This C-terminal region is not conserved in the mammalian TMEM16A, but it contains a long N-terminal cytosolic region that is also able to bind to liposomes containing PI(4,5)P_2 [185] .

- **VAPs** are a conserved family of proteins in eukaryotes with a major sperm protein domain (MSD) as common feature[99,186,187]. Within the MSD there are several binding sites[187], standing out a electropositive face that binds to FFAT and FFAT-like motifs[159,188–190], which are present in many LTPs at ER-PM contact sites (see *Non-vesicular phospholipid transport at ER-PM contact sites*), and in other proteins. Different residues substitutions in the FFAT-like motifs trigger different binding affinity of these LTPs for VAPs[190]. VAPs are highly represented in plants, with 10 members in Arabidopsis[98,99], compared to only two members in yeast (Scs2 and Scs22) and vertebrates (VAP-A and VAP-B)[186]. Yeast and animal VAPs as well as plant VAPs from clades I and III are anchored to the ER through a single C-terminal TMD[99,187]. A cytosolic fragment of diverse sizes, that in some VAPs form a coiled coil domain, link the TMD to the N-terminal MSD[99,187]. Many FFAT containing proteins (as PITP and ORPs) contain additional binding motifs, allowing the complex VAP-LTP to bind simultaneously to the ER and other membranes, including the PM[58,158,168,191].

Yeast cells lacking Scs2 and Scs22 have less ER closely associated to the PM[21,91] but an artificial ER-PM tether is able to recover normal ER-PM attachment[91]. *Scs2/Scs22* cells also present increased total PI4P levels, a phenotype that cannot be complemented by artificial tethers[91]. Scs2 and Scs22 interact with several LTPs, including some members of the ORP family, that mediate the phospholipid and sterol transport at ER-PM contact sites[158,161,192]. The expression of an artificial tether could bring the two membranes close together, but would be unable to recruit LTPs. The plant homolog VAP27-1 (clade I) locate to the ER and ER-PM contact sites in Arabidopsis, and colocalize with Scs2 when expressed in plants[58]. Interestingly, VAP27-1 and VAP27-3 (also clade I) directly bind microtubules and colocalize and interact with NET3C at ER-PM contact sites[58,99]. Networked (NET) is a plant specific superfamily of actin binding proteins mediating the interaction between the actin cytoskeleton and plant endomembranes[193]. Thus, VAP27 and NET3C form a complex together with actin filaments and microtubules coordinating ER-PM contact sites with plant cytoskeleton[58]. Actually, overexpression of VAP27-1 and

NET3C triggers the approximation to the PM of large ER sheets[99]. Additionally, VAP27-3 recruits ORP3a to the ER[164]. Plant VAPs from clade II lack the TMD and therefore are not anchored to the ER membrane. Contrarily, the coiled coil and MSD, which has been demonstrated to bind PI4P and PS[194], target them to punctae at the PM[98,99]. Interestingly, VAP27-8 (also known as VAP-related suppressors of *tmm* 1, VST1) interacts with ER anchored proteins as SRC2 and SYT1 (another ER-PM contact site tether, see below)[98]. Thus, VAPs from clade II in plants also form complexes that link the ER to the PM, but with the opposite topology than the canonical VAPs.

- **E-Syts**, referred as Tricalbins (Tcb) in yeast and Synaptotagmins (SYTs) in plants (I will use E-SYTs when referring to them in general), are conserved ER anchored proteins with a common modular structure. They possess an N-terminal ER anchoring motif which, in the case of E-Syts has been demonstrated to be a hairpin[118]; a SMP domain, and a variable number of C2 domains. The SMP can transfer phospholipids between membranes and always appear in proteins located to MCS[111]. Accordingly, in this thesis we show that plant SYTs also contain this domain and, at least, SYT1[5] and SYT3 also locate to MCS. Previous studies about plant SYTs ignored the presence of the SMP domain, leading to their miss-annotation as homologs of mammalian synaptotagmins[195–197]. The C2 domains (two for plant SYTs) are phospholipid binding domains that bind phospholipids located in the PM[4,5,21,118,198]. Two main types of C2 domains exist according to their Ca^{2+} binding properties: (1) Ca^{2+}-dependent C2 domains generally present conserved aspartate residues that mediate the interaction with Ca^{2+}[199], which in turn modulate C2 binding to phospholipids[4,118,143]; (2) Ca^{2+}-independent C2 domains lack the conserved aspartates and their ability to bind membrane lipids is not dependent on Ca^{2+}[4,200]. The combination of Ca^{2+}-dependent and Ca^{2+}-independent C2 domains and their relative position to other domains within the proteins govern the Ca^{2+} and lipid binding properties of the different E-SYTs[4,5,118,143–145]. Thus, the affinity of SYT1 C2A domain of Arabidopsis for phospholipids increases with Ca^{2+} concentration, without any binding in the absence of Ca^{2+}. Contrarily, the C2B domain binds phospholipids independently of Ca^{2+} concentration. As a result, the tandem C2AB domains are able to bind phospholipids in the absence of Ca^{2+} but the affinity increases with the Ca^{2+} concentration[4]. Among the three mammalian E-Syts, E-Syt2 and E-Syt3 contain three C2 domains (C2A, C2B and C2C)[198] (Figure 5). The C2C domain can strongly bind phospholipids through an exposed basic patch in the absence of Ca^{2+} and this causes a permanent location in ER-PM contact sites[118]. The affinity of the C2A domain of E-Syt2 (and very likely the C2A of E-Syt3) for membrane lipids increase with Ca^{2+}, what is proposed to trigger the conformational change required to diminish the distance between ER and PM under high cytosolic Ca^{2+}[144]. E-Syt1 contains five C2 domains (C2A to C2B)[198] (Figure 5), with C2A and C2C being Ca^{2+}-dependent binding modules, and C2E mimicking the role of C2C in the other E-Syts[143]. However, E-Syt1 presents a subcellular localization clearly different to that of E-Syt2 and E-Syt3. In basal conditions, E-Syt1 is

widely localized throughout the ER, with only a small portion in already formed ER-PM contact sites[113], but after an increase in cytosolic Ca^{2+} it relocalizes to ER-PM contact sites[113,118,143,145]. Accordingly with a role in PM tethering, overexpression of any of the E-Syts diminishes the distance between ER and PM at ER-PM contact sites[113]. Interestingly, the distance in steady-state E-Syt1 tethered ER-PM contact sites (average of 21,8 nm) is bigger than in E-Syt3 tethered ER-PM contact sites (18,8 nm), consistent with a longer cytosolic fragment, but it is highly reduced when the concentration of Ca^{2+} in the cytosol rises (14,8 nm)[113], consistent with a Ca^{2+} triggered conformational change and the binding of C2A and C2C to the PM (Figure 5).

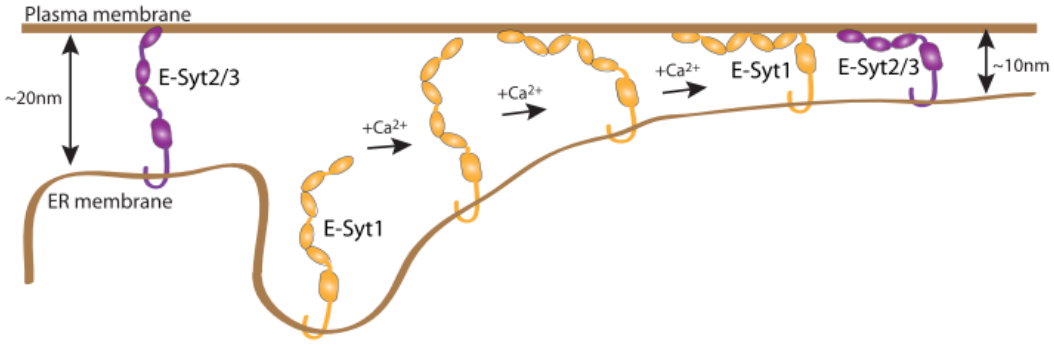


Figure 5: Mechanism of Ca^{2+} dependent ER-PM tethering by E-Syts. From Herdman et al, 2016 ([201]).

In this thesis, we have identified SYT1 and SYT3 of Arabidopsis as ER-PM tethers, homologs of the E-Syt/Tcb family of proteins, and fundamental for the tolerance to diverse abiotic stresses (wounding, osmotic and cold). SYT1 and SYT3 locate in the ER, being highly concentrated at ER-PM contact sites. As E-Syt1, SYT1 and SYT3 bind preferentially to negatively charged phospholipids and the interaction increases with Ca^{2+} concentration. We demonstrated that ER-PM contact sites have a role in the maintenance of PM integrity against mechanical damage, probably by two mechanisms: (1) by reinforcing the basal stability of the PM through its attachment to the ER, and (2) by their rearrangement under a mechanical pressure in the PM to distribute the loads. We also show that both SYT1 and SYT3 are needed for the acquisition of enhanced freezing tolerance during cold acclimation and that they may regulate some of the changes in phospholipid composition during the process. In addition, in the chapter 1 we summarize the current knowledge about MCS in plants.

OBJECTIVES

OBJECTIVES

The general objective of this Thesis Project is **to decipher the roles of plant Synaptotagmins in abiotic stress tolerance**. For that, we have used the model plant *Arabidopsis thaliana* and we have established the following specific goals:

1. Characterization of the Ca^{2+} dependency of the Synaptotagmins-phospholipids interaction.

2. Identification of the molecular mechanism governing SYT1 responses to mechanical stress.

3. Characterization of the function of SYT1 and SYT3 in cold acclimation and freezing tolerance.

CHAPTER 1:

Stitching Organelles: Organization and Function of Specialized Membrane Contact Sites in Plants

Stitching Organelles: Organization and Function of Specialized Membrane Contact Sites in Plants. Jessica Pérez-Sancho, Jens Tilsner, A. Lacey Samuels, Miguel A. Botella, Emmanuelle M. Bayer and Abel Rosado. *Trends in Cell Biology*. **2016**. Vol. 26:9, pags 705-717. http://dx.doi.org/10.1016/j.tcb.2016.05.007 705

Abstract:

The coordination of multiple metabolic activities in plants relies on an inter- organelle communication network established through membrane contact sites (MCS). The MCS are maintained in transient or durable configurations by tethering structures which keep the two membranes in close proximity, and create chemical microdomains that allow localized and targeted exchange of small molecules and possibly proteins. The past few years have witnessed a dramatic increase in our understanding of the structural and molecular organi- zation of plant interorganelle MCS, and their crucial roles in plant specialized functions including stress responses, cell to cell communication, and lipid transport. In this review, we summarize recent advances in understanding the molecular components, structural organization, and functions of different plant- specific MCS architectures.

CHAPTER 2:

Analysis of protein–lipid interactions using purified C2 domains.

Analysis of Protein–Lipid Interactions Using Purified C2 Domains. Jessica Pérez-Sancho, Arnaldo L. Schapire, Miguel A. Botella, and Abel Rosado. Jose R. Botella and Miguel A. Botella (eds.), *Plant Signal Transduction: Methods and Protocols, Methods in Molecular Biology, Springer Science+Business Media New York*, **2016**. Vol. 1363, pag 175-187. DOI 10.1007/978-1-4939-3115-6_14.

Abstract:

C2 domains (C2s) are regulatory protein modules identified in eukaryotic proteins targeted to cell membranes. C2s were initially characterized as independently folded Ca^{2+}-dependent phospholipids binding domains; however, later studies have shown that C2s have evolutionarily diverged into Ca^{2+}-dependent and Ca^{2+}-independent forms. These forms interact and regulate their affinity to diverse lipid species using different binding mechanisms. In this protocol, we describe a biochemical approach to produce, purify, and solubilize functional C2 domains bound to GST for the identification of their putative Ca^{2+}-dependent and Ca^{2+}-independent lipid-binding partners.

CHAPTER 3:

The Arabidopsis synaptotagmin1 is enriched in endoplasmic reticulum-plasma membrane contact sites and confers cellular resistance to mechanical stresses.

The Arabidopsis Synaptotagmin1 Is Enriched in Endoplasmic Reticulum-Plasma Membrane Contact Sites and Confers Cellular Resistance to Mechanical Stresses. Jessica Pérez-Sancho, Steffen Vanneste, Eunkyoung Lee, Heather E. McFarlane, Alicia Esteban del Valle, Victoriano Valpuesta, Jirí Friml, Miguel A. Botella, and Abel Rosado. *Plant Physiology*. **2015**. Vol. 168, pag 132-143. DOI 10.1104/pp.15.00260.

Abstract:

Eukaryotic endoplasmic reticulum (ER)-plasma membrane (PM) contact sites are evolutionarily conserved microdomains that have important roles in specialized metabolic functions such as ER-PM communication, lipid homeostasis, and Ca2+ influx. Despite recent advances in knowledge about ER-PM contact site components and functions in yeast (Saccharomyces cerevisiae) and mammals, relatively little is known about the functional significance of these structures in plants. In this report, we characterize the Arabidopsis (Arabidopsis thaliana) phospholipid binding Synaptotagmin1 (SYT1) as a plant ortholog of the mammal extended synaptotagmins and yeast tricalbins families of ER-PM anchors. We propose that SYT1 functions at ER-PM contact sites because it displays a dual ER-PM localization, it is enriched in microtubule-depleted regions at the cell cortex, and it colocalizes with Vesicle-Associated Protein27-1, a known ER-PM marker. Furthermore, biochemical and physiological analyses indicate that SYT1 might function as an electrostatic phospholipid anchor conferring mechanical stability in plant cells. Together, the subcellular localization and functional characterization of SYT1 highlights a putative role of plant ER-PM contact site components in the cellular adaptation to environmental stresses

CHAPTER 4:

The ER-PM tethers synaptotagmin 1 and 3 regulate cold acclimation associated lipid remodeling in Arabidopsis.

The ER-PM tethers Synaptotagmin 1 and 3 regulate cold acclimation associated lipid remodeling in Arabidopsis.

Jessica Pérez-Sancho[1], Abel Rosado[2], Arnaldo L. Schapire[1], Noemí Ruiz-López[1], Steffen Vanneste[3], Sonia Osorio[1], Lothar Willtmizer[4], Carlos Perea[5], Julio Salinas[5], Miguel Ángel Botella[1].

[1]Departamento de Biología Molecular y Bioquímica, IHSM (Universidad de Málaga-CSIC), Málaga, Spain, [2] Department of Botany, Faculty of Sciences, University of British Columbia, Vancouver, Canada, [3]Department of Plant Systems Biology, VIB-Ghent University, Ghent, Belgium, [4]Central Metabolism Group, Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany, [5]Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas (CSIC), Madrid, Spain.

ABSTRACT

Remodeling of the lipid composition of the PM during cold acclimation is an important milestone of plant adaption to sub-optimal temperature conditions. In Arabidopsis, the endoplasmic reticulum – plasma membrane (ER-PM) tether synaptotagmin1 (SYT1) maintains the plasma membrane (PM) integrity during freezing and is required for the full Ca^{2+}-*dependent* acquisition of freezing tolerance. Despite these important roles, the mechanism governing SYT1 binding to PM, and the *in vivo* effects on lipid composition during cold acclimation have been largely overlooked. In this report, we establish that cold-acclimation causes a relocalization of SYT1 from the ER to ER-PM contact sites. We also show that SYT3, an additional member of the Arabidopsis SYT family, is a Ca^{2+}-responsive phospholipid binding protein located to ER-PM contact sites. *SYT3* is a cold inducible determinant that together with *SYT1* is required for cold acclimation and their loss causes the over-accumulation of their phospholipid binding partners. Overall, our study highlights the role of SYTs as important components linked to the lipid modifications required for cold acclimation and freezing tolerance in plants.

INTRODUCTION

The extent to which plants from temperate climates can prevent freezing damage is a major factor limiting their geographical distribution, growth, and productivity. Since plants lack systems to maintain temperature homeostasis, freezing tolerance is achieved through the induction of physiological and biochemical adaptations upon exposure to low non-freezing temperatures in a process known as cold acclimation. [1,2]. Cold acclimation activates multiple physiological responses including the induction of cold-responsive genes, and the synthesis and accumulation of low-molecular weight molecules and proteins that function as cryoprotectants[3,4]. In addition, during cold acclimation plants adjust their intracellular lipid

composition to prevent the freezing induced damage to intracellular lipid membranes, which is a major factor responsible for freezing injury and cell death[5–11]. The complex changes in lipid composition induced by cold can be grouped into two non-exclusive categories: (1) Changes in structural membrane lipids leading to an enhancement in the physical membrane cryostability, such as the general increase in the degree of unsaturation[6] , and the accumulation of long-chain unsaturated triacylglycerides (TAGs)[11,12], and (2) changes in signalling lipids acting as secondary messengers such as Phosphatidylinositol (PI) and its derived phosphorylated forms[9,13,14]. This new lipid homeostasis requires the massive delivery and removal of lipids[15–18] that can be achieved by two mechanisms: the vesicular transport of bulk lipids, that requires metabolic energy, intact cytoskeleton, and protein transport machineries[19–21], and the non-vesicular lipid trafficking of specific lipids at membrane contact sites, that is facilitated by lipid-transfer proteins (LTPs)[22–24].

Cold acclimation not only triggers dynamic lipid changes in cellular membranes, but also activates Ca^{+2} as a second messenger[25,26]. Thus, after cold stress, Ca^{+2} activates complex signal transduction pathways mainly mediated by three cold inducible transcription factors (*CBF1*, *CBF2* and *CBF3*) that are collectively referred as the CBF hub[27]. The regulation by the CBF hub results in the extensive reorganizations of the transcriptome that elicit the accumulation of low-molecular-weight cryoprotectants (sucrose, raffinose, proline, and hydrophilic polypeptides) to protect cellular structures against cold induced dehydration[3,4,28]. Cold also causes the activation of the phospholipase C (PLC) signaling pathway that modulates the inositol 3-phosphate (IP_3) dependent Ca^{+2} release mechanisms[29,30], and the phospholipase D (PLD) dependent signaling pathway that induces membrane remodeling through the second messenger phosphatidic acid[31].

Previous reports characterizing the founder member of synaptotagmin family in plants (SYT1) demonstrated that *SYT1* is important for PM integrity maintenance, especially under conditions of high potential for membrane disruption[32,33], and it is also involved in the acquisition of Ca^{2+}-dependent freezing tolerance[34]. SYT1 is an ER-PM tether anchored to the ER through a TM domain[33,35]. The cytosolic tail of SYT1 can bind acidic phospholipids in a Ca^{2+} dependent manner through its tandem C2 domains [32,33,36], mediating the interaction with the PM. Additionally, SYTs contain a SMP domain, that, in mammals, target proteins to MCS and mediate non-vesicular lipid trafficking at ER-PM contact sites[37,38].

Interestingly, various reports have shown an increase of SYT1 closely associated to the PM after cold acclimation[39,40] and a hypersensitivity to cold and freezing in the *syt1* mutant[34]. Previous studies in our lab identified a second member of the synaptotagmin family in Arabidopsis, SYT3, to be essential for cold acclimation in *SYT1* deprived backgrounds[41], as *syt1syt3* double mutant plants were more sensitive to freezing than the single *syt1* mutant only

after cold acclimation and *syt3* single mutant did not show any phenotype either with or without cold acclimation[41].

In this study, we characterize *SYT1* as a housekeeping gene in cold acclimation and freezing tolerance, and SYT3 as a functional phospholipid binding protein induced during cold acclimation. Both SYT1 and SYT3 are enriched at ER-PM contact sites. We then focus on the PM delivery mechanisms of SYTs and analyze their roles in the lipidome remodeling during cold acclimation.

RESULTS AND DISCUSSION

SYT1 has a housekeeping role in freezing tolerance

SYT1 is essential for the maintenance of plasma membrane integrity in plants subjected to conditions that compromise cell turgor such as osmotic and ionic stresses[32]. SYT1 is also important for the acquisition of Ca^{2+}-dependent freezing tolerance[34]. However, these studies have based their physiological results on protoplast assays or seedlings grown on agar plates. Therefore, we investigated the occurrence of freezing related phenotypes in four-weeks-old wild type (WT) and *syt1* plants germinated and grown on soil in a controlled environmental chamber, conditions that more closely resemble those found in nature.

We tested how four-weeks-old WT, *syt1-2* (*syt1*), and SYT1 complementing line 9/2 (CL9) plants[32] recovered from a 6 h treatment at different freezing temperatures. Plant survival was recorded as the percentage of plants that were still alive after a 7 days' recovery time. As shown in Figure 1A, *syt1* plants showed reduced freezing tolerance compared to WT or CL9, with a 50 % survival rate at aprox. -3.5 ºC and -5.5 ºC respectively. Next, we investigated whether SYT1 also has a role in cold acclimated freezing tolerance. A similar experiment was performed but, before freezing, plants were exposed to 7 days of cold acclimation at 4 °C. All plants lines were able to tolerate lower temperatures, as expected after cold acclimation, but *syt1* plants continued being less freezing tolerant, with a 50 % survival rate at aprox. -8.5 ºC, compared to the aprox. -10.5 ºC of WT and CL9 (Figure 1B).

Previous proteomics studies of purified PM[39,40] reported the accumulation of SYT1 in PM-detergent resistant membranes (DRM) of Arabidopsis leaves after 1 day of cold acclimation. Using specific antibodies against SYT1 we did not observe significant differences in the total accumulation of SYT1 in WT plants during one week of cold acclimation (Figure 1C), indicating that there are no changes in the total amount of SYT1 protein. Taking in account that SYT1 is an ER resident protein specifically enriched in ER-PM contact sites[33,35], the differences observed in

SYT1 in PM-DRM during the acclimation process could be due to a change in its distribution within the cell. Taking these data together, *SYT1* functions as a housekeeping gene in freezing tolerance.

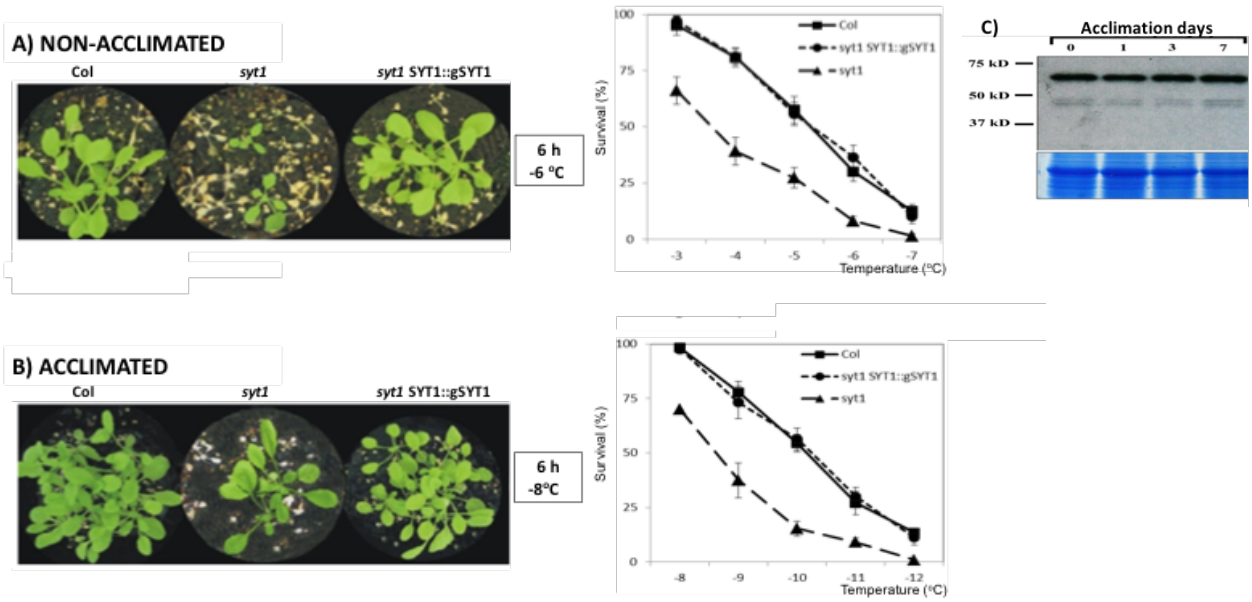


Figure 1: *Syt1* has a housekeeping role in cold acclimation.
Four-weeks-old plants germinated and grown on soil in a controlled environmental chamber were frozen to the indicated temperatures without **A)** or with **B)** one week acclimation at 4°C. The survival rate was measured after one week of recovery at standard growth conditions. Both, NON-ACCLIMATED and ACCLIMATED *syt1* plants, were significantly less freezing tolerant than WT and the complementation line. **C)** SYT1 protein total amount did not change during the cold acclimation process as assessed by western blot

SYT1 accumulates at ER-PM contact sites during cold acclimation

Next, we investigated the SYT1 dynamics during cold acclimation using a SYT1::SYT1-GFP reporter line transformed in the *syt1* background[33], hereafter SYT1-GFP. We first tested whether a SYT1-GFP construct was able to complement the previously reported PM integrity related phenotypes of the *syt1* mutant. We demonstrated that an osmotic shock is able to perturb the PM of *syt1* to a wider extent than that of WT, as showed by a rapid and massive internalization of the lipophilic dye FM4-64[32]. SYT1-GFP seedlings showed similar FM4-64 internalization to WT after 30 min incubation in 150 mM KCl (Figure 2A and Figure S1).

As main changes in SYT1 abundance in PM-DRMs were observed after 1 and 3 days of cold acclimation[39], we chose this time points to assess the dynamics of SYT1-GFP. As shown in Figure 2B, 1 day cold treated plants presented a spotted SYT1-GFP pattern compared to the more reticulated pattern observed in control plants. Plants under a three-day cold treatment showed a similar spotted pattern.

Next, changes in SYT1 location were visualized in control and 1 day and 3 days cold treated plants using a spinning disk microscope controlled by the Volocity® software. We performed three independent experiments (only one for 3 days cold treated plants) and in each of them we imaged one cotyledon per plant of at least 3 plants and at least 3 distinct regions per cotyledon. As previously observed using standard confocal microscopy, 1-day-cold treatment caused a concentration of SYT1-GFP into defined spotted structures that were still visible after 3 days, although not so prominent (Figure 2C). Three-D reconstruction of 200 nm spaced z-stacks from control and 1 day acclimated plants also showed that the SYT1-GFP labeled spots protrude from

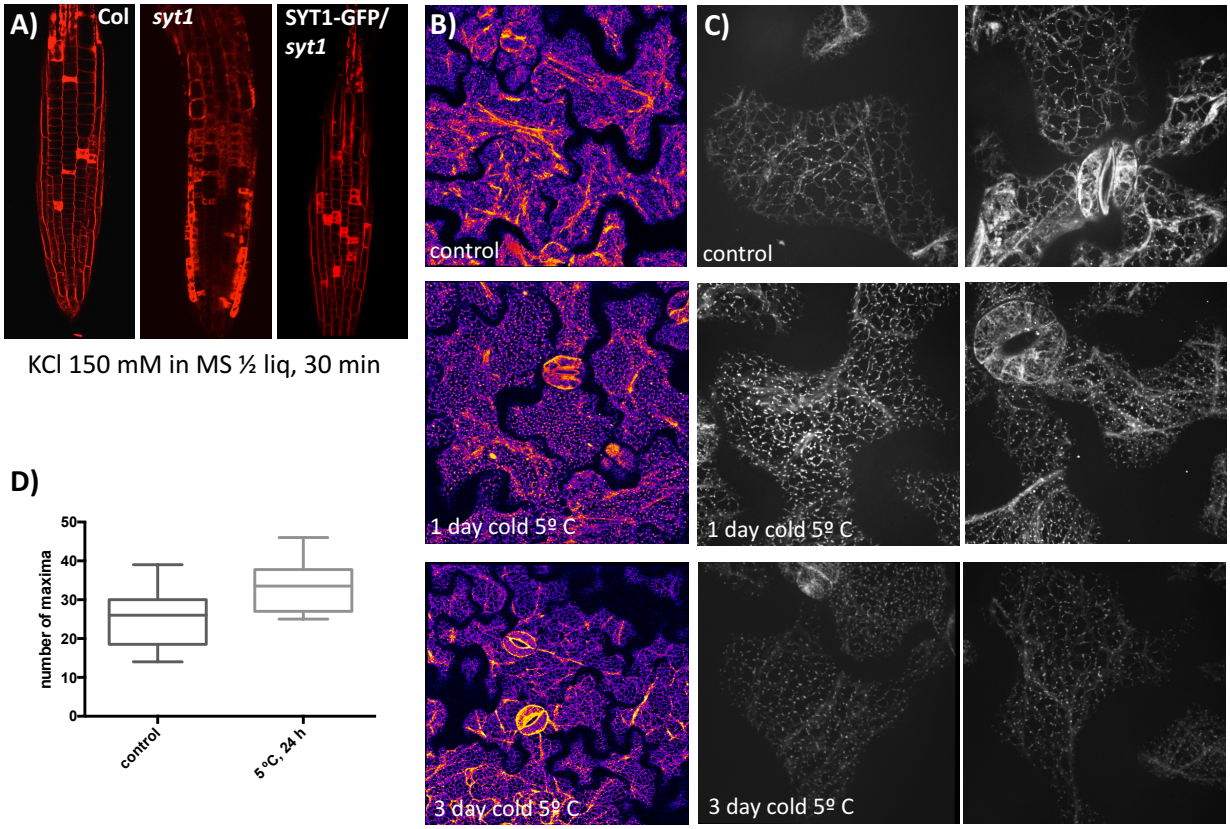


Figure 2: SYT1 accumulation in ER-PM contact sites increases during cold acclimation.
A) Five-days-old seedlings were incubated for 30 min in KCl 150 mM and then stained with FM4-64 4 µM for 10 min. FM4-64 entered massively in *syt1* but stained only a few cells in WT or in *syt1*/SYT1-GFP. **B)** Confocal images of seven-days-old *syt1*/SYT1-GFP seedlings after no cold or one or three days at 5 ºC. Modified LUT: from yellow to purple, from maximal to minimal intensity. **C)** Spinning disk images of seven-days-old *syt1*/SYT1-GFP seedlings after no cold or one or three days at 5 ºC. **D)** Quantification of the number of spots. For each image, spots were localized using *find max* plugging. Graph shows a distribution of the number of spots per 256 mm^2 of leaf in control plants and one-day-cold treated plants.

the ER towards the PM (supplemental video 1 and 2). Quantification with FIJI showed an increase in the number of spots per area in the cold-treated plants (Figure 2D).

Assuming that SYT1-GFP spots represent ER-PM contact sites, the relocalization observed could be caused by a general increase in ER-PM contact sites, a specific redistribution of SYT1-

GFP from the cER towards them or a mix of both. With the aim of answering this question, we used the artificially engineered ER-PM contact site marker MAPPER[42]. This GFP construct contains a TM domain and a signal peptide for its integration in the ER membrane, and a polybasic region that binds lipids in the PM (Figure 3A). MAPPER was originally designed for the visualization of ER-PM contact sites in mammalian cells[42]. We first checked if this construct also labels ER-PM contact sites when transiently or stably expressed in plants. Arabidopsis 5-days old plants stably expressing MAPPER showed a spotted signal reminiscent to that of SYT1 (Figure 3C, control), that became a little more reticulated as plants grew older. MAPPER spots colocalized with SYT1-RFP spots when both constructs were transiently expressed in *Nicotiana Benthamiana* (Figure 3B). Once we validated its proper localization, transgenic Arabidopsis plants carrying MAPPER were subjected to cold treatment. In contrast to SYT1-GFP, cold did not cause obvious changes in MAPPER localization (Figure 3C). This result suggests that the differences of SYT1-GFP signal caused by cold are due to changes in its distribution between cER and ER-PM contact sites rather than a general change in ER-PM contact sites.

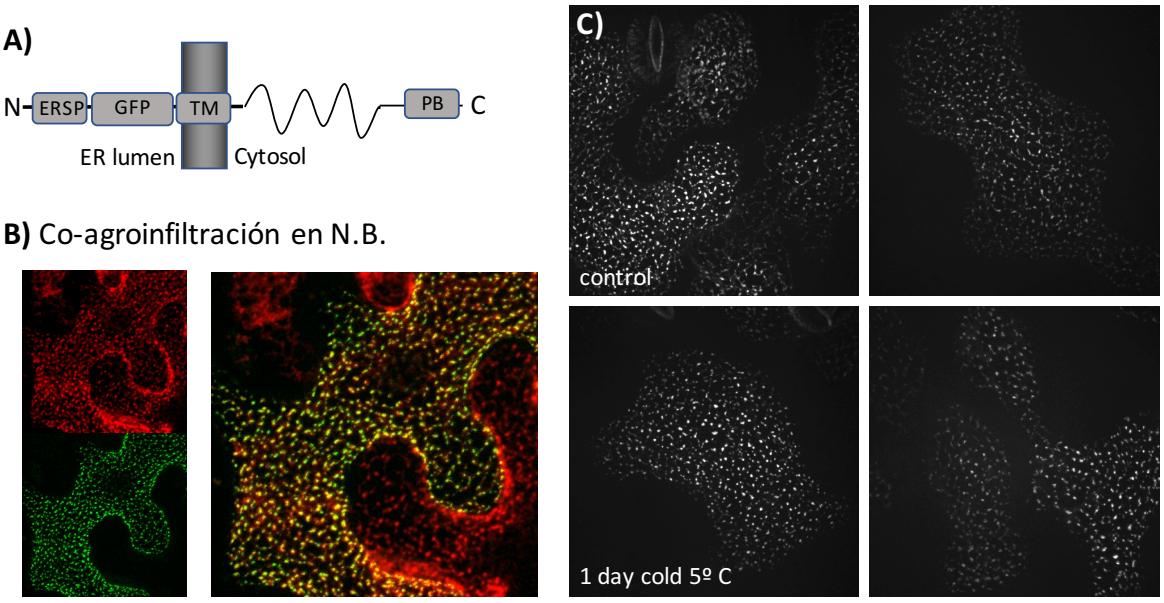


Figure 3: MAPPER accumulation in ER-PM contact sites does not increase during cold acclimation.
A) Schematic representation of MAPPER construct. N= N-terminus; C= C-terminus; ERSP= ER signal peptide; PB= Polibasic region. **B)** Confocal images of *Nicotiana Benthamiana* cells co-agroinfiltrated with SYT1-RFP (red) and MAPPER (green). Merge shows extensive colocalization. **C)** Spinning disk images of seven-days-old MAPPER seedlings.

Identification of SYT3 as a putative cold tolerance determinant

SYT1 belongs to a family of five members in Arabidopsis[43,44]. We questioned whether additional members of the SYT family might be involved in cold acclimation. We first analyzed

the expression profile of the *SYT* genes using the developmental maps of the AtGenExpress database[45,46]. As shown in Figure 4A, *SYT1*, *SYT3 and SYT5* were the only family members expressed ubiquitously, and *SYT1* expression was markedly higher than that of *SYT3 and SYT5*. Conversely, *SYT2* and *SYT4* were only expressed at detectable levels in very specific tissues such as roots, seeds, and pollen. Next we analyzed the expression of all five *SYT* genes using qPCR in control condition and after 24 h of cold acclimation. As shown in Figure 4B, *SYT3* showed a 6-fold induction and *SYT1* showed between 3 and 4-fold induction while the other three *SYT* genes did not change their expression by cold. *SYT3* was previously reported as a pseudogene encoding only a truncated version of the protein[34,47]. However, previous work in our lab demonstrated that *SYT3* presents several splice variants, some of them producing a full-length protein. We also showed that *SYT3* is important for cold acclimation in *SYT1* deprived background[41]. Based on these results we selected *SYT3* as a suitable candidate to further investigate its function during cold acclimation.

SYT3 localizes to ER-PM contact sites and display phospholipid binding activity.

We first analyzed SYT3 expression profile and subcellular localization. To study the expression of *SYT3* at the tissue level, we generated transgenic lines harboring a fused β-glucuronidase (*GUS*) gene under control of a 2.1kb *SYT3 cis*-regulatory region upstream its open reading frame. Histochemical GUS staining in 6-d-old seedlings detected strong GUS activity in stomata and old primary roots and a weaker and more variable expression in the epidermal cells of cotyledons, leaf primordia, and the root elongation zone (Figure 4C). Despite multiple attempts, we were unable to generate a SYT3-GFP fusion driven by the 2.1kb *cis*-regulatory region used for the GUS assays. As an alternative, we generated independent SYT3-GFP transgenic lines driven by the constitutive CaMV 35S promoter (hereafter, 35S::SYT3-GFP). As shown in Figure 4D, the subcellular localization of 35S::SYT3-GFP highly resembled that of SYT1-GFP and endogenous SYT1, with a spotted GFP pattern at the cell periphery both in root and shoot[33]. In addition, SYT3-GFP also showed a highly dynamic movement through transvacuolar strands that was abolished after LatB treatment, similar to SYT1 (Supplemental Videos 3 and 4).

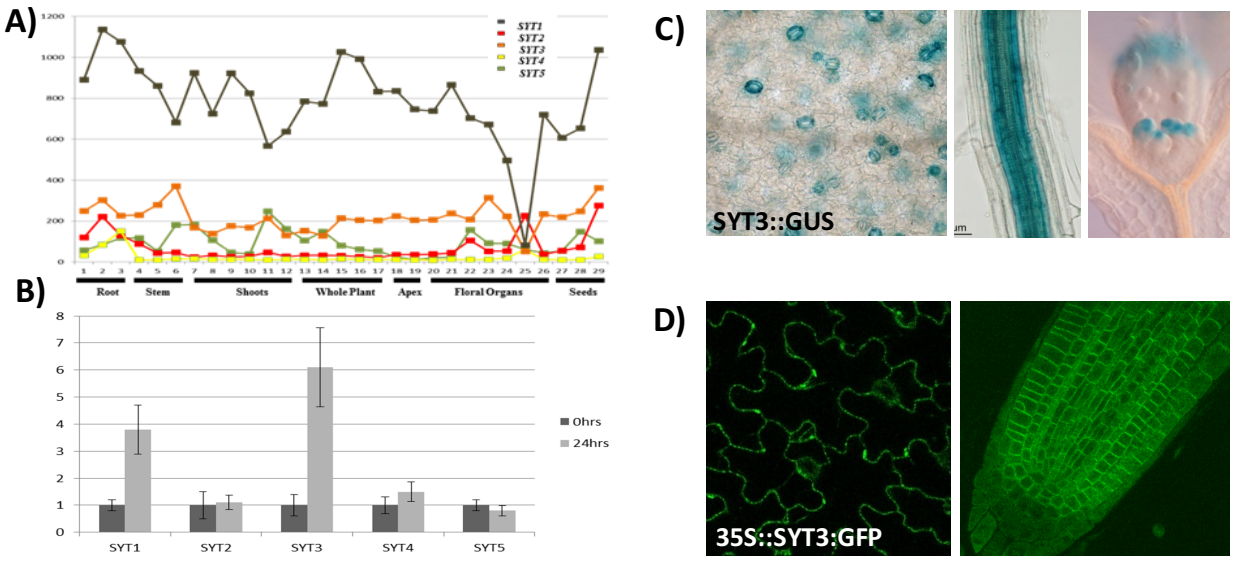


Figure 4: Identification of *SYT3* as a cold inducible gene.

A) Microarray expression profile in different tissues and developmental stages of the five SYT family members in Arabidopsis. **B)** q-RT-PCR of the different *SYTS* showed *SYT1* and *SYT3* induction after 24h of cold treatment at 4 °C. **C)** Histochemical GUS staining in seven-days-old seedlings detected strong GUS activity in stomata and old primary roots and a weaker and more variable expression in leaf primordia. **D)** Confocal images of seven-days-old transgenic 35S::SYT3:GFP plants showing a spotted GFP pattern at the cell periphery (cotyledon, left, and root tip, right).

SYT3 contains two C-terminal C2 domains whose predicted function is the Ca^{2+}-dependent binding to phospholipid membranes. Still, the C2's Ca^{2+} and phospholipid binding properties cannot be reliably predicted from sequence analysis alone[48]. To have a first insight into the Ca^{2+} binding properties of SYT3's C2 domains, we performed 3D molecular modeling of the SYT3 $C2A^{244-402}$ and $C2B^{403-540}$ domains based on the SMART motif database[49,50]. For that purpose, we used the Phyre2 multi-template homology modeling tool coupled to the 3DLigandSite server for protein binding site prediction[51,52]. The resulting PDB coordinates were further refined using the Molprobity modeling algorithms[53]. Finally, the 3D structure was validated by analysis of torsional angles using Rampage[54] and Ramachandran plots were generated (Figure S2). As a result, very robust $C2A^{244-402}$ and $C2B^{403-540}$ 3D models with a high confidence match (>99%) were generated (Figure S2). Three Ca^{+2} coordination pockets with a high confidence score (Cs > 0.5) were identified for $C2A^{244-402}$ while no Ca^{+2} coordination pockets over the 0.5 Cs threshold were identified for $C2B^{403-540}$.

The biochemical interaction of SYT3 C2 domains with phospholipids and its Ca^{2+} dependency was investigated using liposome binding assays[32,36,55]. For that purpose, recombinant C2A and C2B domains of SYT3 were purified as glutathione S-transferase (GST) fusion proteins and incubated with liposomes (25% PS/75% PC) in the presence of different concentrations of free Ca^{2+}. After centrifugation, liposome-bound proteins were separated using

SDS-PAGE and C2 domains were quantified using Coomassie staining. As a result, SYT3-C2A was capable of binding phospholipids in a Ca^{2+}-dependent manner with an estimated half-maximal Ca^{2+} binding value EC_{C2A}= 4.5 ± 0.6µM free Ca^{2+} (Figure 5A). SYT3-C2B showed Ca^{2+}-independent binding to liposomes but contrary to SYT1-C2B[32] its binding affinity increased in the presence of Ca^{2+} (EC_{C2B} = 5.0 ± 0.5µM free Ca^{2+}) (Figure 5B). In both cases, the estimated EC values were within the Ca^{2+} concentration ranges considered physiological in plants and mammals[26,56,57].

Once we established that the SYT3-C2 domains were *bona fide* Ca^{2+} responsive phospholipid binding modules, we determined whether SYT3 is able to bind phospholipids other than PS and PC by protein-lipid overlay assays. We generated a purified SYT3 amino acid fragment containing both C2 domains fused to GST and incubated it with commercial lipid-bound membranes containing various phospholipids (PIP-strips). As a result, we observed that, similarly to SYT1, SYT3 binds phospholipids bearing a phosphorylated head group and two acyl chains, namely, phosphatidic acid (PA) and each of the phosphatidylinositol phosphates (PIP) (Figure C).

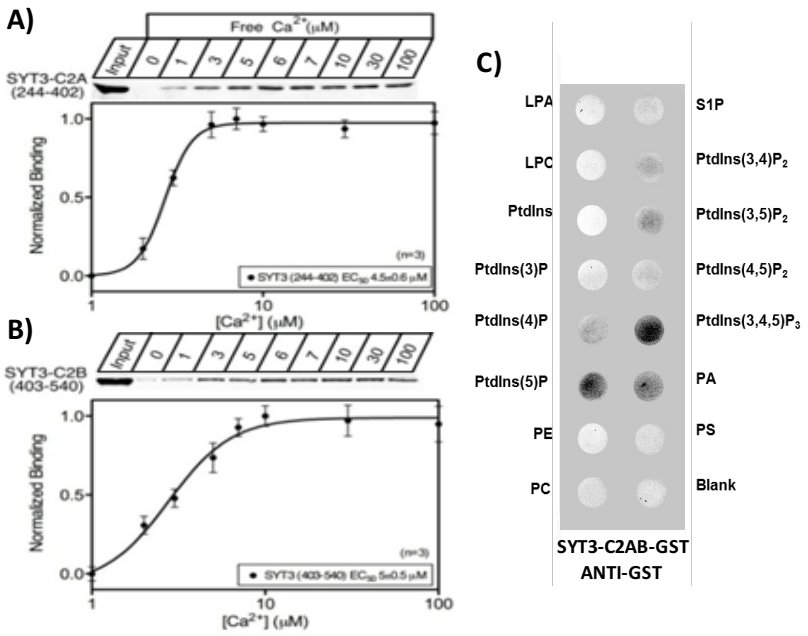


Figure 5: Characterization of SYT3-C2 domains binding to phospholipids.
(A, B) Quantification of SYT3-C2 domains (C2A, **A**, C2B, **B**) binding to artificial liposomes in the presence of different Ca^{+2} concentrations. Cosedimented proteins were separated by SDS/PAGE, stained with CBB and quantified by colorimetry. **C)** PIP STRIPS showing the *in vitro* lipid interactors of SYT3 C2AB. It strongly interacted with phosphatidic acid and several negatively charged PI species. LPA (Lysophosphatidic acid), LPC (Lysophosphocholina), PtdIns (Phosphatidylinositol), PtdIns(3,4)P_2 (Phosphatidylinositol -3,4- diphosphate), PE (Phosphatidylethanolamine), PC (Phosphatidylcholine), S1P (Sphingosine-1-phosphate), PA (Phosphatidic acid), PS (Phosphatidylserine).

***syt1syt3* mutant shows altered levels of SYTs phospholipid binding partners.**

Next we asked whether SYT1 and SYT3 may also have a role in lipid homeostasis in plants, considering that E-Syts function as lipid transfer proteins[37]. For this, we used ultra-performance liquid chromatography coupled to Fourier-transform mass spectrometry (UPLC-FTMS)-based lipidome analyses[58,59] to determine if *syt* mutations induce changes in lipid composition in plants grown under standard conditions. As a result, we obtained a relative quantification of the

contents of 10 major lipid classes in wild type and *syt1syt3* plants. Cold acclimation in WT plants caused a general increase in the unsaturation grade, a decrease in the amount of mono-, di-galactosyl-diacylglycerols (MGDG, DGDG), and sulfoquinovosyl-diacylglycerols (SQDG), specially for the less unsaturated species, and an increase in di- and tri-acylglycerols (DAG and TAG), especially for the highly unsaturated species (Figure 6A), similarly to previous reports[11,12]. As shown in Figure 6B the relative amounts of the most abundant glycolipids in photosynthetic membranes, namely (MGDGs and DGDGs), and the most abundant membrane phospholipid phosphatidylcholine (PC) showed no significant differences between wild type and *syt1syt3*. Interestingly, the most remarkable differences were found in the lipid species that directly interact with the SYT1[33] and SYT3 C2AB domains in the *in vitro* assays, namely phosphatidylglycerols (PGs) and Phosphatidylinositols (PIs), that were under-represented in *syt1syt3* compared to WT. This result suggests that the direct interaction of SYT1 and SYT3 with PGs and PIs regulate the total amount of those lipid species in the plant tissues.

We next assessed any possible functional role for the observed changes in the *syt1syt3* lipid contents that might be associated with the cold acclimation processes. Thus, lipidome analyses were performed in four-weeks-old Arabidopsis plants subjected to 7 days of cold, a time-point where the cold acclimation response leads to a significant increase in the Arabidopsis freezing tolerance. After cold acclimation, PI and PG were over-accumulated in the *syt1syt3* mutant (Figure 6C), supporting an involvement of SYT1 and SYT3 in lipid homeostasis during cold-acclimation in plants.

These results show that both, *SYT1* and *SYT3* are essential to maintain the lipid balance in vegetative tissues, but we cannot discriminate whether the observed PI and PG accumulations in *syt1syt3* plants occur at the ER, at the PM or at different intracellular membranes. In this line, the future characterization of the lipidome of specific organelles will provide a more accurate picture of the specific role of SYTs in the global lipidome profile maintenance.

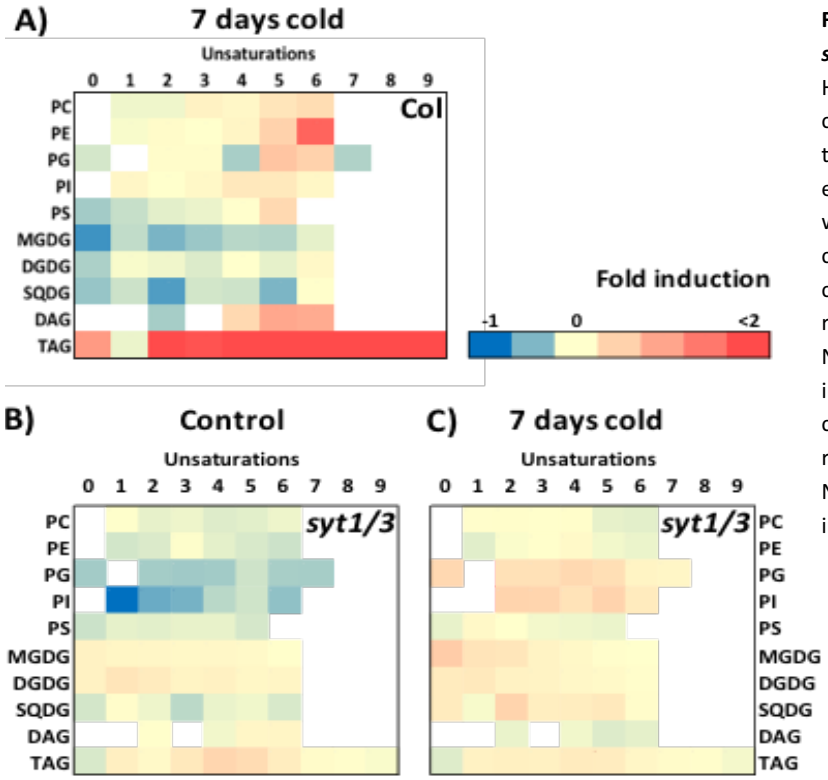


Figure 6: Altered lipid homeostasis in *syt1/syt3* double mutant.

Heat maps displaying the 10 major lipid classes and the number of unsaturations in their fatty acid chains. **A)** Fold induction of each lipid class in Col WT plants after one week at 4 ºC compared to control conditions. **B)** Fold induction of each lipid class in *syt1/syt3* plants in control condition relative to WT plants in control conditions. Note that PGs and PIs are underaccumulated in general. **C)** Fold induction of each lipid class in 7-days-acclimated *syt1/syt3* plants relative to 7-days-acclimated WT plants (A). Note that PGs and PIs are overaccumulated in general.

Figure S1: Related to Figure 2A

KCl 150 mM in MS ½ liq, 30 min

FM4-64 4 uM 10 min

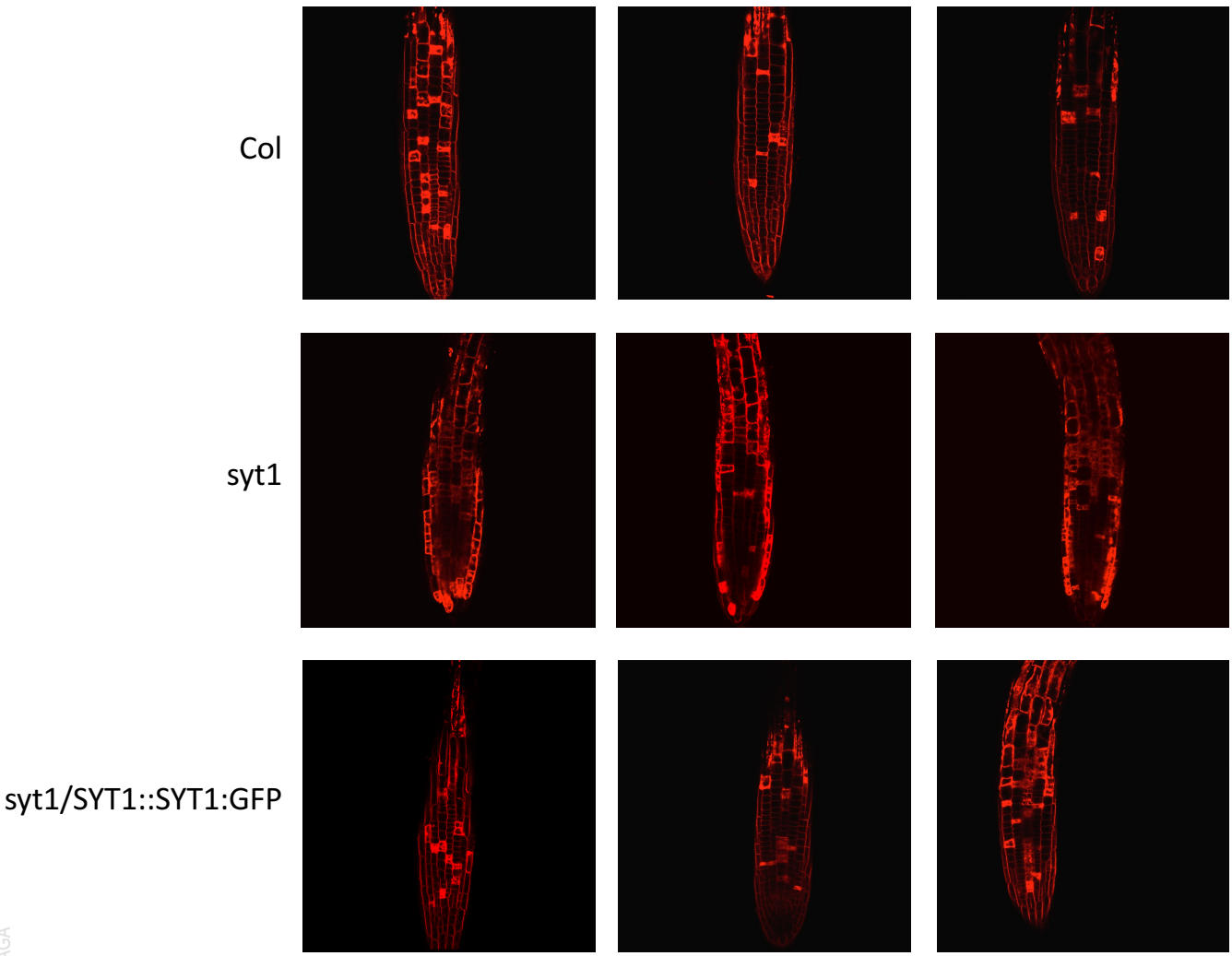
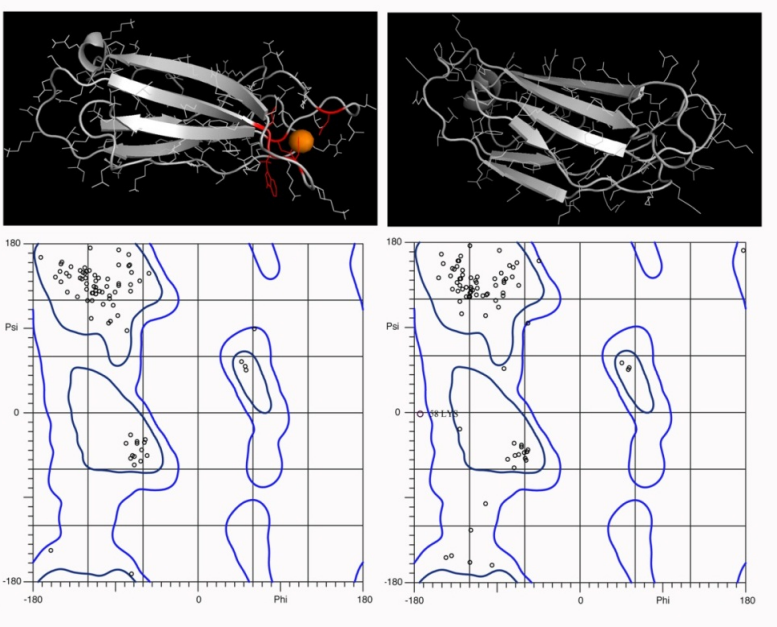
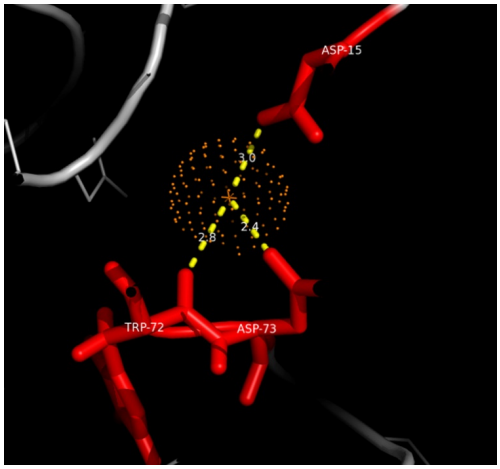


Figure S2: Related to Figure 4

Modelization of the SYT3 C2 domains





Detail of Ca^{+2} binding pocket

CONCLUSIONS

CONCLUSIONS

1. SYT1 and SYT3 localize to ER-PM contact sites in plants.

2. SYT1 and SYT3 bind negatively-charged phospholipids in a Ca^{2+} dependent manner.

3. SYT1 and SYT3 movement depends on actin filaments but their final localization is restricted by cortical microtubules.

4. SYT1 is essential for cellular resistance to mechanical stress, likely by coordinating the cortical ER and cytoskeleton responses to minimize the mechanical loads at the PM.

5. SYT1 is important for non-acclimated and cold-acclimated freezing tolerance without changes in total protein level.

6. Cold acclimation causes the relocalization of SYT1 to ER-PM contact sites.

7. Both SYT1 and cold-induced SYT3 are required for lipidome remodeling in Arabidopsis plants during cold acclimation.

RESUMEN EN ESPAÑOL

RESUMEN EN ESPAÑOL

La Universidad de Málaga requiere un resumen de la tesis en español, de al menos 5000 palabras, cuando ésta ha sido escrita en un idioma diferente. Por tanto, lo que aparece a continuación es dicho resumen.

INTRODUCCIÓN

Las células de todos los organismos están sujetas a estrés, entendiendo éste como condiciones ambientales desfavorables que limitan su normal desarrollo. A diferencia de otros organismos, las plantas no pueden escapar cuando esas condiciones desfavorables se presentan y, por tanto, deben lidiar con ellas.

La membrana plasmática (MP) separa el interior y el exterior celular y actúa como una barrera que protege a la célula de posibles condiciones externas adversas[1,2]. Al mismo tiempo, la célula necesita establecer una comunicación continua con su entorno, percibir estímulos y responder a ellos adecuadamente. De nuevo, la MP es fundamental en esta comunicación, ya que es la parte de la célula en contacto directo con el exterior[3–6]. La visión más extendida de la MP, el Mosaico Fuido de Singer and Nicolson[7], la describe como una bicapa fosfolipídica poblada por algunas proteínas distribuidas de manera relativamente homogénea. Este modelo supuso un gran avance en nuestro entendimiento de las membranas biológicas y ayudó a entender como interacciones hidrofílicas e hidrofóbicas entre grupos polares y apolares de lípidos y proteínas determinan sus características. Hoy en día sabemos que la MP presenta una estructura más compleja y se encuentra subcompartimentalizada en microdominios o compartimentos de distintos tamaños, propiedades y mecanismos de formación[8–12]. El citoesqueleto cortical[13], la pared celular[14] (o matriz extracelular en células animales), interacciones específicas entre ciertos lípidos[15], oligomerizaciones de proteínas[16,17]… son responsables de esta compartimentalización que tiene importantes consecuencias para el desarrollo de la célula y su supervivencia.

Las células eucariotas, además de la MP, presentan un sistema de endomembranas que delimita los distintos orgánulos intracelulares y crea microambientes independientes dentro de la célula. Al mismo tiempo, esta compartimentalización intracelular dificulta el tráfico de metabolitos e información a través de la célula, por lo que son necesarios mecanismos de comunicación entre orgánulos. El tráfico de vesículas ha sido considerado durante mucho tiempo como el único mecanismo posible[18,19], pero hoy en día se sabe que también existe comunicación directa entre orgánulos a través de los llamados “sitios de contacto entre membranas” (MCS, del inglés membrane contact sites)[20–23]. Los MCS pueden definirse como estructuras membranosas o microdominios donde las membranas de dos orgánulos diferentes se aproximan a una distancia menor

de 40 nm sin que haya fusión entre ellas[20,23–25]. Para que esto ocurra, estos sitios poseen proteínas de anclaje, que tienen la capacidad de unirse simultáneamente a las dos membranas[26]. Esta unión puede verse regulada por diversos factores, como la concentración de Ca^{2+} en los distintos orgánulos, la presencia de lípidos específicos en las membranas o la presencia de otras proteínas que refuercen la interacción. Como resultado, los MCS pueden formarse, crecer, disminuir o separarse completamente según las necesidades de la célula.

Prácticamente todos los orgánulos membranosos tienen la capacidad de formar MCS, pero es especialmente destacable el retículo endoplasmático (RE). El RE es un sistema membranoso único y continuo que se distribuye por toda la célula, poseyendo la mayor relación área/volumen de entre todos los orgánulos[27]. Estructuralmente, puede clasificarse en envoltura nuclear y RE periférico, que a su vez está formado por cisternas y túbulos interconectados[28–30]. Además, su forma no está permanentemente definida, sino que se mueve y reorganiza según las necesidades. Cada vez parece más claro que las diferentes formas del RE están optimizadas para desarrollar mejor sus distintas funciones[31,32]. Entre otras muchas, es el principal orgánulo en la síntesis de proteínas y fosfolípidos[18,19] y es un importante almacén intracelular de Ca^{2+}[33]. Además, el RE establece contactos con prácticamente todos los orgánulos, y con el citoesqueleto y la membrana plasmática, lo que también determina su forma en gran medida[27,28].

En esta tesis nos vamos a centrar principalmente en los sitios de contacto entre el RE y la MP (contactos RE-MP), que se definen como zonas donde el RE cortical, aquel que subyace a la membrana plasmática, se aproxima a menos de 30 nm de ésta[34–38], y participan en la transmisión de señales percibidas en la membrana plasmática[1,39–41].

Señalización de Ca^{2+} en los contactos RE-MP.

La señalización de Ca^{2+} en los contactos RE-MP ha sido mucho más ampliamente estudiada en animales que en otros organismos. El RE es el mayor reservorio de Ca^{2+} en metazoos y su liberación como segundo mensajero es activada por la percepción de diversos estímulos en la membrana plasmática[42,43]. Una vez cumplida su función, es necesario recuperar rápidamente los niveles de Ca^{2+} en el RE si queremos que pueda reaccionar ante estímulos venideros. Los contactos RE-MP juegan un papel fundamental en este sentido[44–46].

En células musculares pueden observarse unas estructuras particulares, denominadas díadas y tríadas, en las que profundas invaginaciones de membrana plasmática (T-túbulos) forman extensos contactos, a menos de 15 nm, con el RE[47,48]. La depolarización de la membrana plasmática causa la apertura de canales de Ca^{2+} DHPR localizados en los T-túbulos, por donde entra una pequeña cantidad de Ca^{2+}. Esta pequeña cantidad de

Ca^{2+} queda concentrada en las díadas y tríadas y promueve la apertura de canales de Ca^{2+} RyR en el RE, liberando masivamente el Ca^{2+} luminal en el citosol, lo que promueve la contracción muscular[49–52].

Una vez vaciadas las reservas de Ca^{2+} en el retículo (independientemente del mecanismo de descarga), éstas necesitan rellenarse rápidamente; de ello se encarga principalmente el sistema SOCE (store operated Ca^{2+} entry)[35,45,53–58]. Las STIMs son sensores de Ca^{2+} anclados en la membrana del RE que oligomerizan y se concentran en contactos RE-MP cuando la concentración de Ca^{2+} en el RE baja. Una vez aquí, las STIMs interactúan con canales de Ca^{2+} en la MP (Orai1), promoviendo la entrada de Ca^{2+} extracelular en el citosol. Las bombas de Ca^{2+} del RE (SERCA) también se concentran en contactos RE-MP cuando el Ca^{2+} luminal es bajo, absorbiendo rápidamente hacia el RE el Ca^{2+} introducido al citosol por Orai1.

La Extended-Synaptotagmina1 (E-Syt1) en animales es una proteína anclada al RE que actúa como un sensor de los niveles de Ca^{2+} en el citosol y se concentra en contactos RE-PM cuando la concentración es alta, disminuyendo la distancia entre ambas membranas[37,43,44,59]. Células mutantes en E-Syt1 y sus homólogos E-Syt2 y E-Syt3 presentan muchos menos contactos RE-MP, y los que quedan no son capaces de responder a subidas en la concentración de Ca^{2+}.

Transporte de fosfolípidos sin vesículas en los contactos RE-MP.

Aunque el RE es el orgánulo de mayor importancia en la síntesis de fosfolípidos, cumple esta función en colaboración con otros orgánulos[60–63]. Además, una vez sintetizados, los fosfolípidos tienen que viajar desde su lugar de síntesis a su localización definitiva dentro de la célula. Hay dos mecanismos principales para el transporte de lípidos, el tráfico a través de vesículas y el tráfico sin vesículas en los MCS, con ayuda de proteínas transportadoras de lípidos (LTP, del inglés lipid transfer proteins)[61,64–67].

Las LTP poseen un dominio de transporte de lípidos (LTD, lipid transfer domain) que se pliega formando un bolsillo hidrofóbico donde puede albergar lípidos y transportarlos entre membranas[65,68]. Este transporte es más eficiente cuanto más cerca entre sí se encuentran las membranas, pues el citosol que atravesar es menor, por lo que muchas LTP se localizan en los MCS.

Las ORP/Osh son proteínas conservadas capaces de mediar el transporte coordinado de fosfatidilserina (PS) y fosfatidilinositol-4-fosfato (PI4P) en los contactos RE-MP. Para ello se unen a la MP a través de interacciones específicas de su domino PH con fosfolípidos de membrana y al RE a través de la interacción con proteínas VAP, que reconocen el dominio FFAT de las ORPs[69–71]. La proteína transportadora de fosfatidilinositol (PITP, del

inglés phosphatidylinositol transfer proteins) RGDBα, más conocida como Nir2 en mamíferos, es capaz de intercambiar ácido fosfatídico (PA) por PI en los contactos RE-MP, donde se localizan a través de la interacción de su dominio FFAT con las VAP. ORP/Osh y RGDBα actúan de forma similar: el LTD coge un fosfolípido (PI4P o PA) de la MP, lo transporta al RE y lo intercambia por otro (PS o PI) que lleva de vuelta a la MP.

Además, el transporte lipídico en MCS también puede mediarse con proteínas distintas de las LTP canónicas, como las E-Syt/Tcb/SYT[72,73]. Estudios de cristalización de la proteína E-Syt2 muestran que el dominio SMP dimeriza formando una estructura tubular que puede albergar lípidos en un túnel central hidrofóbico e intercambiarlos entre membranas[72]. A pesar de ello, células mutantes para las tres E-Syt de mamíferos, no tienen alterados los niveles de fosfolípidos[73], indicando que las E-Syt no regulan la homeostasis de fosfolípidos en condiciones basales. Teniendo en cuenta que las E-Syt también funcionan como sensores de los niveles de Ca^{2+} en el citosol y que su capacidad para transferir lípidos entre liposomas *in vitro* depende de la concentración de Ca^{2+} en el medio[73], una hipótesis atractiva es que las E-Syt ayuden a recuperar los niveles basales de lípidos tras una modificación importante causada por una cascada de señalización de Ca^{2+}.

Proteínas de anclaje RE-MP.

De entre todas las proteínas que localizan en contactos RE-MP, al menos 4 tienen capacidad de anclaje entre las membranas: **(1)** Las **junctofilinas** son proteínas exclusivas de animales que mantienen la unión entre el RE y la MP en díadas y tríadas y son fundamentales para el acoplamiento entre excitación y la contracción de las células musculares[74–77]. **(2)** Las **TMEM16/Ist2** son proteínas conservadas en eucariotas. Están ancladas al RE mediante múltiples dominios transmembrana (TM) y pueden interaccionar con la membrana plasmática a través de un dominio polibásico[78–80]. Las levaduras mutantes de Ist2 presentan hasta un 40 % menos de MP anclada al RE a una distancia menor de 30 nm[81]. **(3)** Las **VAP** es una familia conservada en eucariotas, pero con mucha mayor representación en plantas (10 miembros) que en animales (VAP-A y VAP-B) y levaduras (Scs2 y Scs22)[82,83]. Se caracterizan por la presencia de un dominio MSD (major sperm domain) y la mayoría de ellas están ancladas al RE[41]. El MSD tiene capacidad de unión a fosfolípidos *per se*, pero además, posee una zona de reconocimiento del motivo FFAT, muy abundante entre LPT[84,85]. Las LTP, a su vez, suelen tener otros motivos de unión a fosfolípidos, pudiendo unirse a diversas membranas simultáneamente. Así, el complejo VAP/LTP actúa como anclaje entre membranas en los MCS. **(4)** Las **E-Syt/Tcb/SYT** son proteínas de retículo conservadas en eucariotas. Además del dominio TM, poseen un dominio SMP y varios dominios C2 (dos en SYT de Arabidopsis)[22,86]. Los dominios C2 son dominios de unión a fosfolípidos y se encargan de la interacción con la MP, que puede estar o no regulada por Ca^{2+} en función de las

características particulares de cada C2[59,87]. Hasta la fecha, todas las proteínas con dominio SMP que se han probado se localizan en MCS[88], entre ellas las E-Syt/Tcb, que se localizan en los contactos RE-MP y participan en el transporte lipídico sin vesículas (ver apartado anterior). Gracias a estas características estructurales, las E-Syt/Tcb pueden actuar como anclajes entre el RE y la MP regulados por Ca^{2+} y por la composición lipídica de las membranas[43,73,89].

En esta tesis, hemos identificado a la SYT1 de Arabidopsis como una proteína de anclaje RE-MP homóloga a las E-Syt de mamíferos y las Tcb de levaduras y con un papel fundamental en la tolerancia a diversos estreses abióticos. Su capacidad de unión a membranas también está regulada por Ca^{2+} y la homeostasis lipídica ante condiciones de estrés se encuentra comprometida. Además, hemos caracterizado a otra proteína de la familia, SYT3, y hemos comprobado que también se acumula en contactos RE-MP y participa en la adquisición de una nueva homeostasis lipídica tras la exposición de las plantas a frío.

Por otro lado, aunque los MCS se han visualizado en plantas desde antaño, su caracterización es mucho más escasa que en otros organismos. Además, normalmente su caracterización no ha sido dirigida por el interés que despertaran estos sitios de contacto propiamente, sino como observación secundaria en el estudio de otras cuestiones. Por eso hemos creído conveniente la realización de una revisión sobre el estado actual del conocimiento sobre MCS en plantas.

CAPÍTULO 1: STITCHING ORGANELLES: ORGANIZATION AND FUNCTION OF SPECIALIZED MEMBRANE CONTACT SITES IN PLANTS. (UNIENDO ORGÁNULOS: ORGANIZACIÓN Y FUNCIÓN DE SITIOS DE CONTACTO ENTRE MEMBRANAS EN PLANTAS).

Las características principales de los MCS en distintos organismos pueden resumirse en (1) complejos proteicos se encargan de aproximar las dos membranas, (2) las membranas implicadas no llegan a fusionar (aunque se pueden dar intermedios de hemifusión), (3) hay ciertos lípidos y proteínas que son especialmente abundantes en los MCS y (4) los MCS regulan procesos de tráfico y señalización[20,90–94].

En plantas también existen microdomios de membrana con estas características y funciones conservadas, aunque nuestro conocimiento sobre ellos es mucho menor que en otros organismos. La búsqueda de homólogos en plantas de las proteínas de anclaje RE-MP de mamíferos y levaduras fue fundamental en la identificación de dos familias de proteínas de anclaje en plantas: VAP27[82,95] y SYT[1]. A pesar de ello, la existencia de homólogos a otros organismos no descarta la existencia de proteínas de anclaje específicas de plantas.

VAP27-1 es el miembro mejor caracterizado de la familia VAP27 en Arabidopsis. Se encuentra anclado al retículo mediante un dominio TM e interacciona con la membrana plasmática a través de un complejo con NET3C y con los microtúbulos y filamentos de actina corticales[95]. VAP27 es una familia de 10 miembros en Arabidopsis; los miembros de los clados I y III tienen dominios TM y, al igual que VAP27-1 (clado I), se anclan al RE y están enriquecidas en contactos RE-MP. Por el contrario, los miembros del clado II no poseen dominio TM y se localizan en la membrana plasmática[82]. En Arabidopsis, la familia SYT posee al menos 5 miembros (*SYT1-SYT5*), de los cuales SYT1 es el único que ha sido caracterizado como proteína de anclaje RE-MP[1]. En posteriores secciones describiré SYT1 en profundidad.

Además, las plantas poseen MCS con arquitecturas y funciones únicas:

Plasmodesmos: Contactos RE-MP que regulan la comunicación intercelular.

Los plasmodesmos son canales que atraviesan la pared celular conectando los citoplasmas de células vegetales adyacentes[96,97]. Tienen una estructura única, con el RE y la MP formando dos tubos concéntricos de unos 40 nm de diámetro máximo conectados entre si (~10 nm de distancia entre ambas membranas) mediante estructuras proteicas a lo largo de todo el plasmodesmo[96]. Estos anclajes proteicos continúan sin ser caracterizados, pero cabe destacar que tanto VAP27-1 como SYT1 se localizan en plasmodesmos. La permeabilidad de los plasmodesmos está regulada por el tamaño de la manga citoplasmática, que a su vez depende de la separación entre el RE y la MP[86]. Las proteínas de anclaje de los contactos RE-MP podrían determinar el tamaño de la manga citoplasmática y, por tanto, participar en la regulación de la permeabilidad de los plasmodesmos. En este sentido, la permeabilidad de los plasmodesmos determina la capacidad de los virus para dispersarse por la planta[98,99] y se ha observado que SYT1 es reclutado a los plasmodesmos durante la infección con varios virus y que la movilidad de estos es reducida en plantas mutantes *syt1*[100,101].

Contactos cloroplasto-RE: módulos de homeostasis lipídica en plantas.

Los cloroplastos participan en la biosíntesis de muchos productos esenciales para el funcionamiento de la planta, como aminoácidos, tocoferoles, carotenoides y ácidos grasos; para lo que necesita estar en continua comunicación con el resto de la célula. Esta comunicación es particularmente importante en el caso del RE, ya que la mayoría de los ácidos grasos sintetizados en los cloroplastos son exportados al RE para la síntesis de glicerolípidos y posteriormente devueltos a los cloroplastos, donde se transforman en galactolípidos, los lípidos mayoritarios de las membranas plastidiales. Ya en los años 80, imágenes de microscopía electrónica de transmisión (MET) mostraron la existencia de zonas de gran aproximación entre cloroplastos y el RE[102]; y más recientemente, el uso de "pinzas ópticas" demostró que 400 pN de fuerza no son suficientes para romper estas interacciones[103]. La cercanía entre las membranas plastidiales y la membrana del RE (denominada PLAM, del inglés plastid associated membrane of the ER) en los contactos RE-cloroplasto puede facilitar el transporte de lípidos sin vesículas entre estos dos orgánulos[63,104–106]. Además, se ha propuesto que enzimas localizadas en la PLAM

pueden actuar en *trans* sobre sustratos en la membrana plastidial externa y viceversa a través de la hemifusión de las membranas implicadas[107,108].

Contactos inducibles por estrés: Estrómulos, peróxulos y contactos mitocondria-cloroplasto.

Además, los cloroplastos pueden formar estrómulos (túbulos de estroma) de unos 0.35-1.5 mm de diámetro que se alargan y retraen constantemente, estableciendo contactos con otros orgánulos[109–111]. La formación y la morfología de los estrómulos depende de factores intrínsecos, como el tamaño, la densidad o el estadio de desarrollo de los cloroplastos[112]; pero también se induce su formación bajo condiciones que modifiquen el estado redox del cloroplasto[111,113,114]. Se cree que la formación de estrómulos en respuesta a estrés puede ayudar a una rápida translocación de proteínas del cloroplasto al núcleo en sitios de contacto estrómulo-núcleo[111]. Esta hipótesis se ve apoyada por la observación de que ciertas proteínas nucleares son secuestradas en los cloroplastos, pero son movilizadas al núcleo en respuesta a estrés, como por ejemplo los factores de transcripción PMT y WHIRLY1 o la proteína interactora del receptor N, NRIP1[115–117].

La modificación del estado redox de los cloroplastos también induce la formación de peróxulos, o extensiones del peroxisoma, que establecen contactos con otros orgánulos y participan en la detoxificación de especies reactivas de oxígeno[118–121]. Por ejemplo, la exposición a luz de alta intensidad favorece la formación de contactos a tres bandas entre peroxisomas, cloroplastos y mitocondrias e incrementa la adhesión entre peroxisomas y cloroplastos, que se encuentran unidos mediante peróxulos[119,121].

Por último, las mitocondrias también forman extensiones y establecen contactos con los cloroplastos en respuesta a estrés[122,123].

CAPÍTULO 2: ANALYSIS OF PROTEIN–LIPID INTERACTIONS USING PURIFIED C2 DOMAINS (ANÁLISIS DE INTERACCIONES LÍPIDO-PROTEÍNA USANDO DOMINIOS C2 PURIFICADOS).

Los dominios C2 (C2) son dominios proteicos de unos 130 aminoácidos que se pliegan de forma autónoma e independiente en forma de β- sándwich[124]. Los C2 se identificaron inicialmente como sensores de Ca^{2+} en la proteína kinasa C (PKC)[125] y posteriormente fueron también caracterizados como dominios de unión a fosfolípidos en la synaptotagmina1 (Syt1)[126]. Actualmente, los C2 son reconocidos como el segundo dominio más abundante de unión a lípidos y el segundo más abundante de unión a Ca^{2+} en eucariotas[127], aunque también hay C2 que no tienen capacidad de unir Ca^{2+}[87]. La caracterización de la unión a lípidos y Ca^{2+} de los C2 proporciona una información muy útil para la identificación de la función *in vivo* de estos dominios[1,2]. Por eso, como parte de esta tesis doctoral, desarrollamos un método para caracterizar la unión a lípidos y Ca^{2+} de C2, tanto solubles como insolubles, de las Synaptotagminas de plantas (aunque virtualmente puede aplicarse a cualquier dominio C2). Como ejemplo de C2 soluble usamos el dominio C2B de SYT3 y como ejemplo de C2 poco solubles, el tándem C2AB de SYT3.

Los C2 se expresaron fusionados a GST en *Escherichia coli* y se purificaron por afinidad usando resina de glutatión. El paso previo a la purificación es la obtención del extracto proteico, que es diferente en función de la solubilidad de los C2 que estemos purificando. En el caso de C2AB (poco soluble) fue necesario añadir un 1 % del detergente Sarkosyl[128] al tampón de lisis para solubilizarlo. El Sarkosyl provoca una desnaturalización de las proteínas que puede revertirse añadiendo CHAPS y Tritón X-100[129]. Esta renaturalización es fundamental para que la GST interaccione con el glutatión de la resina en la purificación subsiguiente y para que los dominios C2 unan Ca^{2+} y fosfolípidos. La interacción con fosfolípidos y su dependencia con Ca^{2+} se determinó cualitativamente incubando los C2 con PIP strips™ (membranas comerciales con distintos fosfolípidos), de forma que el C2 solo se pega en las zonas de la membrana que contienen fosfolípidos a los que unirse. Realizando el ensayo a distintas concentraciones de Ca^{2+}, evaluamos la dependencia de estas interacciones con Ca^{2+}. Por último, se realizó una evaluación semicuantitativa de la unión a fosfolípidos en función de la concentración de Ca^{2+}. Para ello se prepararon vesículas multilamelares y se realizaron ensayos de interacción lípido-proteína por centrifugación a distintas concentraciones de Ca^{2+}.

CAPÍTULO 3: THE ARABIDOPSIS SYNAPTOTAGMIN1 IS ENRICHED IN ENDOPLASMIC RETICULUM-PLASMA MEMBRANE CONTACT SITES AND CONFERS CELLULAR RESISTANCE TO MECHANICAL STRESSES (LA SYNAPTOTAGMINA1 DE ARABIDOPSIS ESTÁ ENRIQUECIDA EN LOS CONTACTOS ENTRE LA MEMBRANA PLASMÁTICA Y EL RETÍCULO ENDOPLASMÁTICO Y CONFIERE RESISTENCIA A LA CÉLULA FRENTE A ESTRÉS MECÁNICO).

Las plantas están continuamente sometidas a fuerzas físicomecánicas, tanto externas (viento, gravedad, contacto con insectos, gotas de agua, otras plantas vecinas…) como internas (por ejemplo, presión de turgencia)[130–134]. Las plantas sienten y responden a estas señales mecánicas[135], aunque tenemos un conocimiento muy escaso de los mecanismos que usan para ello. Basado en el conocimiento sobre sensores de señales mecánicas y mecanismos de transducción en bacterias y mamíferos[136], se han establecido dos modelos de señalización mecánica en plantas: el modelo de canales iónicos y el modelo de "tensegridad". En el modelo de canales iónicos, homólogos de estos canales en plantas se abrirían o cerrarían en respuesta a estímulos mecánicos, dejando entrar Ca^{2+} extracelular al citosol e iniciando una cascada de señalización[137–139]. En el modelo de tensegridad, la célula actúa como una estructura en autoequilibrio estable, formado por elementos que soportan compresión y elementos que soportan tracción[140]. La modificación mecánica de cualquiera de estos elementos se transforma en una pérdida de ese equilibrio y en la rápida transmisión de esa señal mecánica hasta zonas muy distantes de la célula respecto de donde ha sido percibida inicialmente[141]. En plantas, la pared celular actúa como soporte estructural y el citoesqueleto cortical actúa como sensor de tensegridad[142–144]. En este artículo, caracterizamos a la Synaptotagmina1 (SYT1) de Arabidopsis como una proteína de anclaje entre el RE y la membrana plasmática implicada en la resistencia a estrés mecánico en plantas.

SYT1 de Arabidopsis se localiza tanto en RE como en MP y está enriquecida en los contactos RE-MP.

Mediante el uso microscopía confocal, inmunotinción con anticuerpos específicos frente a SYT1 y la colocalización con proteínas residentes de RE y MP fusionadas a la proteína reportera GFP (del inglés, green fluorescent protein), determinamos la localización subcelular de SYT1 en células del meristemo de la raíz. En el interior celular, SYT1 se localiza en el RE; mientras que en la periferia celular presenta una señal punteada que colocaliza tanto con los marcadores de RE como con los de MP. Por otro lado, generamos una construcción SYT1::SYT1:GFP (me referiré a ella como SYT1-GFP), cuya localización en raíz es muy similar a la observada para SYT1 por inmunolocalización. En la superficie de células epidérmicas de hoja, SYT1-GFP presenta una señal reticulada en forma de cuerdas y cuentas, diferente a la presentada por marcadores de RE. En el plano ecuatorial, SYT1-GFP presenta un patrón punteado en la periferia celular y movimiento a través de hebras transvacuolares. Para mejorar la resolución en la localización de SYT1, usamos microscopía electrónica de transmisión (MET) de muestras fijadas por congelación a alta presión, criosustituidas e inmunomarcadas con partículas de oro y observamos que SYT1 se acumula en los sitios donde el RE está muy próximo a la MP y entre los microtúbulos corticales. Además, la expresión transitoria en Nicotiana Bentamiana de SYT1-GFP y VAP27-RFP (un marcador de contactos RE-MP en plantas) mostró una alta colocalización de ambos marcadores.

Los contactos RE-MP marcados por SYT1 son predominantemente estacionarios.

A continuación, usamos la toma de imágenes a intervalos y FRAP (del inglés, fluorescence recovery after photo-bleaching) en células epidérmicas de hoja para determinar la dinámica de SYT1. Observamos que la señal interna es muy dinámica mientras que la señal cortical es estacionaria. Tras el fotoblanqueo, la señal se recuperó en tan solo 5 min y principalmente en las mismas posiciones en que se encontraba previamente.

Los contactos RE-MP localizan en zonas de la periferia celular libres de microtúbulos.

Mediante inmunolocalización en células de raíz en división activa, observamos que SYT1 migra desde el RE al huso mitótico durante la metafase y se transporta al fragmoplasto durante la citocinesis. Por eso, decidimos estudiar la relación de la localización de SYT1 con los microtúbulos. Observamos que la señal de SYT1 es muatuamente excluyente con la de MAP4 y TUA6 (marcadores de microtúbulos). Además, estudiamos el efecto de inhibidores de la polimerización del citoesqueleto sobre la señal de SYT1-GFP. El tratamiento con latrunculina B (que provoca la despolimerización de los filamentos de actina F) causó el fin del movimiento a través de hebras transvacuolares y la acumulación de la señal interna, mientras que la señal cortical permaneció invariable. En cambio, el tratamiento con orizalina (que provoca la despolimerización de los microtúbulos), no causó ningún cambio en la señal de SYT1-GFP en las células del pavimento.

Los dominios C2 de SYT1 se localizan en la MP y unen fosfatidilinositol fosfatos.

SYT1 posee un dominio TM y dos dominios C2, por lo que podría unirse al RE y a la MP simultáneamente. Para descifrar la orientación de la proteína, expresamos los dominios C2AB de SYT1 fusionados a GFP (C2AB-GFP) y comprobamos que se localizan principalmente en la MP, con una señal continua y homogénea, a diferencia de la señal reticulada de SYT1-GFP. Para identificar los lípidos de la MP con los que interaccionan los C2AB, realizamos ensayos in vitro de interacción lípido-proteína. Los dominios C2AB purificados se incubaron con membranas con distintos lípidos en presencia o ausencia de Ca^{2+} y, en ambas condiciones, se observó interacción con fosfatidilserina (PS), ácido fosfatídico (PA), ácido lisofosfatídico (LPA) y fosfatidilinositol fosfatos (PIP). Cabe destacar que la presencia del grupo fosfato es esencial para la unión, ya que no se detectó interacción alguna con fosfatidilinositol (PI). La diferencia más acuciada en ausencia de Ca^{2+} fue una gran disminución de la interacción con PS.

SYT1 otorga resistencia celular a estrés mecánico.

Generamos plantas transgénicas expresando una construcción del gen de la β-glucuronidasa dirigido por el promotor de SYT1 (SYT1::GUS) y comprobamos que su expresión fue inducida en zonas dañadas mecánicamente. A continuación, estudiamos la respuesta de la señal de SYT1-GFP al aplicar una presión constante (6750 Pa) sobre los cotiledones y comprobamos que cambiaba rápidamente (menos de 20 s) desde la red habitual en condiciones control a una estructura mucho más punteada. Una vez comprobado que tanto la expresión de *SYT1* como la localización de la proteína responden a daño mecánico, estudiamos el comportamiento del mutante *syt1* frente a este estrés. En hojas presionadas con un hemostato se observó mayor parada del crecimiento, mayor necrosis celular y mayor tinción con azul de tripano en torno a la zona presionada en plantas *syt1* que en plantas silvestres. Esta diferencia en la tolerancia a estrés mecánico se cuantificó mediante *ion leakage*. Una disolución en la que fueron sumergidos discos de hoja de *syt1* presentó una conductividad un 45 % mayor que la de una disolución con discos de hoja de plantas silvestres. Además, protoplastos *syt1* presentaron menor resistencia a centrifugación que protoplastos silvestres, indicando que la función de SYT1 en la tolerancia a estrés mecánico es, al menos parcialmente, independiente de la pared celular.

Discusión de los resultados.

La homología estructural de las SYT de plantas con las E-Syts/Tcbs[145], la localización de SYT1 en contactos RE-MP y su colocalización con VAP27 (homólogo de plantas de la proteína de levaduras Scs2, que interacciona con las Tcbs[41]) apoyan que el mecanismo de anclaje entre la MP y el RE en plantas sea similar a los ya descritos para animales y levaduras.

CAPÍTULO 4: THE ER-PM TETHERS SYNAPTOTAGMIN 1 AND 3 REGULATE COLD ACCLIMATION ASSOCIATED LIPID REMODELING IN ARABIDOPSIS (LAS PROTEÍNAS DE ANCLAJE ENTRE EL RE Y LA MP SYNAPTOTAGMINA 1 Y 3 REGULAN LA REMODELACIÓN LIPÍDICA ASOCIADA A LA ACLIMATACIÓN AL FRÍO).

La capacidad de las plantas para adaptarse a climas templados es uno de los mayores factores limitantes en su distribución geográfica, crecimiento y productividad. La exposición a frío provoca una serie de cambios en la planta, colectivamente conocidos como aclimatación a frío, que mejoran su tolerancia a congelación[146,147]. Uno de estos cambios es el establecimiento de una nueva homeostasis lipídica para prevenir el daño que la congelación provoca en las membranas celulares[148–154]. Para alcanzar esta nueva homeostasis lipídica es necesario retirar los lípidos presentes y aportar nuevos[60,155–157]. El transporte de lípidos en masa ocurre mediante transporte vesicular[61,158,159], mientras que ciertos lípidos pueden ser transportados por LTP en los MCS[66,67,160]. Además, la aclimatación al frío inicia una cascada de señalización de Ca^{2+} que culmina en una reprogramación a nivel transcripcional[161].

SYT1 de plantas es una proteína de anclaje RE-MP[1]. Es esencial para mantener la integridad de la MP en condiciones de estrés[1,2] y participa en la adquisición de tolerancia a congelación dependiente de Ca^{2+}[162]. Además, se ha observado que SYT1 se asocia más fuertemente a la MP tras la aclimatación a frío[163,164] y que los mutantes *syt1* son hipersensibles a frío[162]. Estudios previos en nuestro laboratorio, identificaron a otro miembro de la familia, *SYT3*, involucrado en la aclimatación a frío[165]. En esta tesis, nosotros caracterizamos *SYT1* como un gen constitutivo en la aclimatación a frío. También caracterizamos SYT3 como una proteína con capacidad de unión a lípidos, inducible por frío, localizada en contactos RE-MP y esencial para la tolerancia a congelación en ausencia de SYT1.

SYT1 actúa como gen constitutivo en la tolerancia a congelación.

Los estudios previos sobre la tolerancia a congelación del mutante *syt1* se han realizado en protoplastos o plántulas jóvenes crecidas en placa. Nosotros evaluamos la tolerancia a congelación de plantas de 4 semanas, germinadas y crecidas en suelo. Comparamos la tasa de supervivencia tras 6 horas de congelación y una semana de recuperación en condiciones control, con y sin aclimatación previa, de plantas silvestres, *syt1-2* (*syt1*) y una línea de complementación (CL9). La aclimatación permitió a todas las plantas tolerar temperaturas más bajas, pero en ambas condiciones el mutante *syt1* presentó menor tolerancia que las demás plantas. A pesar de ello, mediante *western blot* no se observaron diferencias significativas en la cantidad total de proteína SYT1 a lo largo de una semana de aclimatación.

SYT1 se acumula en los contactos RE-MP durante la aclimatación a frío.

A continuación, estudiamos la dinámica de SYT1 durante 1 o 3 días de aclimatación a frío usando la línea SYT1-GFP previamente descrita[1]. La exposición a 1 día de frío causó la reducción de la señal reticulada de SYT1-GFP y la agregación en estructuras punteadas definidas. Las plantas tratadas con frío durante 3 días mostraron un fenotipo similar. Cuantificamos el número de puntos por área usando el *plugging* "find máxima" de FIJI y comprobamos que había más tras el tratamiento de frío que en condiciones control. Este aumento en el número de puntos marcados por SYT1-GFP podría deberse a un aumento de los contactos RE-MP, a una mayor acumulación de SYT1-GFP en estos sitios o a ambos. Para evaluar el comportamiento de los contactos RE-MP, usamos el

marcador MAPPER, diseñado inicialmente para marcar contactos RE-MP en animales[42]. Primero comprobamos que también marca contactos RE-MP en Arabidopsis y después evaluamos su dinámica con frío. Contrariamente a lo observado para SYT1-GFP, el tratamiento de frío no provocó cambios obvios en la localización de MAPPER; lo que sugiere que los cambios observados en SYT1-GFP se deben a su redistribución entre RE cortical y contactos RE-MP.

Identificación de *SYT3* como un gen determinante en la aclimatación a frío.

SYT1 pertenece a una familia de 5 miembros en Arabidopsis[166,167], de los cuales solo *SYT1, SYT3* y *SYT5* presentan una expresión ubicua. Mediante experimentos de qPCR observamos que solo *SYT1* y *SYT3* se indujeron con 24 horas de tratamiento de frío. Estudios previos en nuestro laboratorio ya habían demostrado que *SYT3* es importante en el proceso de aclimatación a frío cuando *SYT1* no está presente[165].

SYT3 se localiza en contactos RE-MP y tiene capacidad de unión a fosfolípidos.

Generamos líneas transgénicas con el promotor de *SYT3* fusionado al gen reportero de la ß-glucuronidasa (SYT3::GUS) y observamos fuerte actividad GUS en estomas y en el centro de la raíz primaria. Por otra parte, la localización subcelular de 35S::SYT3-GFP fue muy similar a la de SYT1-GFP. Al igual que SYT1, SYT3 contiene dos dominios C2 terminales. Mediante ensayos de interacción lípido-proteína determinamos que el dominio C2A de SYT3 une lípidos de una manera dependiente de Ca^{2+}, mientras que C2B fue capaz de unir lípidos incluso en ausencia de Ca^{2+}, aunque la interacción aumentó al aumentar la concentración de Ca^{2+}. Similar a lo observado para SYT1, los dominios C2AB de SYT3 interaccionaron principalmente con PIP y PA.

Los mutantes *syt1syt3* presentan niveles alterados de los fosfolípidos interactores.

Puesto que los homólogos de las SYT de plantas tienen capacidad de transportar lípidos entre membranas y controlan la homeostasis lipídica de la MP[72,73], nosotros evaluamos el papel de SYT1 y SYT3 en la adquisición de una nueva homeostasis lipídica en plantas tras el proceso de aclimatación al frío. No se encontraron diferencias significativas entre plantas silvestres y dobles mutantes *syt1syt3* para la mayoría de los lípidos. En cambio, las plantas *syt1syt3* presentaron niveles alterados de fosfatidilinositoles y fosfatidilgliceroles, que son precisamente los lípidos a los que se unen, sugiriendo que la interacción de las SYT con estos lípidos regula su acumulación.

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