IDENTIFICATION OF A NOVEL PROTEIN TETHER ENRICHED AT ENDOPLASMIC RETICULUM-PLASMA MEMBRANE CONTACT SITES AND INVOLVED IN SALT STRESS TOLERANCE IN ARABIDOPSIS

by

Francisco Benítez de la Fuente

B.Sc., Universidad de Málaga, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies
(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)
June 2018

© Francisco Benítez de la Fuente, 2018
Committee Page

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

IDENTIFICATION OF A NOVEL PROTEIN TETHER ENRICHED AT ENDOPLASMIC RETICULUM-PLASMA MEMBRANE CONTACT SITES AND INVOLVED IN SALT STRESS TOLERANCE IN ARABIDOPSIS

submitted by FRANCISCO BENITEZ in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in BOTANY

Examing Committee:

ABEL ROSADO, BOTANY
Supervisor

LJERKA KUNST, BOTANY
Supervisory Committee Member

GEOFFREY WASTENEYS, BOTANY
Supervisory Committee Member

ELIZABETH CONIBEAR, BIOCHEMISTRY
Additional Examiner

Additional Supervisory Committee Members:

XIN LI, BOTANY
Supervisory Committee Member

Supervisory Committee Member
Abstract

Plants are sessile and are exposed to environmental changes that can compromise their survival and yield. Salt stress is one of the most common environmental stresses. Arabidopsis SYT1 is a key player in plant response to salt stress mediated by Ca$^{2+}$, and acts as protein bridge, tethering the endoplasmic reticulum to the plasma membrane. These regions of close contact between organelles, called membrane contact sites, are conserved in eukaryotes, and are involved in functions such as Ca$^{2+}$ signaling and lipid homeostasis. However, their relationship with salt stress in plants is unknown. In this project, I aimed to discover new contact site tethers in the model organism Arabidopsis thaliana and shed light on the relation of endoplasmic reticulum-plasma membrane contact sites (EPCS) and salt stress tolerance. I used bioinformatic, phylogenetic and cellular biological approaches and I related an Arabidopsis protein family, the N-terminal transmembrane C2 domain (NTMC2) family, to contact sites. A putative tether called Arabidopsis thaliana Ca$^{2+}$-dependent Lipid Binding (AtCLB) belongs to the NTMC2 family. AtCLB has a subcellular localization pattern of beads and strings, which resembles the pattern found for the plant EPCS tether SYT1. AtCLB pattern becomes more punctate under depletion of cytosolic Ca$^{2+}$, suggesting that intracellular Ca$^{2+}$ is important for AtCLB contact with the plasma membrane. Mutant plants lacking functional AtCLB did not show any visible phenotype in salt stress conditions. Under salt stress treatments, however, the normal subcellular pattern of AtCLB was altered, EPCS formation was increased and the intermembrane distance was decreased. This result suggests a role for AtCLB in salt stress tolerance that might be phenotypically masked by functional redundancy. This subcellular alteration and the reduction of intermembrane distance was mimicked when the amount of phosphatidylinositol-(4,5)-bisphosphate was artificially increased at the plasma membrane. These results point towards a model of EPCS tethering involving cytosolic Ca$^{2+}$, salt stress and negatively charged phosphoinositides. Further study will be required to fully understand EPCS regulation. In summary, these discoveries clarify some mechanistic aspects of EPCS tethering in plants and open a door to the discovery of new contact site protein tethers from the plant protein family NTMC2.
Lay Summary

Plants are sessile and are exposed to environmental changes that can compromise their survival and yield. Among all environmental factors that affect plants, drought is one of the most extended. Drought produces an increase in the concentration of salts in the soil, causing salt stress. Understanding how plants respond to salt stress is fundamental to improve crop yield and to secure food for the rapidly growing world population. In this project, I used the model organism *Arabidopsis thaliana* and bioinformatic and microscopy techniques to find new proteins important for plant tolerance to salt stress. I discovered the protein AtCLB, which belong to the family NTMC2 and it is implicated in the communication between different parts of the cell during salt stress, although it is not required for plant tolerance to salt. Future studies will decipher the precise role of AtCLB in plant response to salt stress.
Preface

In Chapter III, a preliminary SMP consensus sequence and a preliminary phylogenetic analysis was performed by Dr. Sunjoo Joo, research assistant in Dr. Jae-Hyeok Lee’s lab, Department of Botany, UBC. I carried out the final SMP consensus sequence and the subsequent phylogenetic analysis, with guidance by Dr. Abel Rosado. I performed the rest of the work described in Chapter III, including the structural prediction, study and alignment of the putative tethers, and the gene expression analysis.

In Chapter IV, I generated AtCLB-GFP, SYTF-GFP and 2TMCLB-GFP transgenic lines, with early technical support from Dr. Makoto Shirakawa, post doctoral fellow in Dr. Abel Rosado’s lab. I obtained the insertional mutant atclbl-3 and performed the phenotypic analysis and subsequent quantification. I performed the qRT-PCR analysis with technical support of Dr. Eun Kyoung Lee, research associate in Dr. Abel Rosado’s lab. The co-immunoprecipitation analysis that detected AtCLB as an interactor of SYT1 was performed by Dr. Jessica Pérez Sancho, a former PhD student at Dr. Miguel Ángel Botella’s lab, in the University of Málaga. The chemical treatments used in confocal microscopy experiments were conceived by Dr. Abel Rosado, Dr. Eun Kyoung Lee, and myself. I performed AtCLB-GFP and SYT1-GFP-related confocal microscopy experiments, and subsequent quantification with early guidance and technical support by Dr. Eun Kyoung Lee. Dr. Eun Kyoung Lee performed the experiments and quantifications related to CITRINE-2xPHPLC, HDEL and MAPPER fluorescent markers, with exception of the quantification of GFP-HDEL reticulated structures areas in NaCl stress, which was performed by me.

Preparation of UBQ10:AtCLB-tRFP, UBQ10:SYTF-tRFP, UBQ10:2TMCLB-RFP, pAtCLB:AtCLB-GFP, pSYTF:SYTF-GFP, p2TMCLB:2TMCLB-GFP and pAtCLB:GUS constructs was performed by myself with the subsequent transformation into Arabidopsis Col-0 background. I also transformed UBQ10:AtCLB-tRFP, UBQ10:SYTF-tRFP and UBQ10:2TMCLB-RFP constructs into transgenic plants expressing SYT1-GFP, for co-localization analysis. We thank Dr. Geoffrey Wasteneys for the gift of the UBQ10 promoter-driven C terminal tagRFP fusion destination binary vector and Dr. Mathias Schuetz for
the gift of the C4H-GFP transgenic plants. I generated the double mutants \textit{atclb1-3 syt1} and \textit{atclb1-3 syt5} and the triple mutant \textit{atclb1-3 syt1/5} while the double mutant \textit{syt1/5} was previously generated in Dr. Abel Rosado’s lab.
Table of Contents

Abstract ........................................................................................................................................... iii

Lay Summary .................................................................................................................................. iv

Preface .............................................................................................................................................. v

Table of Contents .............................................................................................................................. vii

List of Tables .................................................................................................................................. xi

List of Figures ................................................................................................................................. xii

List of Abbreviations ...................................................................................................................... xiv

Acknowledgements ......................................................................................................................... xvi

Dedication ....................................................................................................................................... xviii

Chapter I: Introduction .................................................................................................................. 1

1.1. Salt stress sensing and response in plants ................................................................................. 1

1.1.1. Ion transporters and channels in NaCl tolerance ................................................................. 1

1.1.2. ABA signaling during NaCl stress ......................................................................................... 3

1.1.3. Phospholipid signaling during NaCl stress ........................................................................... 4

1.2. Intracellular communication during NaCl stress .................................................................... 5

1.3. Structure of membrane contact sites and their associated functions .................................. 6
1.3.1. MCS tethers in non-vesicular lipid transfer ............................................................................. 7
1.3.2. Organelle inheritance and autophagy at MCSs ........................................................................... 9
1.3.3 EPCS tethers in Ca\textsuperscript{2+} signaling and homeostasis ......................................................... 10
1.4. EPCS tethers in plants ....................................................................................................................... 11
1.5 Structure and function of the SMP domain ....................................................................................... 14
1.6. Research objectives .......................................................................................................................... 15

Chapter II: Methods ................................................................................................................................. 17

2.1 Bioinformatic Analysis ....................................................................................................................... 17
2.2 Plant material and growth conditions ............................................................................................... 18
2.3 Molecular cloning .............................................................................................................................. 18
2.4 Generation of transgenic plants and transformants selection .......................................................... 20
2.5 Chemical treatments, image acquisition and quantifications .......................................................... 20
2.6 Physiological treatments ................................................................................................................... 21
2.7 Quantitative RT-PCR ....................................................................................................................... 21
2.8 Accession numbers ........................................................................................................................... 21

Chapter III: The search for MCS tethers ................................................................................................. 22

3.1. Results ............................................................................................................................................... 22
3.1.1. AtCLB, a putative MCS tether, belongs to the NTMC2 family ...................................................... 22
3.1.2. The NTMC2 tethers have Ca\textsuperscript{2+}-binding and lipid-binding domains ............................... 25
3.1.3. The SMP domain has conserved residues in eukaryotes .............................................................. 29
3.1.4. The expression pattern of the putative tethers indicates roles in abiotic stress

3. Discussion

3.2.1. The Arabidopsis NTMC2 family might contain more MCS tethers

3.2.2. Different subfamilies of NTMC2 proteins have evolved differentially in plants

3.2.3. Conserved residues in eukaryotic SMP domains suggest that SMP-protein dimerization and lipid-transport may also occur in plants.

3.2.4. Putative tether expression patterns suggest functions in abiotic and biotic stresses

Chapter IV: Characterization of the putative tethers

4.1. Results

4.1.1. AtCLB and SYTF show a “beads and strings” pattern at the cortical ER

4.1.2. SYT1 and AtCLB reticulations increase under salt stress

4.1.3. atclb1-3 does not show Ca²⁺-mediated NaCl stress phenotypes

4.1.4. Depletion of cytosolic Ca²⁺ increases SYT1 and AtCLB puncta pattern

4.1.5. SYT1 and AtCLB subcellular pattern expands after increases of PtdIns(4,5)P₂ at the plasma membrane

4.2. Discussion

4.2.1. AtCLB and SYTF subcellular distribution patterns resemble SYT1 and suggest EPCS localization

4.2.2. Decreases in cytosolic Ca²⁺ promote EPCS formation by SYT1 and AtCLB

4.2.3. AtCLB is not required for plant tolerance to NaCl stress
4.2.4. Salt stress and PtdIns(4,5)P₂ increases induce SYT1 and AtCLB EPCS and reduce intermembrane distance .................................................................56

Chapter V: Conclusions ................................................................................................................58

Bibliography ..................................................................................................................................62

Appendices .....................................................................................................................................71

Appendix A: Expression level of NTMC2 genes according to RNAseq data .........................71
Appendix B: Alignment of 2TMCLB model with ESyt2 SMP and C2 domains .........................73
Appendix C: Alignment of SYTF model with ESyt2 SMP and C2 domains ............................74
Appendix D: SYT1 co-immunoprecipitation analysis shows AtCLB as interactor in vitro ......75
Appendix E: SYT1-GFP and AtCLB localization under KCl, MgCl₂ and Na₂SO₄ stresses .......76
Appendix F: PtdIns(4,5)P₂ increases under NaCl stress and after blockage of PLC ...............77
Appendix G: HDEL-GFP subcellular localization under depletion of cytosolic Ca²⁺ ............78
Appendix H: SYT1-GFP and HDEL-GFP localization upon increases of cytosolic Ca²⁺ .......79
Appendix I: SYT1 and HDEL co-localization under NaCl stress ............................................80
Appendix J: MAPPER shows decrease of intermembrane distance upon salt stress ............81
List of Tables

Table 2.1: List of primers used in the thesis. ................................................................. 19
Table 2.2: List of constructs generated. ........................................................................... 20
Table 3.1: Protein members of the NTMC2 family .......................................................... 23
Table 3.2: Expression levels of the tether candidates in different tissue. ....................... 32
List of Figures

Figure 1.1: Model for Na\textsuperscript{+} compartmentalization by SOS pathway.......................... 3

Figure 1.2: Model for TMEM24 non-vesicular lipid transport at EPCSs............................... 8

Figure 1.3: Model for STIM1-Orai1 Ca\textsuperscript{2+} signaling at EPCSs................................. 10

Figure 1.4: Model for SYT1 binding at EPCSs.................................................................. 13

Figure 3.1: Phylogenetic tree of SMP-containing proteins in plants.................................... 25

Figure 3.2: Ribbon diagrams of the alignment between the ESyt2 SMP domain and the consensus SMP model........................................................................................................ 27

Figure 3.3: Ribbon diagrams of the alignment between AtCLB model and the SMP and C2 domains of ESyt2 .................................................................................................................. 28

Figure 3.4: Functional domains of the AtCLB, 2TMCLB and SYTF tether candidates.............. 29

Figure 3.5: Alignment of the consensus SMP domain and the SMP domain of the MCS tethers... 30

Figure 3.6: Microarray-based expression levels of SYT1, AtCLB, SYTF and 2TMCLB in different developmental stages ........................................................................................................... 31

Figure 3.7: Up and downregulation of the putative tethers under different treatments............ 33

Figure 3.8: Crystal structure of human ESyt2 SMP and C2 domains ..................................... 36

Figure 3.9: Alignment of the consensus SMP and the SMP domains of the MCS tethers and Arabidopsis NTMC2 proteins.................................................................................................. 37

Figure 4.1: Subcellular localization of C4H-GFP, SYT1-GFP, AtCLB-GFP and SYTF-GFP....... 42

Figure 4.2: Subcellular localization of C4H and 2TMCLB-GFP............................................ 43

Figure 4.3: Subcellular localization of SYT1-GFP and AtCLB-GFP under NaCl, K\textsubscript{2}SO\textsubscript{4} and sorbitol stress..................................................................................................................... 44

Figure 4.4: Quantification of closed reticulated structures of SYT1-GFP and AtCLB-GFP under NaCl, K\textsubscript{2}SO\textsubscript{4} and sorbitol treatments......................................................................................... 45
Figure 4.5: Distribution of HDEL-GFP reticulated structures area under NaCl stress........................................................................... 46

Figure 4.6: Phenotypical characterization of atclb1-3 mutant in NaCl stress and different Ca$^{2+}$ conditions.................................................................................................................................................................................................................................. 47

Figure 4.7: Relative AtCLB mRNA abundance in atclb1-1 and atclb1-3 mutants ........................................................................... 48

Figure 4.8: Subcellular localization of SYT1-GFP and AtCLB-GFP after depletion of Ca$^{2+}$cyt by EGTA and LaCl3. .................................................................................................................................................................................................................................. 49

Figure 4.9: Quantification of puncta number of SYT1-GFP and AtCLB-GFP in cytosolic Ca$^{2+}$ depletion treatments. .................................................................................................................................................................................................................................. 50

Figure 4.10: Subcellular localization of SYT1-GFP and AtCLB-GFP after PLC inactivation by U73122.................................................................................................................................................................................................................................. 51

Figure 4.11: Quantification of closed reticulate structures of SYT1-GFP and AtCLB-GFP under U73122 treatment.................................................................................................................................................................................................................................. 52
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA</td>
<td>Abscisic Acid</td>
</tr>
<tr>
<td>ABI1</td>
<td>Abscisic Acid Insensitive 1</td>
</tr>
<tr>
<td>AtCLB</td>
<td><em>Arabidopsis thaliana</em> Calcium-dependent Lipid-binding</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>CBL</td>
<td>Calcineurin B-like</td>
</tr>
<tr>
<td>CIPK</td>
<td>CBL-interacting Protein Kinase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EPCS</td>
<td>Endoplasmic Reticulum-Plasma Membrane Contact Site</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERMES</td>
<td>ER-mitochondria Encounter Structure</td>
</tr>
<tr>
<td>ESyt</td>
<td>Extended Synaptotagmin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>MCS</td>
<td>Membrane Contact Site</td>
</tr>
<tr>
<td>MSP</td>
<td>Major Sperm</td>
</tr>
<tr>
<td>NVJ</td>
<td>Nucleus-Vacuole Junction</td>
</tr>
<tr>
<td>ORP</td>
<td>OSBP-related Proteins</td>
</tr>
<tr>
<td>OSBP</td>
<td>Oxysterol-binding Protein</td>
</tr>
</tbody>
</table>
OSCA1  Reduced Hyperosmolarity-induced Calcium Increase
PI      Phosphatidylinositol
PI4K    Phosphatidylinositol 4 Kinase
PIP5K   Phosphatidylinositol 4-phosphate 5 Kinase
PKC     Protein Kinase C
PLC     Phospholipase C
PM      Plasma Membrane
PP2B    Protein Serine/threonine Phosphatase 2B
PtdIns(4,5)P_2  Phosphatidylinositol-(4,5)-bisphosphate
SMP     Synaptotagmin-like Mitochondrial Lipid-binding Protein
SOS     Salt Overly Sensitive
StART   Steroidogenic Acute Regulatory Transfer
STIM    Stromal Interaction Molecule
SYT     Synaptotagmin
TMEM24  Transmembrane Protein 24
TULIP   Tubular Lipid
VAP     VAMP-associated Protein
VST     VAP-related Suppressor of TMM
[Ca^{2+}]_{cyt}  Cytosolic concentration of Ca^{2+} ions
Acknowledgements

First, I would like to thank my supervisor, Dr. Abel Rosado, who gave me the opportunity to come to UBC and who supported me during these years, financially, intellectually, and personally. My settling in Vancouver was much easier thanks to him, who really understood how important sunshine can be for an Andalusian.

I also would like to thank my supervisory committee, Dr. Xin Li, Dr Geoffrey Wasteneys and Dr. Ljerka Kunst, for their guidance and useful suggestions.

Thanks to Dr. Makoto Shirakawa, whose guidance and support at the beginning of the project introduced me to research. I would never be grateful enough to Dr. Eun Kyoung Lee: she has been indispensable in my learning of molecular cloning, confocal microscopy and research in general. This project would not have been possible without her guidance, mentorship, scientific discussions, and answers to my never-ending questions. I also want to thank the undergraduate students that helped me through the project: Melissa, Geety and Lydia.

I am also grateful to Dr. Robin Young. She was a great guidance and support during my teaching assistantships, and she helped me to understand how important teaching is and how much I love it. Her advice will have a profound influence on my future teaching.

I would like to thank Dr. Ljerka Kunst and Dr. George Haughn lab members for their helpful advice through my learning and their comradeship. There was always a good atmosphere thanks to them.

I am immensely grateful to UBC, for the financial support, and to the Botany office staff, specially Isabel Ferens and Alice Liou, who were always willing to help with the ins and outs of bureaucracy.
Last, but not least, I want to thank my friends and my Spanish and Vancouverite families because they gave me the support and joy every scientist needs to overcome the bad moments. This thesis is specially dedicated to them.
La patience est amère,
mais son fruit est doux

Jean-Jacques Rousseau. Philosopher and botanist.
Chapter I: Introduction

1.1. Salt stress sensing and response in plants

Being sessile, plants must cope with adverse environmental conditions, such as soil salinity, drought, and extreme temperatures, which reduce plant growth and yield (Cramer et al., 2011). Among these physical and/or chemical factors, NaCl stress is one of the most important environmental constraints limiting crop productivity worldwide (Parre et al., 2007; Zhu, 2016). NaCl is the most common compound causing stress and its physiological effects in plants include, among others, ion toxicity (Luan et al., 2009), restriction of water availability (Zhu, 2016), damage to cellular lipid membranes (Schapire et al., 2008), oxidative stress and metabolic alterations such as photosynthesis inhibition (Zhu, 2016). Despite the important physiological alterations caused by NaCl in plants, the molecular components responsible for sensing and responding to NaCl are largely uncharacterized (Zhu, 2016). In this context, one of the best-characterized cellular responses to NaCl stress, the cytosolic accumulation of Ca$^{2+}$ ions ([Ca$^{2+}]_{cyt}$), has been used to identify components of the NaCl signaling pathway and highlighted the roles of Na$^{+}$ transporters, hormones and lipids in the NaCl stress response in *Arabidopsis thaliana* (de Zelicourt et al., 2016; Zhu, 2016).

1.1.1. Ion transporters and channels in NaCl tolerance

The analysis of loss- and gain-of-function mutants with altered Ca$^{2+}$ signaling responses to osmotic and/or ionic stress has been essential for the identification of the molecular components involved in NaCl tolerance. For example, the analysis of mutants with reduced [Ca$^{2+}]_{cyt}$ after hyperosmotic stress in Arabidopsis enabled the identification of the hyperosmolarity-gated Ca$^{2+}$-channel OSCA1 (reduced hyperosmolarity-induced calcium increase 1). OSCA1 is located at the plasma membrane (PM). Therefore, this identification also highlighted the important role of PM-localized Ca$^{2+}$ channels as putative NaCl sensors (Yuan et al., 2014). Along the same line, other studies proposed that PM-localized mechano-
sensitive Ca\(^{2+}\) channels could act as sensors for osmotic and salt stress, since both stresses reduce cell turgor and affect membrane tension (Swarbreck et al., 2013). However, the functional redundancy of these mechano-sensitive Ca\(^{2+}\) channels has hindered their functional characterization as NaCl stress signaling components.

A mechanism used by plants to tolerate salt stress is ion compartmentalization (Figure 1.1), which involves the active transport of Na\(^{+}\) ions (Luan, 2009). In this process, the [Ca\(^{2+}\)]\(_{cyt}\) increase produced by NaCl stress is detected by Ca\(^{2+}\)-binding proteins which trigger the Salt Overly Sensitive (SOS) protein kinase signaling pathway (Zhu, 2002, 2016). In this pathway, SOS3, a member of the Ca\(^{2+}\)-binding Calcineurin B-like (CBL) protein family, acts as a Ca\(^{2+}\) sensor that binds and activates the CBL-interacting protein kinase (CIPK) SOS2 upon [Ca\(^{2+}\)]\(_{cyt}\) increase (Luan, 2009). The SOS3/SOS2 complex, now active, phosphorylates and activates the PM-localized Na\(^{+}\)/H\(^{+}\) antiporter SOS1 (Kim et al., 2007). SOS1 uses the positive H\(^{+}\) gradient between the cytosol and the apoplast to shuttle Na\(^{+}\) from the cytosol to the apoplast while PM ATPases maintain the pH gradient, pumping H\(^{+}\) back to the apoplast (Kim et al., 2007; Luan, 2009; Zhu, 2000). SOS3 is expressed in roots and directs the expulsion of Na\(^{+}\) to the soil but other SOS3 paralogs such as CBL10 are expressed mainly in shoots and leaves indicating that Na\(^{+}\) compartmentalization occurs in all plant tissues (Kim et al., 2007). In this case, the CBL10/SOS2 complex is targeted to the tonoplast, where it might activate tonoplast Na\(^{+}\) transporters such as NHX1 that pump Na\(^{+}\) into the central vacuole against its concentration gradient (Figure 1.1) (Deinlein et al., 2014; Kim et al., 2007). Finally, an additional Na\(^{+}\)/H\(^{+}\) antiporter, HKT1, is expressed in parenchyma and vascular cells. HKT1 links the Na\(^{+}\) detoxification mechanisms in shoots and roots by loading Na\(^{+}\) from leaves to the vascular system and unloading Na\(^{+}\), in combination to SOS1 from the roots to the soil (Figure 1.1) (Mäser et al., 2002; Zhu, 2000).
ABA signaling during NaCl stress

In plants, the cellular responses to NaCl stress generally involve an increase of abscisic acid (ABA), an important phytohormone activated in response to multiple environmental stresses. ABA signalling requires three main components: a member of the PYR/PYL/RCAR family of ABA receptors, a member of the protein phosphatase PP2C family that acts as a negative regulator, and the protein kinase SnRK2 that displays auto-phosphorylation activity and acts as a positive regulator of the ABA signaling pathway.
In the absence of ABA, the autophosphorylation activity of SnRK2 is inhibited by the binding of the phosphatase PP2C, negatively regulating the signaling pathway. The binding of ABA to the PYR/PYL/RCAR receptor promotes the interaction of the receptor with PP2C and leads to the inhibition of PP2C phosphatase activity. The inactivation of PP2C allows SnRK2 autophosphorylation, capable now of phosphorylating its targets and activating the ABA signaling pathway (Boudsocq et al., 2007). SnRK2s have been shown to phosphorylate transcription factors and membrane proteins such as transporters and channels (Sato et al., 2009; de Zelicourt et al., 2016). Members of the ABA signalling pathway have been recently related to the activation of proteins from the MAP kinase pathway, a phosphorylation pathway widely reported in the regulation of biotic stress responses, starting to clarify the role of MAP kinases in abiotic stress response (Boudsocq et al., 2015; Danquah et al., 2015; Mitula et al., 2015).

1.1.3. Phospholipid signaling during NaCl stress

In plants, PM lipids that increase in response to ABA, such as phosphatidic acid (PA), have been postulated to act as important signaling molecules during NaCl stress. For example, ABA increases PA levels by regulating the activity of the PA biosynthesis enzyme phospholipase D (Zhang et al., 2004). PA interacts with the abscisic acid insensitive 1 (ABI1) PP2C, anchoring it to the plasma membrane, reducing its movement in the cytosol and decreasing its activity as negative regulator of ABA signaling (Zhang et al., 2004). Salt and osmotic stress also cause an increase of PA and phosphoinositides such as phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) (DeWald et al., 2001; Meijer et al., 2001; Munnik et al., 2000). In salt stress conditions, PA activates MAP kinase 6 which leads to the phosphorylation of SOS1 (Yu et al., 2010). On the other hand, PtdIns(4,5)P2 serves as substrate for the phospholipase C (PLC), a peripheral protein that is anchored to the cytosolic side of the plasma membrane in a Ca2+-dependent manner (Singh et al., 2015). PLC catalyzes a reaction producing inositol-1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) from PtdIns(4,5)P2. IP3 is a well-known signalling lipid in animals, which also produces a increase in [Ca2+]cyt in plants (DeWald et al., 2001; Singh et al., 2015; Zhu, 2016). Intriguingly,
tomato plants with reduced levels of IP$_3$ showed higher tolerance to drought and metabolic alterations, suggesting a complex involvement of IP$_3$ in plant tolerance to abiotic stress (Khodakovskaya et al., 2010).

1.2. Intracellular communication during NaCl stress

In response to stress, plant cells need to regulate the activity of different organelles that spatially separate incompatible biochemical reactions (Lunn, 2007). To coordinate such segregated activities, plant cells use highly dynamic inter-organelle communication networks regulated by either vesicular exchange, that relies on the formation of vesicles that transport proteins, solutes and lipids from the ER and/or Golgi apparatus to destination organelles, including the plasma membrane or the vacuole (Jürgens, 2004) or through non-vesicular mechanisms involving the establishment of transient and/or stable physical attachments between closely apposed organelle membranes, known as membrane contact sites (MCS) (Schrader et al., 2015).

In Arabidopsis, the role of vesicular transport in NaCl tolerance is illustrated by transgenic plants overexpressing RabG3e, a protein involved in endocytic processes, whose overexpression leads to the accumulation of Na$^+$ in the vacuoles and to an improved plant tolerance to salt and osmotic stresses (Mazel et al., 2004). PIP aquaporins internalization rate increases during salt stress (Li et al., 2011; Luu et al., 2012). These endocytic and internalization responses could facilitate the removal of ion transporters at the plasma membrane and the prevention of water loss (Baral et al., 2015).

The role of non-vesicular transport in salt stress tolerance has been recently proposed and most mechanistic information has been gathered through the physiological characterization of the Arabidopsis Synaptotagmin 1 mutant (syt1). SYT1 is a molecular tether that bridges the endoplasmic reticulum (ER) and the plasma membrane (PM), the loss-of-function of which causes Ca$^{2+}$-dependent NaCl stress hypersensitivity, through a largely unknown mechanism involving ER-PM membrane contact sites (Pérez-Sancho et al., 2015; Schapire et al., 2008). This research project aims at identifying additional molecular
Chapter I: Introduction

components putatively involved in non-vesicular communication in Arabidopsis and characterize their putative function in ionic stress response.

1.3. Structure of membrane contact sites and their associated functions

Membrane contact sites (MCS) can be defined as intracellular regions where the membranes of two different organelles are in close proximity (Prinz, 2014). These regions were first identified in the 1970s thanks to electron micrographs of onion stems and rat liver (Morré et al., 1971). In eukaryotes, the MCS intermembrane distance ranges between 10 and 30 nm due to the linkage of specific protein tethering complexes that prevent membrane fusion (McFarlane et al., 2017; Prinz, 2014).

In eukaryotes, membrane contact sites involving almost every organelle have been identified. Because the ER is an extensive and active organelle, which needs to communicate with the rest of the cell, it is involved in most of the contact sites discovered so far. ER-PM contact sites (EPCS) have been found in plants, yeast and mammals (Besprozvannaya et al., 2018; Giordano et al., 2013; Lees et al., 2017; Pérez-Sancho et al., 2015; Toulmay and Prinz, 2012; Wang et al., 2014) and are the most studied ones. Membrane contact sites between the ER and the mitochondria (Hamasaki et al., 2013; Lackner et al., 2013; Staehelin, 1997) and the ER and the Golgi complex (B. E. Juniper, 1982; Liu et al., 2017; Sohn et al., 2016) have also been identified in eukaryotes. Contact sites between the ER and the vacuole (Staehelin, 1997; Toulmay and Prinz, 2012) are present in yeast and plants. Interestingly, the existence of contact sites involving the chloroplast (Andersson et al., 2007; Tan et al., 2011) and EPCS at plasmodesmata (Bayer et al., 2017) point towards a specific role of these MCS in plants, absent in its eukaryotic counterparts.

The protein tethers at MCS are not only structural components that bridge two membranes, but also have important roles in non-vesicular lipid transport, Ca^{2+} homeostasis and signalling, autophagy, and organelle division. However, the molecular components involved in those processes at plant MCS remain largely unknown and most of our knowledge derives from the mammalian and yeast literature (Pérez-Sancho et al., 2016; Prinz, 2014).
Chapter I: Introduction

1.3.1. MCS tethers in non-vesicular lipid transfer

Several mammal- and yeast-based studies have shown that MCS tethers are involved in lipid homeostasis, mainly in ER-PM and ER-mitochondria (Ghai et al., 2017; Jeong et al., 2017; Kim et al., 2015; Lees et al., 2017; Yu et al., 2016).

Glycerophospholipids such as phosphatidylcholine (PC), PA, phosphatidylglycerol (PG) and phosphatidylserine (PS) can be potentially transported, in a non-vesicular manner, through the yeast ER-mitochondria encounter structure (ERMES) complex, localized at ER-mitochondria contact sites (Jeong et al., 2017). Mammalian ORPs (OSBP-related proteins) are ER proteins which are recruited to EPCS by binding to PtdIns(4,5)P$_2$ and are suggested to transport phosphoinositides between those membranes (Ghai et al., 2017). Similarly, the mammalian ER protein extended-synaptotagmin 1 (ESyt) is also localized at EPCS and it is capable of selectively transporting glycerophospholipids through its lipid-binding synaptotagmin-like mitochondrial-lipid-binding protein (SMP) domain (Giordano et al., 2013; Yu et al., 2016).

The function of the mammalian ER membrane protein transmembrane protein 24 (TMEM24) as negative regulator of Ca$^{2+}$ signaling exemplifies the lipid transport roles associated with EPCS tethers (Lees et al., 2017) (Figure 1.2). TMEM24 is present at EPCS as a dimer, anchored to the ER membrane through a N-terminal TM domain and attached to the PM by a C-terminal poly-basic domain, thanks to electrostatic interactions with negatively charged phosphoinositides in the cytosolic side of the PM. TMEM24 also has a SMP domain that preferentially binds phosphatidylinositol (PI). In resting cells TMEM24 transports PI (labelled as a red glycerolipids) from the ER, where it is synthesized, to the PM, where it can be sequentially phosphorylated by phosphatidylinositol 4 kinase (PI4K) and phosphatidylinositol 4-phosphate 5 kinase (PIP5K) to produce PtdIns(4,5)P$_2$ (Figure 1.2a). In response to a stimulus, [Ca$^{2+}$]$_{cyt}$ increases (Figure 1.2b), triggering PLC activity (Figure 1.2c). PLC hydrolyzes PtdIns(4,5)P$_2$ into DAG and IP$_3$, further increasing [Ca$^{2+}$]$_{cyt}$ (Figure 1.2d). The increase in [Ca$^{2+}$]$_{cyt}$ also activates the protein kinase C (PKC), that phosphorylates the poly-basic domain of TMEM24 (Figure 1.2e). The poly-basic domains of TMEM24
now have a negative charge due to the acquisition of the phosphate groups, that repel the negatively charged phosphoinositides, breaking the ER-PM bridge and stopping the lipid transfer (Figure 1.2f). Eventually, the activity of the PLC reduces PtdIns(4,5)P$_2$ pool at the PM and the high [Ca$^{2+}$]$_{cyt}$ cannot be maintained anymore. When [Ca$^{2+}$]$_{cyt}$ levels return to resting state, the protein serine/threonine phosphatase 2B (PP2B) de-phosphorylates TMEM24 poly-basic domains (Figure 1.2g), that recover the PM binding and, consequently, the PI transport to the PM, indirectly restoring the PtdIns(4,5)P$_2$ pool for a new round of Ca$^{2+}$ signaling event (Lees et al., 2017) (Figure 1.2h).

![Figure 1.2](image)

**Figure 1.2: Model for TMEM24 non-vesicular lipid transport at EPCSs.** The TM domain anchors TMEM24 to the ER membrane while the polybasic (PB) domain is attached to negatively charged phosphoinositides at the PM. PI (labelled as red and black glycerolipids) are transported from the ER to the PM when the contact site is established under low [Ca$^{2+}$]$_{cyt}$. Increments in [Ca$^{2+}$]$_{cyt}$ break TMEM24 contact site and stop PI transport through phosphorylation of the PB domain of TMEM24. Modified from Lees et al., (2017).

Lipid transfer at MCSs has not been reported yet in plants. However, putative tethers in plants have annotated lipid-binding domains similar to those found in yeast and mammalian MCS tethers with lipid-transfer roles, including the oxysterol binding (OSB) domain or the SMP domain. This suggests that non-
vesicular lipid transfer could also occur in plants (Pérez-Sancho et al., 2016). Additionally, trans-organellar complementation studies involving the ER and the chloroplast suggested that nonpolar substrates can be moved between the ER and the chloroplast (Mehrshahi et al., 2013). This may be explained by the presence of protein tethers forming contact sites and/or the presence of membrane hemifusion. In the membrane hemifusion model a hybrid between the ER and the outer chloroplast lipid membranes is formed, allowing enzymes in the ER lumen to access substrates in the outer membrane of the chloroplast (Mehrshahi et al., 2014).

1.3.2. Organelle inheritance and autophagy at MCSs

A number of MCS tethers has been associated with the functions related to autophagy and organelle division (Hamasaki et al., 2013; Knoblach et al., 2013; Lackner et al., 2013; Park et al., 2016; Wijdeven et al., 2016). In yeast, proteins such as Num1, Inp1p, and Pex3p, form contact sites between the ER and the mitochondria. These proteins have been related to mitochondria inheritance, division and distribution (Knoblach et al., 2013; Lackner et al., 2013). Similarly, the yeast protein Vps13 localizes to endosome-mitochondria contact sites and to nucleus-vacuole junctions (NVJ), where it is thought to regulate mitophagy (mitochondria autophagy) in response to nitrogen starvation conditions (Park et al., 2016). In mammals, autophagosomes for mitochondria autophagy form at ER-mitochondria contact sites. Autophagosome marker ATG14 is recruited by the ER-resident protein STX17 to form ER-mitochondria contact sites in response to starvation (Hamasaki et al., 2013).

The presence of contact sites involving the ER, the vacuole and plastids in plants (Andersson et al., 2007; Staehelin, 1997; Tan et al., 2011) suggests that MCSs could also regulate organelle inheritance and autophagy in plant cells. A recent study found that the *Physcomitrella patens* ER protein MELL1 has a dual ER-mitochondria localization. MELL1 is involved in ER-mitochondria associations and in the mitochondria shape and number, reinforcing the idea of organelle inheritance as MCS function in plants (Mueller and Reski, 2015).
1.3.3 EPCS tethers in Ca\(^{2+}\) signaling and homeostasis

The function of EPCS in Ca\(^{2+}\) homeostasis is well-exemplified by the mammalian protein complex formed by the stromal interaction molecule (STIM) and Orai (Derler et al., 2016) (Figure 1.3). STIM1 is a protein located in the ER membrane thanks to a transmembrane (TM) domain, where it is usually found as a homodimer. In this inactive state, the STIM1 N-terminal domain faces the lumen of the ER where its EF-hand and SAM domains are bound to Ca\(^{2+}\), abundant in the ER lumen in the cell resting state (Figure 1.3a). Depletion of ER Ca\(^{2+}\) stores change STIM1 conformation (Figure 1.3b), causing its multimerization and activation (Derler et al., 2016). The conformational change is transmitted to the C-terminal polybasic

Figure 1.3: Model for STIM1-Orai1 Ca\(^{2+}\) signaling at EPCSs. The TM domain anchors STIM1 to the ER membrane while the N-terminal domains, in the ER lumen, are bound to Ca\(^{2+}\) in resting state. Depletion of ER luminal Ca\(^{2+}\) cause STIM1 conformation to change, activating it and recruiting it to EPCSs. STIM1 then interacts with and activates Orai1, a Ca2+ channel at the PM. Orai1 allows then the entrance of Ca2+ from the extracellular space, further increasing [Ca\(^{2+}\)]\(_{cyt}\). Adapted from Derler et al., (2016).
domain which binds PtdIns(4,5)P$_2$ at the PM (Figure 1.3c). STIM1, in its inactive state, moves along the microtubules and has a homogeneous localization. However, after the conformational change, STIM1 is recruited to EPCS and its localization becomes more punctate and stable (Derler et al., 2016). STIM1, now in contact with the PM, interacts with Orai1 (Figure 1.3d). Orai1 is a Ca$^{2+}$-selective ion channel that is activated upon interaction with STIM1, allowing the passive entrance of Ca$^{2+}$ through the PM into the cytosol and further increasing [Ca$^{2+}$]$_{cyt}$ (Derler et al., 2016; Prakriya and Lewis, 2015) (Figure 1.3e).

In plants, the Arabidopsis EPCS tether SYT1 has demonstrated functions related to Ca$^{2+}$ signalling. SYT1 possesses two Ca$^{2+}$-dependent lipid binding C2 domains (Figure 1.4), that can bind phosphoinositides in vitro (Pérez-Sancho et al., 2015; Schapire et al., 2008). syt1 mutant plants show hypersensibility to salt and cold stress mediated by Ca$^{2+}$ (Schapire et al., 2008; Yamazaki et al., 2008). However, how the Ca$^{2+}$-dependent lipid binding properties of SYT1 affect its roles in salt and cold stress tolerance requires clarification.

### 1.4. EPCS tethers in plants

Despite the early identification of MCS in plants, no molecular components involved in their establishment have been described until recently.

In this context, Perez-Sancho and coworkers (2016) used homology searches for putative MCS tethers in Arabidopsis and divided them into two groups: transmembrane and cytosolic tethers. Among the cytosolic tethers are the ORPs and the steroidalogenic acute regulatory transfer (StART) proteins; in the group of transmembrane tethers are the VAMP-associated protein (VAP) and the SMP families (Pérez-Sancho et al., 2016). These four protein families have homologs in yeast and mammals that are known MCSs tethers (Ghai et al., 2017; Giordano et al., 2013; Wilhelm et al., 2017). The most studied MCSs in plants are the EPCS. Two transmembrane proteins have been demonstrated to be EPCS tethers in Arabidopsis: VAP27-1, a member of the VAP family, and SYT1, member of the SMP family (Pérez-Sancho et al., 2015, 2016; Wang et al., 2014).
Chapter I: Introduction

VAP27-1 contains a TM domain and an MSP (major sperm) domain. The TM domain attaches VAP27-1 to the ER membrane while the MSP domain is used for protein-protein interaction. The EPCS is formed due to the association of VAP27-1 MSP domain with NET3C, a member of the plant NET family of actin-binding proteins. These contact sites also include actin filaments and microtubules, which influence NET3C and VAP27-1 turnover, respectively (Wang et al., 2014). More recently, it has been shown that the VAP27 plant protein family includes nine more members, some of them with TM domains and EPCS localization (Wang et al., 2016). Interestingly, several studies have related VAP27 tethers with lipid-binding proteins as demonstrated for their eukaryote homologues (Barajas et al., 2014; Ho et al., 2016; Petersen et al., 2009; Saravanan et al., 2009). The sphingosine transfer protein ACD11 interacts with VAP27-1; considering the EPCS localization of VAP27-1, sphingosine transfer by ACD11 might take place at those sites (Petersen et al., 2009; Wang et al., 2014). Arabidopsis sterol-binding protein ORP3a has an ER localization dependent on VAP27-3 (Saravanan et al., 2009). VAPs and ORPs have also been related to virus infection in plant cells, suggesting that plant MCS proteins might be involved in biotic stress (Barajas et al., 2014). Recently, VAP27-8 (named by Ho et al., 2016 as VAP-related suppressor of TMM; VST1), a PM protein (Wang et al., 2016) has been reported to be involved in stomatal development by interacting with ERL2 receptor kinase (Ho et al., 2016). Bimolecular fluorescence complementation studies confirmed the interaction of VAP27-8 and SYT1. Furthermore, SYT1 EPCS localization and requires functional VAP27-8/-9 and -10. These data suggest that SYT1, ERL2 and at least VAP27-8 might be required for stomata signalling, adding another process to the MCSs functions in plants (Ho et al., 2016).

The Arabidopsis SYT1 belongs to the SMP family, with a total of five members known so far. While SYT1 and SYT5 are ubiquitously expressed, SYT2, SYT3 and SYT4 are specifically expressed in anthers, senescent and mature leaves, and dry seeds, respectively (unpublished; appendix A). Arabidopsis SYTs contain an SMP domain, a TM domain and a minimum of two Ca\(^{2+}\)-dependent lipid-binding C2 domains (Pérez-Sancho et al., 2016). SYT1 is localized at EPCSs, with its N-terminal TM domain anchored to the ER membrane and its C-terminal C2 domains in contact with the PM (Pérez-Sancho et al., 2015).
Unpublished data from our lab suggest an EPCS localization also for SYT5, following the SYT1 tethering model. Additionally, unpublished data from a collaborator indicate that SYT1 can form homodimers, and heterodimers with SYT5 (appendix D) (Figure 1.4). SYT1 C2 domains bind glycerophospholipids and negatively charged phosphoinositides in vitro, but only the C2A domain does it in a Ca\(^{2+}\) dependent manner (Pérez-Sancho et al., 2015; Schapire et al., 2008). The lipid-binding activity of the SYT1 C2 domains resembles the results obtained in its mammalian counterparts, the ESyts (Giordano et al., 2013). Loss of function mutant syt1-2 shows hypersensitivity to mechanical stress, a process that has been related to the role of SYT1 in membrane maintenance (Pérez-Sancho et al., 2015; Schapire et al., 2008). Membrane integrity is also important in freezing tolerance. Studies have shown that SYT1 is important for the Ca\(^{2+}\)-dependent freezing tolerance in Arabidopsis protoplasts. Furthermore, Arabidopsis SYT1 RNAi plants were hypersensitive to freezing stress, presumably because of a lack of membrane resealing due to SYT1 absence (Yamazaki et al., 2008). syt1-2 also shows hypersensitivity to salt stress, but only in conditions

![Figure 1.4: Model for SYT1 binding at EPCSs.](image) The TM domain anchors SYT1 to the ER membrane while the lipid binding C2 domains are attached to negatively charged phosphoinositides at the PM (labelled in blue). Note that only the C2A domain has Ca\(^{2+}\)-binding activity. The SMP domains interact, forming the SYT1 homodimer and the SYT1/SYT5 heterodimer.
with low Ca\(^{2+}\) in the medium, suggesting that Ca\(^{2+}\) is involved in SYT1 role in salt stress tolerance (Schapire et al., 2008). SYT1 was reported to be co-localize with VAP27-1 (Pérez-Sancho et al., 2015) although a more recent study shows that SYT1- and VAP27-1-resident contact sites are distinct (Siao et al., 2016). Absence of SYT1 increased VAP27-1 turnover and destabilized the ER network, suggesting that SYT1 might indirectly stabilize VAP27-1 contact sites by restraining ER movement (Siao et al., 2016). Accordingly, reticulon proteins RTNLB3 and 6, involved in the regulation of ER curvature, interact with SYT1 (Kriechbaumer et al., 2015). Interestingly, RTNLB3/6 are present in plasmodesmata, specialized plant channels that allow the connection of the ER network between cells (Kriechbaumer et al., 2015; Nicolas et al., 2017a). Mutant plants lacking functional SYT1 showed less viral movement between cells and abolition in the accumulation of viral proteins in plasmodesmata, suggesting that SYT1 could promote cell-to-cell movement of viral proteins through plasmodesmata. This data reinforces the function of plant MCS in biotic stress (Levy et al., 2015; Uchiyama et al., 2014). At plasmodesmata, the ER, the PM and actin filaments converge and it has been suggested that specialized EPCS could exist there (Nicolas et al., 2017b; Pérez-Sancho et al., 2016). Like VAP27-1, SYT1 also shows association with the cytoskeleton: SYT1 puncta localize to microtubule-depleted regions and actin filaments depolymerizing drugs such as latrunculin-B cause aggregation of SYT1 in equatorial ER regions of epidermal cells, without affecting cortical regions. These data suggest that established SYT1 EPCSs do not depend on the cytoskeleton while delivery of SYT1 to contact sites might required a functional cytoskeleton. Furthermore, depolymerization of microtubules after oryzalin treatments did not affect SYT1 localization (Pérez-Sancho et al., 2015).

1.1.5 Structure and function of the SMP domain

The SMP domain is present in eukaryote MCS tethers, including the ESyts and the tricalbins, the EPCS tethers homologous to Arabidopsis SYT1 in mammals and yeast, respectively (Lees et al., 2017; Pérez-Sancho et al., 2015; Toulmay and Prinz, 2012). The SMP domain was first identified in yeast proteins belonging to the ERMES complex, a complex forming contact sites between the ER and the mitochondria
and important for inter-organelle phospholipid interchange (Kornmann et al., 2009). A bioinformatic study showed that the SMP domain of the ERMES proteins had similarity to an annotated domain called TULIP (tubular lipid). The TULIP domain was reported to fold in a hydrophobic tunnel shape that, presumably, could bind and transport lipids (Kopec et al., 2010). The SMP domain has been demonstrated to bind and transport glycerophospholipids across membranes at MCSs in yeast and mammals, showing some degree of specificity depending on the protein (Jeong et al., 2017; Lees et al., 2017; Yu et al., 2016). The lipid-transport function of the SMP domain has not been probed yet in plants.

Interestingly, the SMP domain is present in tethers located at different contact sites, such as ER-mitochondria, ER-PM and nucleus-vacuole junctions (NVJ) (Giordano et al., 2013; Kornmann et al., 2009; Lees et al., 2017; Pérez-Sancho et al., 2015; Toulmay and Prinz, 2012). Furthermore, a study demonstrated that human proteins containing the SMP domain were directed to MCS also in yeast, suggesting that the SMP domain could be important for a MCS localization (Toulmay and Prinz, 2012).

2.1.6. Research objectives

The information about MCS and the tethers forming them in plants is scarce, compared to yeast and mammals (Pérez-Sancho et al., 2016). MCS are formed by protein complexes. The existence of distinct contact sites and the different functions of MCS tethers in yeast and mammals make us suggest that more MCS tethers might exist in plants (Pérez-Sancho et al., 2016; Prinz, 2014; Siao et al., 2016). Also, SYT1, a plant EPCS tether, has been to be required for abiotic stress tolerance, specially salt stress tolerance, an important function for plants as sessile organisms (Pérez-Sancho et al., 2015; Schapire et al., 2008; Yamazaki et al., 2008). Thus, we hypothesized that more MCS tethers could exist in plants and that they might have roles in salt stress tolerance. Following this hypothesis, my M.Sc. focuses on two objectives:

1. Identification of membrane contact sites tethers in Arabidopsis thaliana using reverse genetic approaches (Chapter 3 and 4). Based on Prinz and Toulmay (2004), who proposed that the SMP domain may be indicative of MCS tethers, and the EPCS localization of SYT1 (Pérez-Sancho
Chapter I: Introduction

et al., 2015), I hypothesized that proteins containing the SMP domain could be localized at MCS in plants. To test this hypothesis, I used bioinformatic data and phylogenetic analysis to find new SMP-containing proteins as putative MCS tethers.

2. **Functional characterization of the putative tethers in response to salt stress (Chapter 4).** Focusing on NaCl stress tolerance among the SYT1 tether functions (Schapire et al., 2008), I hypothesized that the putative tethers could have functions in NaCl tolerance and/or signaling mediated by Ca$^{2+}$. To test this hypothesis, I performed physiological treatments in insertional mutants of the putative tethers looking for hypersensitivity or resistance phenotypes to ionic stress in different Ca$^{2+}$ conditions. I also studied the localization of the putative tethers in the physiological conditions mentioned above using live-cell imaging.
Chapter II: Methods

2.1 Bioinformatic Analysis

The annotated SMP domain sequences from Nvj2p (Uniprot accession: Q06833; amino acid 300 to amino acid 505), Mmm1 (P41800; 190-409), Tcb1 (Q12466; 150-375), Tcb2 (P48231; 150-375), Tcb3 (Q03640; 150-375), SYT1 (Q9SKR2; 62-248), SYT5 (Q8L706; 70-248), ESyt1 (Q9BSJ8; 135-313), ESyt2 (A0FGR8; 191-370), ESyt3 (A0FGR9114-290) were aligned and further used for obtaining the SMP consensus sequence. All alignments were performed using Seaview through ClustalW2 algorithm (Gouy et al., 2010). Jalview software was used for obtaining the SMP consensus sequence from the SMP alignment by selection of the residues with the highest identity in each position, and for the visualization of the alignments (Waterhouse et al., 2009). The Arabidopsis Information Resource (TAIR) protein BLAST tool was used for the search of SMP-containing proteins in *Arabidopsis thaliana*, applying an e-value threshold of 0.0001 and using TAIR10 protein dataset. The National Center for Biotechnology Information (NCBI) BLAST tool was used for searching SMP-proteins in different plant species. Representative species of different phyla, classes, orders and families were selected for the phylogenetic tree, applying a e-value threshold of 10-20. MEGA7 (Kumar et al., 2016) was used for the creation of the phylogenetic tree, using the maximum likelihood method and 100 replicates (bootstrap). TraVA (Klepikova et al., 2016) and GENEVESTIGATOR (Zimmermann, 2004) were used to obtain the mRNA expression information for the putative tethers from RNAseq and micro-array data, respectively. The RNAseq data by TraVA show the absolute read counts normalized by TMM (Trimmed mean of M-values). Expression changes in different conditions were studied using ePlant and GENEVESTIGATOR (Waese et al., 2017; Zimmermann, 2004). TMHMM and PSIPRED bioinformatic tools were used for the prediction of transmembrane domains (Buchan et al., 2013; Sonnhammer et al., 1998) while Uniprot and Metallopred were used for the identification of the C2 domains and metal-binding ability, respectively (Bateman et al., 2017; Naik, 2011).
Chapter II: Methods

Structures of the SMP consensus and the putative tethers were predicted using Phyre2 and subsequently visualized and aligned using Chimera (Kelly et al., 2015; Pettersen et al., 2004); structure of the SMP and C2 domain of ESyt2 were obtained from PDB (ID 4P42) (Schauder et al., 2014).

2.2 Plant material and growth conditions.

_Arabitopsis thaliana_ Columbia (Col-0) ecotype was used as wild type and as genetic background for transgenes. Previously published lines used in this M.Sc. Thesis are SYT1-GFP (Pérez-Sancho et al., 2015), syt1-2 (SAIL_775_A08) (Schapire et al., 2008) and atclb1-1 (Salk_006298) (De Silva et al., 2011). TDNA insertion line atclb1-3 (Salk_076501) was also used in this thesis and obtained from ABRC (Arabidopsis Biological Resource Center, Columbus, Ohio, USA). Seeds were sterilized using a solution containing 4:1 of 87.5% ethanol and 30% hydrogen peroxide, respectively, for 1 minute. Seedlings were grown on half strength Murashige and Skoog (MS) medium (Caisson Labs, Smithfield, Utah, USA) at 21°C/24h-light, after 48h/4°C stratification. They were subsequently transferred, if needed, to soil (Sunshine mix #4, Sun Gro Horticulture Canada Ltd., Hadashville, Manitoba, Canada) and grown at 22°C under 16-h light/8-h dark cycle.

2.3 Molecular cloning

DNeasy Mini Kit and RNeasy Plant Mini Kit (Qiagen, Montreal, Canada) were used for the extraction of Arabidopsis DNA and RNA, respectively. SuperScript II reverse Transcriptase (Invitrogen, Waltham, Massachusetts, USA) was used for the reverse transcription of RNA to cDNA. Phusion High-fidelity Taq polymerase (New England Biolabs, Whitby, Ontario, Canada) and specific primers (Table 2.1) were used for the amplification of the CDS, promotor and genomic sequences of _AtCLB_, _SYTF_ and _2TMCLB_. pENTR-D-TOPO (Life Technologies, Carlsbad, California, USA) was used to generate entry vectors containing _AtCLB_, _SYTF_ and _2TMCLB_ CDS, as well as pAtCLB:AtCLB, while entry vectors containing the promoter region of _SYTF_ and _2TMCLB_ were created using pENTR-5’-TOPO (Life
Technologies). R4 Gateway Binary Vector technology (Nakanawa et al., 2008) was used for obtaining pSYTF:SYTF(CDS) and p2TMCLB:2TMCLB (CDS) entry vectors. Gateway LR Clonase II (Life Technologies) was used for the recombination of inserts from entry vectors to UBQ10 promoter-driven C-terminal GFP/TagRFP fusion destination binary vectors or pGWB (for GUS fusion or native promoter-driven C-terminal GFP fusions) binary destination vectors. Entry and destination vectors were transformed into NEB 10-β competent Escherichia coli cells (New England Biolabs) by heat shock (37°C for 5 minutes) and plated onto selective lysogeny broth (LB) solid medium (Fisher Bioreagents, Pittsburgh, Pennsylvania, USA) containing 100μg/ml kanamycin (entry vectors) or 100 μg/ml hygromycin (destination vectors) (Sigma Aldrich, Oakville, Ontario, Canada). Positive colonies were confirmed by colony PCR, digestion

### Table 2.1: List of primers used in the thesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward AtCLB CDS</td>
<td>CACCATGGGTGTTTGATTCTGGGATTCTG</td>
</tr>
<tr>
<td>Reverse AtCLB CDS</td>
<td>CTGCTGTTTTCACCATCGATTCTGG</td>
</tr>
<tr>
<td>Forward Prom. AtCLB</td>
<td>CACCAATAATTATGACGCTT</td>
</tr>
<tr>
<td>Reverse Prom. AtCLB</td>
<td>TTTTCTGCAACCGAAACAAAGATA</td>
</tr>
<tr>
<td>Forward SYTF CDS</td>
<td>CACCATGGGTGCTGAGAATAAGAGGA</td>
</tr>
<tr>
<td>Reverse SYTF CDS</td>
<td>AGAATCTCGAGACGCGGGCTAGC</td>
</tr>
<tr>
<td>Forward Prom. SYTF</td>
<td>AAGTAAATTGTAGAGCT</td>
</tr>
<tr>
<td>Reverse Prom. SYTF</td>
<td>CTCTTCGATTACGGTCCTCA</td>
</tr>
<tr>
<td>Forw. At1g53590 CDS</td>
<td>CACCATGGAGTCTTCTTTGAATTCA</td>
</tr>
<tr>
<td>Rev. At1g53590 CDS</td>
<td>CTTTGAAGATGATCCTTTTCCT</td>
</tr>
<tr>
<td>Forw. Prom. At1g53590</td>
<td>AATTATGGAAGCAATGATTTTTATCAG</td>
</tr>
<tr>
<td>Rev. Prom. At1g53590</td>
<td>CGTATCCTTTTCATGATCAGABC</td>
</tr>
<tr>
<td>Forw. qRT-PCR ACT2</td>
<td>CTGGATCGGTGTCCAATTC</td>
</tr>
<tr>
<td>Rev. qRT-PCR ACT2</td>
<td>CCTGGATCGGTTCATCATAC</td>
</tr>
<tr>
<td>Forw. qRT-PCR AtCLB</td>
<td>TGGCTGCTGAGTCTAGTATG</td>
</tr>
<tr>
<td>Rev. qRT-PCR AtCLB</td>
<td>ACCTGTTCATAATCGTGAAACGA</td>
</tr>
</tbody>
</table>
with restriction enzymes and sequencing. A list of all final constructs and the vectors used are listed in Table 2.2.

Table 2.2: List of constructs generated. These constructs were subsequently transformed into Arabidopsis plants, Col-0 ecotype.

<table>
<thead>
<tr>
<th>Constructs Generated</th>
<th>Overexpression constructs</th>
<th>Endogenous expression constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUB10:AtCLB(CDS)-GFP (AtCLB-GFP)</td>
<td>pAtCLB:AtCLB-GFP</td>
<td></td>
</tr>
<tr>
<td>pUB10:AtCLB(CDS)-tRFP</td>
<td>pAtCLB:GUS</td>
<td></td>
</tr>
<tr>
<td>pUB10:SYTF(CDS)-GFP (SYTF-GFP)</td>
<td>pSYTF:SYTF(CDS)-GFP</td>
<td></td>
</tr>
<tr>
<td>pUB10:SYTF(CDS)-tRFP</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pUB10:2TMCLB(CDS)-GFP (2TMCLB-GFP)</td>
<td>p2TMCLB:2TMCLB(CDS)-GFP</td>
<td></td>
</tr>
<tr>
<td>pUB10:2TMCLB(CDS)-RFP</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

2.4 Generation of transgenic plants and transformants selection

Destination vectors were transformed into *Agrobacterium tumefaciens*, strain GV3101 carrying the PMP90 Ti plasmid, by electroporation. Floral dip technique was used for Arabidopsis transformation (Clough and Bent, 1998). Transgenic lines were selected using half strength MS medium containing 15µg/mL glufosinate-ammonium or 25µg/mL hygromycin (*pUB10* and pGWB constructs, respectively; Sigma-Aldrich). A minimum of 5 lines was selected and the presence of the insertion was confirmed by PCR. Homozygous transformants with a single insertion were obtained after analyzing the T2 ratio of resistant plants.

2.5 Chemical treatments, image acquisition and quantifications

5-days-old Arabidopsis seedlings grown vertically on half strength MS solid medium were transferred into tenth strength MS liquid medium containing 100mM NaCl, 100mM KCl, 50mM MgCl₂, 50mM Na₂SO₄, 50mM K₂SO₄, 200mM sorbitol, 75µM U73122, 1mM LaCl₃ or 5mM EGTA (all from Sigma Aldrich) and incubated for 16h in darkness. Pictures were taken using a Nikon C1 confocal laser scanning microscope equipped with 488 and 515/30nm emission filter and Nikon Plan Apochromat oil
immersion objectives (40 × 1.0 NA and 60 × 1.4 NA, respectively). Image analysis was performed using FIJI (Schindelin et al., 2012). Puncta and close reticulate structures numbers were quantified on 225µm² cortical regions of a minimum of 20 epidermal cells from 5 independent seedlings. Statistical differences were identified by analysis of variance (ANOVA) test using Excel 10 software (Microsoft).

2.6 Physiological treatments

5-days-old Arabidopsis seedlings grown vertically in tenth strength MS solid medium were transferred into tenth strength MS solid medium containing 100mM NaCl, 3mM CaCl₂, or 100mM NaCl plus 3mM CaCl₂ (all from Sigma Aldrich) and incubated for 4 days. When transferred, all seedlings had a root length of 130mm. Root length was measured using FIJI (Schindelin et al., 2012). At least 15 biological replicates for each mutant were used in two independent experiments. Statistical differences were identified by analysis of variance (ANOVA) test using Excel 10 software (Microsoft).

2.7 Quantitative RT-PCR

cDNA used in qRT-PCR was obtained as explained in section 2.2, using Arabidopsis 10-days old seedlings and 1µg of RNA for the retro-transcriptase reaction per sample. qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and a iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad). Relative expression was calculated by the 2^ΔΔCt method (Livak and Schmittgen, 2001), using ACT2 as a control. Primers used for the qRT-PCR are listed in Table 2.1.

2.8 Accession numbers

The Arabidopsis Genome Initiative locus identifiers for genes mentioned in this thesis are SYT1 (At2g20990), HDEL (At1g29330), C4H (At2g30490), ACT2 (At3g18780), AtCLB (At3g61050), SYTF (At3g18370) and 2TMCLB (At1g53590).
Chapter III: The search for MCS tethers

3.1. Results

3.1.1. AtCLB, a putative MCS tether, belongs to the NTMC2 family

In yeast and mammals, MCS components are usually multigene families of proteins involved in the physical tethering of the apposed membranes. Apart from this structural function, MCS components also have additional regulatory functions in non-vesicular transfer of lipids and Ca\(^{2+}\) signaling (Prinz, 2014). In plants, the two MCS tethers characterized so far, SYT1 and VAP27-1, also belong to multigene protein families (Pérez-Sancho et al., 2015, 2016; Wang et al., 2014). Given the multiplicity of MCS functions described in yeast and mammals, I hypothesized that some as yet uncharacterized Arabidopsis proteins might represent additional MCS tethers that, as in other organisms, establish multiprotein tethering complexes (Giordano et al., 2013; Jeong et al., 2017; Pérez-Sancho et al., 2016).

To identify the most probable MCS tether candidates in Arabidopsis, I performed homology studies using the SMP domain as query. The SMP domain is conserved in all eukaryotes and it is a functional domain found in MCS-localized proteins in mammals, yeast and plants (Giordano et al., 2013; Lees et al., 2017; Pérez-Sancho et al., 2015; Toulmay and Prinz, 2012).

First, I built a SMP consensus sequence to be used as a query against the Arabidopsis genome. To build the SMP consensus sequence, the SMP domain sequences from experimentally validated MCS tethers were aligned and the most common residues were selected. These sequences included the human EPCS tethers ESyt1/2 and 3 (Giordano et al., 2013; Schauder et al., 2014; Toulmay and Prinz, 2012), the yeast EPCS tethers tricalbin1/2 and 3 (Toulmay and Prinz, 2012) and the plant EPCS tethers SYT1 and SYT5 (Pérez-Sancho et al., 2015; unpublished data). Additionally, to identify MCS components outside the ER-PM interface I included the SMP domain sequence of the yeast Nvj2p tether, a protein localized in the
nucleus-vacuole junctions (Toulmay and Prinz, 2012), and of Mmm1, a protein localized at the ER-mitochondria contact sites (Jeong et al., 2017; Toulmay and Prinz, 2012).

The SMP consensus sequence was used as query in a protein BLAST search, looking for SMP-containing proteins in Arabidopsis thaliana. Apart from members of the SYT family (SYT1 to SYT5) the only protein obtained with an *e*-value above the selected threshold (see section 2.1.1. Bioinformatic analysis) was At3g61050, annotated as the Arabidopsis thaliana calcium-dependent lipid-binding protein (AtCLB). AtCLB belongs to a protein family called N-terminal transmembrane C2 domain (NTMC2), which includes the SYT family (SYT1 to SYT5 plus a new putative member, SYTF/6) and several proteins with unknown function (Table 3.1). Proteins from the NTMC2 family are characterized by the putative presence of similar domains: one or more N-terminal TM domains, a SMP domain and at least one C-terminal C2 domain. The NTMC2 family is divided into subfamilies or types, from type 1 (T1) to type 6 (T6) (Table 3.1). Two ubiquitously expressed NTMC2 proteins (appendix A) not identified in our BLAST search, a type 3 NTMC2 At3g18370 (SYT6/SYTF), and a type 6 NTMC2 At1g53590 (named 2TMCLB due to the prediction of 2 transmembrane domains; see next section) were used as controls to assess the

Table 3.1: Protein members of the NTMC2 family. The column on the right shows the subfamily type of each protein.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>NTMC2 Subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>At2g20990 (SYT1)</td>
<td></td>
</tr>
<tr>
<td>At1g20080 (SYT2)</td>
<td></td>
</tr>
<tr>
<td>At5g04220 (SYT3)</td>
<td>Type 1</td>
</tr>
<tr>
<td>At5g11100 (SYT4)</td>
<td></td>
</tr>
<tr>
<td>At1g05500 (SYT5)</td>
<td>Type 2</td>
</tr>
<tr>
<td>At3g18370 (SYT6/F)</td>
<td>Type 3</td>
</tr>
<tr>
<td>At3g61050 (AtCLB)</td>
<td>Type 4</td>
</tr>
<tr>
<td>At1g50260</td>
<td></td>
</tr>
<tr>
<td>At3g19830</td>
<td>Type 5</td>
</tr>
<tr>
<td>At1g53590 (2TMCLB)</td>
<td>Type 6</td>
</tr>
<tr>
<td>At3g14590</td>
<td></td>
</tr>
</tbody>
</table>
specificity of the approach and to determine whether the SMP consensus sequence used in the analysis was too restrictive for the identification of putative MCS candidates.

In this study, I also analyzed how SMP proteins evolved in plants by performing a phylogenetic analysis of SMP-containing proteins from representative species across the plant kingdom. For this analysis, I selected SMP-containing proteins from non-vascular plants including algae (Klebsormidium nitens and Micromonas commode), a moss (Physcomitrella patens) and a liverwort (Marchantia polymorpha); and vascular plants including pteridophytes (Selaginella moellendorffii, fern), gymnosperms (Picea sitchensis, conifer), monocotyledons (Elaeis guineensis, palm tree; Ananas comosus, pineapple; Zea mays, maize and Oryza sativa, rice) and different families of dicotyledons, such as Solanaceae (Solanum lycopersicum, tomato) and Rosaceae (Fragaria vesca, wild strawberry and Prunus persica, peach).

The phylogenetic analysis of the selected SMP-containing proteins shows that the SMP proteins can be separated in three branches (Figure 3.1). In the first branch (Figure 3.1, blue) non-annotated and SYT-like proteins from algae, moss, fern, monocotyledons and different dicotyledon species appear related to Arabidopsis SYT1, SYT2 and SYT3; in the second branch (Figure 3.1, orange), SYT5 and SYT4 related-proteins are the predominant ones; in the third branch (Figure 3.1, green) proteins from an alga, tomato and liverwort are related to the putative tether, AtCLB. However, in some cases, the protein annotations do not match this distribution as, for example, the S. lycopersicum SYT1 appears in the AtCLB branch, and the O. sativa CLB, appears in SYT4 and SYT5 branch. The fact that CLB-like proteins were identified in such diverse species across the plant kingdom, indicates that members of the family have an early evolutionary origin and have been conserved during plant evolution (Figure 3.1).
Chapter III: The search for MCS tethers

3.1.2. The NTMC2 tethers have Ca\(^{2+}\)-binding and lipid-binding domains

Contact sites tethers are able to maintain two membranes at close distance thanks to the presence of protein domains capable of docking to one of the membranes such as transmembrane domains or lipid-binding domains, and/or protein-protein interaction domains capable of establishing multiprotein tethering.

Figure 3.1: Phylogenetic tree of SMP-containing proteins in plants. The blue background marks the SYT1/2/3 branch; the orange region marks the SYT4/5 branch and the green background marks the CLB branch. The SYT family is labelled in colored bold; AtCLB is labelled in green bold.
Chapter III: The search for MCS tethers

complexes. (Giordano et al., 2013; Pérez-Sancho et al., 2015; Toumay and Prinz, 2012; Wang et al., 2014). In this study, I analyzed the presence of such domains in the three putative NTMC2 tethers.

A bioinformatic search on Uniprot showed annotated transmembrane domains for SYTF (30aa-50aa) and 2TMCLB (5-22; 28-45) (Bateman et al., 2017). Those predictions were reinforced by software such as TMHMM (Sonnhammer et al., 1998) and PSIPRED (Buchan et al., 2013). However, the predictions are not clear for AtCLB: PSIPRED shows a transmembrane domain close to the N-terminus (2-22); TMHMM also detects a hydrophobic, transmembrane domain but advises of the possible presence of a signal peptide in that region, due to the short distance between this hydrophobic region and the N-terminus of AtCLB. On the other hand, all three tether candidates have Ca²⁺-dependent lipid-binding C2 domains: AtCLB (250-361), 2TMCLB (283-383) and SYTF (292-403; 482-576; 606-701), that might be involved in the docking function. Additionally, Metallopred, a bioinformatic tool that predicts metal-binding activity based on the protein sequence (Naik, 2011) showed that AtCLB, 2TMCLB and SYTF can bind divalent cations, concurring with the Ca²⁺-requirement for the phospholipid binding mediated by SYT C2A domain. The SMP domain was annotated in Uniprot for AtCLB (69-247) and 2TMCLB (62-268) (Bateman et al., 2017). However, no data were available for the SMP domain in SYTF. The position of the SMP domain in SYTF (94-280) was later identified by aligning the consensus SMP domain to the protein sequence of SYTF.

To generate more evidence supporting the presence of C2 and SMP domains in the putative tethers, Phyre2 online tool (Kelly et al., 2015) was used to predict the 3D folding of the consensus SMP and of AtCLB, SYTF and 2TMCLB proteins. Those 3D structures were then compared to the crystalized SMP and C2 domains of Esyt2 (Schauder et al., 2014; Xu et al., 2014), looking for structural similarities. The output models of the consensus SMP, AtCLB, SYTF and 2TMCLB had an 84%, 57%, 72% and 38% of residues modelled with a 90% of accuracy, respectively. The consensus SMP was first aligned to the SMP domain of ESyt2 (Figure 3.2a). The structural alignment had a quality score (Q-score) of 0.3. The Q-score varies from 1 (identical structures) to 0 (completely different structures), indicating that the two SMP domains have 70% of their residues unaligned. However, the folding of the SMP consensus resembled
Chapter III: The search for MCS tethers

ESyt2 SMP domain, including the arrangement of α-helixes and β-sheets and the characteristic hydrophobic tunnel of the SMP domain, with lipid-transport function in yeast and mammals (Kopec et al., 2010; Schauder et al., 2014; Yu et al., 2016) (Figure 3.2b,c). Afterwards, the SMP and C2 domains of ESyt2 were aligned to the predicted models of AtCLB (Figure 3.3), 2TMCLB and SYTF (appendices B and C, respectively).

The SMP and C2 domains of ESyt2 aligned regions within the AtCLB predicted model with Q-scores of 0.84 and 0.68, respectively. The structural alignment of ESyt2 SMP domain showed the tunnel folding of AtCLB SMP domain, having with similar arrangement of α-helixes and β-sheets (Figure 3.3b). The structural alignment also reinforced the annotated position of the SMP domain (69-247). The alignment of

Figure 3.2: Ribbon diagrams of the alignment between the ESyt2 SMP domain and the consensus SMP model. a, front view. b, top view, showing the tunnel-like conformation typical of the SMP domain. c, top view, including lipids passing through the domain. ESyt2 SMP: yellow; consensus SMP: purple; lipid ligand: grey.
Chapter III: The search for MCS tethers

The C2 domains showed some unaligned regions corresponding to the turns between β-strands (Figure 3.3c) but showed similarities in the folding of the C2 domain, formed by a pair of four β-strands facing each other and connected by an α-helix (Figure 3.3c). The alignment of the C2 domains indicated a different region corresponding the C2 domain of AtCLB with respect to the annotated one (263-390). The alignment of the SMP and C2 domains of ESyt2 into 2TMCLB model (Q-scores of 0.82 and 0.67, respectively) (appendix B) indicated new positions for the SMP and C2 domains of 2TMCLB (68-260 and 282-397, respectively). Additionally, a disorder region was identified from the end of the C2 domain to the C-terminus of the protein (appendix B), accordingly with the absence of domains annotated in Uniprot. On the other hand, alignment of SYTF model with SMP domain of ESyt2 (Q-score of 0.8) confirmed the position of SYTF SMP domain already suggested by sequence alignment (94-280) (appendix C). The alignment of the C2 domain of ESyt2 with SYTF model indicated the presence of 3 C2 domains with Q-scores of 0.40 (C2A),

**Figure 3.3: Ribbon diagrams of the alignment between AtCLB model and the SMP and C2 domains of ESyt2.**

- **a**, front view.
- **b**, close view of the C2 domain alignment.
- **c**, top view of the SMP domain alignment. AtCLB model: grey; ESyt2 SMP: red; ESyt2 SMP: yellow; AtCLB TM predicted region: blue.
Chapter III: The search for MCS tethers

0.70 (C2B) and 0.63 (C2C) (appendix C), as previously annotated by Uniprot, and suggested new positions (C2A: 292-446; C2B: 480-593; C2C: 606-716). An additional short disordered region was identified close to the C-terminus (appendix C). This information, together with the prediction of transmembrane domains, was used to establish the domains’ organization of the tether candidates (Figure 3.4).

Figure 3.4: Functional domains of the AtCLB, 2TMCLB and SYTF tether candidates. Blue, yellow and red ovals represent predicted transmembrane, SMP and C2 domains, respectively. Total residues’ number is shown at the C-terminus of each putative tether.

3.1.3. The SMP domain has conserved residues in eukaryotes

I next considered whether the SMP domains from different organisms across eukaryotes have conserved residues, which could be important for their function and therefore conserved through evolution. For this purpose, I created a new alignment including the consensus SMP sequence, the SMP domain sequences of the MCS (yeast tricalbins, plant SYTs, human ESys and yeast Mmm1 and Nvj2p) and the SMP domain sequences of the putative tethers (AtCLB, SYTF and 2TMCLB).

Three residues were conserved in all proteins: a tryptophan (W) at position 128 in the alignment, an asparagine (N) at position 130, and a glycine (G) at position 186 (Figure 3.5, red squares and arrows). We noticed that the residue 129, located between the conserved tryptophan and the conserved asparagine, was hydrophobic in all proteins, varying between leucine (L), the most common, and alanine (A), isoleucine (I), methionine (M) or phenylalanine (F) (Figure 3.5, yellow square and arrow). Other hydrophobic residues such as tryptophan, valine (V) and proline (P) and hydrophilic residues such as glutamate (E), lysine (L)
and arginine (R), located along the SMP domain sequences, were conserved in most of the proteins (Figure 3.5).

3.1.4. The expression pattern of the putative tethers indicates roles in abiotic stress

MCS tethers have been associated with many different functions including lipid and Ca\(^{2+}\) transport, Ca\(^{2+}\) signalling, organelle inheritance and organelle division in yeast and mammals (Prinz, 2014). The few plant EPCS tethers have been shown to be involved in root hair development to salt, cold and mechanical stress tolerance (Kim et al., 2016; Pérez-Sancho et al., 2015; Schapire et al., 2008; Uchiyama et al., 2014; Wang et al., 2016).

Figure 3.5: Alignment of the consensus SMP domain and the SMP domain of the MCS tethers. Residues are coloured based on the percentage of identity, where bluer residues have a higher identity among the sequences; only residues with a minimum of 60% of identity are coloured. Red squares and arrows show residues conserved in all sequences while the yellow square and arrow show the hydrophobic residues conserved in position 129. Numbers indicate residues’ position within the alignment of the SMP domains. ESyt: Human extended synaptotagmin; Tcb: yeast tricalbin; Mmm1: yeast maintenance of mitochondrial morphology 1; Nvj2p: yeast nucleus-vacuole junction 2 protein.

3.1.4. The expression pattern of the putative tethers indicates roles in abiotic stress

MCS tethers have been associated with many different functions including lipid and Ca\(^{2+}\) transport, Ca\(^{2+}\) signalling, organelle inheritance and organelle division in yeast and mammals (Prinz, 2014). The few plant EPCS tethers have been shown to be involved in root hair development to salt, cold and mechanical stress tolerance (Kim et al., 2016; Pérez-Sancho et al., 2015; Schapire et al., 2008; Uchiyama et al., 2014; Wang et al., 2016).
To obtain more information about the possible function of the tether candidates I analyzed their transcriptional profiles at different developmental stages. For this study I used a publicly available RNAseq database, TraVA (Klepikova et al., 2016) and a microarray database, GENEVESTIGATOR (Zimmermann, 2004). As a result, I established that the transcriptional levels of \textit{AtCLB} and especially \textit{SYTF} and \textit{2TMCLB} were much lower than those of \textit{SYT1} in all tissues analyzed (Table 3.2) These RNAseq data were somewhat supported by microarray analyses showing that \textit{SYT1} and \textit{AtCLB} were highly expressed compared to the average expression levels of Arabidopsis genes (Figure 3.6) while \textit{SYTF} and \textit{2TMCLB} expression was lower and close to the average values (Table 3.2; Figure 3.6). Interestingly, \textit{SYT1} and \textit{AtCLB} are highly expressed

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.6.png}
\caption{Microarray-based expression levels of \textit{SYT1}, \textit{AtCLB}, \textit{SYTF} and \textit{2TMCLB} in different developmental stages. Expression showed in log2. On the left side, a comparison with the expression of Arabidopsis genes show how much are expressed \textit{SYT1}, \textit{AtCLB}, \textit{SYTF} and \textit{2TMCLB} respect to the average. \textit{SYT1}, yellow dots; \textit{AtCLB}, red dots; \textit{SYTF}, blue dots; \textit{2TMCLB}, green dots.}
\end{figure}
Chapter III: The search for MCS tethers

Table 3.2: Expression levels of the tether candidates in different tissue. Values expressed as absolute read counts. SYT1 is shown as comparison. Higher expression values have deeper green colours while lower values have deeper red colours. Intermediate values are shown in white and lighter colours. Data modified from TraVA.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SYT1</th>
<th>AtCLB</th>
<th>SYTF</th>
<th>2TMCLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthers (Opened)</td>
<td>1976</td>
<td>1409</td>
<td>517</td>
<td>504</td>
</tr>
<tr>
<td>Anthers (Mature flow.)</td>
<td>1214</td>
<td>2065</td>
<td>327</td>
<td>320</td>
</tr>
<tr>
<td>Carpels (Mature flow.)</td>
<td>3313</td>
<td>2209</td>
<td>803</td>
<td>661</td>
</tr>
<tr>
<td>Stamen filaments (Mature flow.)</td>
<td>6873</td>
<td>2976</td>
<td>1228</td>
<td>917</td>
</tr>
<tr>
<td>Petals (Mature flow.)</td>
<td>4678</td>
<td>2330</td>
<td>1759</td>
<td>688</td>
</tr>
<tr>
<td>Sepals (Mature flow.)</td>
<td>4519</td>
<td>1937</td>
<td>1464</td>
<td>749</td>
</tr>
<tr>
<td>Flower</td>
<td>3451</td>
<td>2020</td>
<td>1313</td>
<td>645</td>
</tr>
<tr>
<td>Internode</td>
<td>5050</td>
<td>1983</td>
<td>1324</td>
<td>1796</td>
</tr>
<tr>
<td>Senescence internode</td>
<td>3553</td>
<td>2436</td>
<td>2522</td>
<td>2900</td>
</tr>
<tr>
<td>Whole leaf (Mature)</td>
<td>5029</td>
<td>2081</td>
<td>2051</td>
<td>1574</td>
</tr>
<tr>
<td>SAM 7-d</td>
<td>4058</td>
<td>2979</td>
<td>992</td>
<td>878</td>
</tr>
<tr>
<td>Meristem 10-d</td>
<td>6011</td>
<td>3316</td>
<td>1047</td>
<td>1420</td>
</tr>
<tr>
<td>Inflorescence meristem 15-d</td>
<td>3839</td>
<td>2169</td>
<td>571</td>
<td>844</td>
</tr>
<tr>
<td>Pod of the senescent silique</td>
<td>6432</td>
<td>2060</td>
<td>843</td>
<td>1103</td>
</tr>
<tr>
<td>Pedicel</td>
<td>5876</td>
<td>2578</td>
<td>1968</td>
<td>972</td>
</tr>
<tr>
<td>Root without apex</td>
<td>3152</td>
<td>1391</td>
<td>858</td>
<td>988</td>
</tr>
<tr>
<td>Seedling cotyledons</td>
<td>6650</td>
<td>2633</td>
<td>1081</td>
<td>593</td>
</tr>
<tr>
<td>Seedling hypocotyl</td>
<td>6494</td>
<td>1999</td>
<td>2039</td>
<td>2145</td>
</tr>
<tr>
<td>Seedling root</td>
<td>4454</td>
<td>1745</td>
<td>837</td>
<td>1094</td>
</tr>
<tr>
<td>Dry seeds</td>
<td>8251</td>
<td>3650</td>
<td>1965</td>
<td>888</td>
</tr>
<tr>
<td>Germinating seeds</td>
<td>7145</td>
<td>3195</td>
<td>3698</td>
<td>1038</td>
</tr>
</tbody>
</table>

during senescence and in seeds (Figure 3.6). This agrees with RNAseq data, where SYT1 and AtCLB have the highest expression values in dry and germinating seeds (Table 3.2). SYTF and 2TMCLB have lower expression values in most tissues. SYTF is more highly expressed in senescent internodes, mature leaves, germinating seeds, pedicels, and seedling hypocotyls while 2TMCLB is more expressed in senescent internodes, internodes, and seedling hypocotyls (Table 3.2).

The expression levels of the different candidates in response to changes in environmental conditions was also evaluated. For these analyses I used the microarray data available from ePlant (Waese et al., 2017). In the analysis, the expression data was represented as “fold” values, indicating the ratio of expression obtained compared to control conditions: values below 1 indicate downregulation and values above 1 indicate upregulation. Only treatments with a minimum of 30% expression change were considered (Figure 3.7).
My results show that AtCLB is downregulated in Arabidopsis plants exposed to abiotic stresses, such as salt (0.66-fold) and osmotic stress (0.48-fold). Moreover, expression of AtCLB in Arabidopsis leaves after inoculation of ABA decreases to 0.15-fold (Pandey et al., 2010). Interestingly, Arabidopsis leaves inoculated with Pseudomonas syringae or bacterial-derived elicitors also suffer a decrease of AtCLB expression (0.47-fold and 0.67-fold, respectively) (Waese et al., 2017). In contrast, SYTF is upregulated after osmotic stress treatments (2.25-fold) as well as salt (1.56-fold) and cold stresses (1.74-fold) (Kilian et al., 2007). Expression of SYTF in leaves decreases to 0.15-fold after inoculation of ABA (Pandey et al., 2010). SYTF is upregulated (1.8-fold) when Arabidopsis seedlings are incubated with AgNO₃, an ethylene inhibitor (Goda et al., 2008). SYTF is also upregulated in biotic stress-related treatments: incubated of seedlings with salicylic acid (SA) and infection of leaves with Phytophthora infestans, a eukaryotic microorganism causing the plant disease known as “potato blight” (1.77-fold and 1.97-fold, respectively) (Goda et al., 2008; Waese et al., 2017). Like SYTF, 2TMCLB is upregulated after osmotic (2.59-fold), salt (1.76-fold) and cold stresses (2.78-fold) (Kilian et al., 2007). Inoculation of ABA causes downregulation of SYTF (0.16 fold) (Pandey et al., 2010). Additionally, incubation of SA downregulates of 2TMCLB down
to 0.6-fold in Arabidopsis seedlings (Goda et al., 2008). Gibberellic acid (GA) synthesis inhibitor propiconazole also downregulates 2TMCLB seedlings (0.6-fold) (Goda et al., 2008).

3.2. Discussion

3.2.1. The Arabidopsis NTMC2 family might contain more MCS tethers

The finding of AtCLB, a SMP-containing protein and a putative MCS tether, revealed a connection between a protein family, NTMC2, and MCSs. The Arabidopsis SYT family also belongs to the NTMC2 family (Table 3.1), reinforcing the relation between the NTMC2 protein family and MCS. This also gives an additional interest to the several proteins with unknown function present in the NTMC2 family (Table 3.1), since these proteins could also be located at MCS. The NTMC2 family has also been identified in rice (Oryza sativa), as a family with sequence and domain similarity to animal synaptotagmins (members of the animal NTMC2) and ESyts tethers (Craxton, 2007, 2010; Giordano et al., 2013; Huang et al., 2017). Remarkably, all the Arabidopsis NTMC2 family members analyzed posses transmembrane, SMP and C2 domains, resembling those characterized in SYT1 (Figure 3.4; Figure 3.3; appendix B; appendix C) (Pérez-Sancho et al., 2015). The domains’ similarity between the tether candidates AtCLB, SYTF and 2TMCLB, and the EPCS tether SYT1 reinforces the relation between the NTMC2 family and MCSs, specifically EPCSSs. On the other hand, the majority of tethers used in the creation of the SMP consensus sequence were EPCS: tricalbins (Toulmay and Prinz, 2012), ESyts (Giordano et al., 2013) and SYT1 (Pérez-Sancho et al., 2015). This could have enriched the bioinformatic analysis towards the selection of EPCS candidates, preventing the discovery of other types of contact site tethers. Therefore, the SMP consensus approach might not be adequate to identify other types of contact sites components. Nevertheless, my work suggests that among the unknown members of the NTMC2 family, new protein tethers at MCS could be identified.
3.2.2. Different subfamilies of NTMC2 proteins have evolved differentially in plants

The phylogenetic analysis described in Figure 3.1 shows that the SMP proteins are not grouped by species but separated into three main clades: one clade for SYT1, SYT2 and SYT3-related proteins (Figure 3.1, blue), a second clade for SYT4 and SYT5-related proteins (Figure 3.1, orange) and a third clade for AtCLB-related proteins (Figure 3.1, green). This distribution also represents the early evolutionary divergence of the different NTMC2 subfamilies as SYT1, SYT2 and SYT3 belong to the NTMC2 type 1 subfamily, SYT4 and SYT5 to the NTMC2 type 2 subfamily, and AtCLB belongs to the NTMC2 type 4 subfamily (Table 3.1). In my analysis I also uncovered that some genes could be mis-annotated in different plant genomes. For example, a closer analysis of the annotated S. lycopersicum SYT1 shows a higher degree of similarity with the Arabidopsis AtCLB than with the Arabidopsis SYT1, and the annotated O. sativa CLB was more similar to the Arabidopsis SYT5 than to AtCLB. I therefore conclude that my phylogenetic analysis can be used as a tool to identify the most likely EPCS tether orthologs in crops.

3.2.3. Conserved residues in eukaryotic SMP domains suggest that SMP-protein dimerization and lipid-transport may also occur in plants.

The alignment of SMP domains of the MCS tethers and putative tethers used in this study shows that three amino acid residues are conserved in all proteins: Trp128, Asn130, and Gly186 (Figure 3.5, red squares, and arrows). Also, the residue 129, located between the conserved Trp128 and Asn130, was hydrophobic in all proteins (Figure 3.5, yellow square and arrow). The conservation of these residues despite the phylogenetical distance separating these organisms (human, Arabidopsis and yeast) and the different contact site localization of the tethers (EPCS, NVJ and ER-mitochondria), suggest that they are important for the integrity of the SMP structure and/or function.

Trp128, Asn130 and Gly186 are located at the N-terminus of the SMP domain. Crystallization of the mammalian and yeast (Zygosaccharomyces rouxii) SMP-containing tethers ESyt-2 and Mmm1 (Jeong et al., 2017; Schauder et al., 2014) showed that these proteins dimerize by interaction of the SMP domains,
forming a hydrophobic tunnel which is capable of transporting glycerophospholipids. The lipid-transport property of the SMP domain was bioinformatically predicted (Kopec et al., 2010) and later, experimentally demonstrated in vitro by incubation of the protein tethers with vesicles of a known lipid composition and by subsequent analyses in the change of the vesicles’ composition (Lees et al., 2017; Yu et al., 2016). The N-terminal region of the SMP domains is involved in the SMP-SMP interaction, as shown by the crystal structures (Jeong et al., 2017; Schauder et al., 2014), suggesting that Trp128, Asn130 and Gly186 might be important for SMP dimerization. Moreover, Trp128, Asn130 and Gly186 are also conserved in zrMmm1. Interestingly, residues Trp221 (corresponding to Trp128 in the SMP alignment) and Phe222 (corresponding to the conserved hydrophobic residue in position 129 of the alignment) of zrMmm1, located at the N-terminus region of the SMP domain, are important to stabilize zrMmm1 dimer by hydrophobic interactions between SMP domains (Jeong et al., 2017).

**Figure 3.8: Crystal structure of human ESyt2 SMP and C2 domains.** Different ESyt2 proteins within the dimer are coloured in blue and orange. SMP domains are highlighted in lighter colours, showing the tunnel structure capable of lipid transport. Residues Trp197, Leu198 and Asn199 are highlighted in red, green and magenta, respectively. **Inset I:** the conserved Trp197 and the juxtaposed hydrophobic residue, Leu198, are in the junction between SMP domains. **Insets II and III:** Asn199 residues appear facing a β-strand of their respective SMP domains. Crystal structure modified from Schauder et al., 2014.
Chapter III: The search for MCS tethers

The conserved Trp and the juxtaposed hydrophobic residue on the crystal structure of ESyt2 (Trp197 and Leu198) are also at the junction between SMP domains (Schauder et al., 2014) (Figure 3.8, Inset I), suggesting that they may be important for stabilizing the interaction of SMP domains. However, the position of the conserved Asn in ESyt2 (Asn199), facing a β-strand within the structure of the SMP domain (Figure 3.5, Inset II and III), do not seem to support a role in tether dimerization by SMP interaction, but SMP folding instead. The conservation of the Trp and its juxtaposed hydrophobic residue in yeast and mammals (Figure 3.5) and their position at the junction between SMP domains in yeast Mmm1 and mammalian ESyt2 tethers (Jeong et al., 2017) (Figure 3.8) suggest that the hydrophobic effect of these two residues and especially, the steric properties of the Trp, are important for the dimerization and for the lipid-transfer properties of SMP-tethers in yeast and mammal. It is unclear, however, what is the function of the conserved Asn.

Remarkably, the conservation of the Trp128, Asn130 and Gly186 residues can be expanded to the SMP domains of the NTMC2 family proteins, as shown in Figure 3.9. Given the amino acid conservation in the N-terminus of eukaryotic SMP domains I hypothesize that the Arabidopsis NTMC2 proteins may be able to dimerize and transfer lipids following the same mechanisms described for yeast and mammal SMP-

Figure 3.9: Alignment of the consensus SMP and the SMP domains of the MCS tethers and Arabidopsis NTMC2 proteins. Residues are coloured based on the percentage of identity, where bluer residues have a higher identity among the sequences; only residues with a minimum of 60% of identity are coloured. Red squares and arrows show residues conserved in all sequences while the yellow square and arrow show the hydrophobic residues conserved at position 129. Numbers indicate residue position within the alignment of the SMP domains. ESyt: Human extended synaptotagmin; Tcb: yeast tricalbin; Mmm1: yeast maintenance of mitochondrial morphology 1; Nvj2p: yeast nucleus-vacuole junction 2 protein.
tethers (Jeong et al., 2017; Schauder et al., 2014). This idea is further supported by co-immunoprecipitation analyses using the Arabidopsis SYT1 as a bait, indicating that SYT1 can form homodimers and heterodimers with SYT5 and AtCLB (appendix D).

3.2.4. Putative tether expression patterns suggest functions in abiotic and biotic stresses

The study of the putative tether expression indicated that AtCLB has expression levels similar to the ones of SYT1, while SYTF and 2TMCLB have lower expression levels (Table 3.2 and Figure 3.6). This may suggest that SYTF and 2TMCLB function primarily in response to certain stimuli and could be upregulated by such or that they need a lower protein turn-over with respect to SYT1 and AtCLB. Although the three putative tethers are ubiquitously expressed, SYTF and 2TMCLB have more specific expression patterns compared to AtCLB (Table 3.2) reinforcing the idea of a more tissue-specific function while AtCLB may have a more general function since it is highly expressed in all tissues and developmental stages (Table 3.2; Figure 3.6). SYT1 and AtCLB have their highest expression in dry and germinating seeds (Table 3.2). This correlates with an increase of their expression in senescent tissues, specifically dry seeds (Figure 3.6), suggesting that AtCLB function might be especially important in that process.

The study of the expression changes of the putative tethers under different stresses and treatments indicated different up and downregulation patterns for AtCLB, on one hand, and SYTF and 2TMCLB, on the other hand (Figure 3.7). AtCLB is downregulated in abiotic stress treatments and after the inoculation of Arabidopsis leaves with ABA (Figure 3.7). ABA is a phytohormone whose levels increase during abiotic stress responses (de Zelicourt et al., 2016); therefore, AtCLB might work in the negative regulation of abiotic stress tolerance. AtCLB is also downregulated in bacteria-related biotic stress treatments (Figure 3.7). Interestingly, SYT1 plays a role in biotic stress as negative regulator of cell-to-cell virus movement (Uchiyama et al., 2014). The downregulation of AtCLB under biotic stresses, although only in bacterial-related infections, suggests that AtCLB may also have a role in the negative regulation of biotic stresses. AtCLB is downregulated under inhibitions of ethylene. The higher expression levels of AtCLB in seeds and
the function of ethylene in the promotion of germination in dormant seeds support that AtCLB might be especially important in that developmental stage (Table 3.2) (Corbineau et al., 2014). In addition, the downregulation of AtCLB after incubation with ABA agreed with the antagonist function of ABA and ethylene in seed dormancy (Figure 3.7) (Corbineau et al., 2014). Considering this, AtCLB might have roles in the positive regulation of seed dormancy, being positively regulated by ethylene and negatively regulated by ABA.

SYTF and 2TMCLB are both highly upregulated in abiotic stress treatments. In addition, they are strongly downregulated after inoculation of ABA (Figure 3.7). Given the role of ABA as general modulator of abiotic stress responses (de Zelicourt et al., 2016), it is plausible that SYTF and 2TMCLB have functions in the abiotic stress signalling pathway, working upstream of ABA and being regulated by it through a negative feed-back. The additional function of ABA in leaf senescence (Zhang et al., 2012) could also indicate that SYTF and 2TMCLB, genes that exhibit a peak of expression in mature leaves (Table 3.2), might function as negative regulators of leaf senescence, being downregulated by ABA once the senescence process starts.

SYTF is also upregulated is some biotic stress treatments: it is upregulated after incubation with salicylic acid, a phytohormone involved in the response to biotic stress, and infection with Phytophthora infestans, an eukaryote pathogen (Figure 3.7). However, it is not upregulated after infection with Pseudomonas syringae or incubation with bacterial-derived elicitors, suggesting that SYTF may be working in the response against specific pathogens. Inhibition of ethylene, a phytohormone involved in senescence, upregulates SYTF. Considering that SYTF exhibit a higher expression in senescent internodes and mature leaves (Table 3.2), this data suggests a role for SYTF in senescence. However, ethylene was shown to work in later stages of leaf development rather than in seedlings (Fischer, 2012), where SYTF is also highly expressed (Table 3.2) suggesting that SYTF may have different functions in different tissues. On the other hand, ethylene can also break dormancy in seeds, working antagonistically with ABA and
inhibiting its synthesis (Corbineau et al., 2014). *SYTF* is highly expressed in seeds and germinating seeds (Table 3.2) suggesting it may function with ethylene in the negative regulation of seed dormancy.

Finally, *2TMCLB* is downregulated after the inhibition of GA synthesis. *2TMCLB* has a peak of expression in seedling hypocotyls and internodes, where cell elongation is promoted (Daviere and Achard, 2013), suggesting a role for *2TMCLB* downstream of GA, acting in cell elongation. Salicylic acid also causes *2TMCLB* downregulation. Accumulation of SA in Arabidopsis plants results in a dwarf phenotype while plants with mutations in SA synthesis have an elevated growth rate (Rivas-San Vicente and Plasencia, 2011). These data suggest an antagonistic role for *2TMCLB* and salicylic acid in cell elongation. However, *2TMCLB* also has a peak of expression in senescent internodes, implying a more complex role for *2TMCLB* in different tissues and developmental stages.
Chapter IV: Characterization of the putative tethers

4.1. Results

4.1.1. AtCLB and SYTF show a “beads and strings” pattern at the cortical ER

An important characteristic of MCS tethers is their dual localization in the membranes that are bridged by them. To determine whether the localization of the MCS candidates identified in this study is compatible with a bridging function we performed subcellular localization analyses using confocal microscopy. For that purpose, we generated transgenic Arabidopsis lines harbouring translational fusions of the putative tethers with the green fluorescent protein (GFP). Since the putative tethers have predicted transmembrane domains at the N-terminus (figure 3.4) we attached the GFP tag on the C-terminus of the proteins to minimize protein mislocalization. Due to the low expression levels of SYTF and 2TMCLB (Table 3.2; Figure 3.6) all the constructs were driven by a constitutive promoter (UBI10) that ensures a strong ectopic expression of Arabidopsis genes (Grefen et al., 2010). The Arabidopsis database TAIR was used to identify splice variants of the putative tethers. As a result, no splice variants were identified for 2TMCLB and SYTF and a single splice variant, lacking the last intron and a small part of the 3’-UTR was detected for AtCLB (At3g61050.2) (Figure 4.6a, striped box and dashed line).

To assess the subcellular localization of the markers at the cortical ER we compared their localization profiles to those of C4H-GFP, a well-established ER-membrane marker (Ro et al., 2001), and pSYT1:SYT1-GFP (hereafter, SYT1-GFP), a previously described EPCS marker (Pérez-Sancho et al., 2015).

As shown in Figure 4.1a, C4H-GFP fluorescence showed the typical ER-membrane localization pattern with clear reticulate structures in the cortical region of epidermal cells, a continuous signal in equatorial regions, and strong fluorescence in the transvacuolar strands (Figure 4.1, white arrows). SYT1-GFP localized as a network of beads and strings in the cortical region of epidermal cells and a punctate and
Chapter IV: Characterization of the putative tethers

Chapter IV: Characterization of the putative tethers

A discontinuous signal in the equatorial regions. A weak fluorescent SYT1-GFP signal was also visible in transvacuolar strands (Figure 4.1b). The subcellular localization analyses of the tether candidates show that the pattern of UB10:AtCLB-GFP (hereafter, AtCLB-GFP) resembles that of SYT1-GFP marker, as it shows open, reticulate structures formed by puncta and tubules in the cortical regions of epidermal cells. In the equatorial plane, a clear punctate pattern and weak transvacuolar strand signals were observed (Figure 4.1c). Similarly, UB10:SYTF-GFP (hereafter, SYTF-GFP) had a beads and strings pattern in the cortical region of epidermal cells while showing a punctate pattern in the equatorial region. Like for AtCLB-GFP, transvacuolar strands were occasionally visible in seedlings expressing SYTF-GFP (Figure 4.1d).

Remarkably, the subcellular localization of UB10:2TMCLB-GFP (hereafter 2TMCLB-GFP) resembled that of the C4H-GFP marker with completely closed reticulate structures in cortical regions of epidermal cells, and strong labelling of the transvacuolar strands (Figure 4.2b), as also seen for C4H-GFP (Figure 4.2a). In equatorial regions, 2TMCLB-GFP showed a continuous signal (Figure 4.2b), distinct from the one observed in AtCLB-GFP and SYTF-GFP (Figure 4.1b, c, d) but similar to that of C4H-GFP (Figure 4.2a).

Figure 4.1: Subcellular localization of C4H-GFP, SYT1-GFP, AtCLB-GFP and SYTF-GFP. a, C4H-GFP. b, SYT1-GFP. c, AtCLB-GFP. d, SYTF-GFP. Upper panels: cortical region; bottom panels: equatorial region. White arrows indicate transvacuolar strands. White squares show 2x zoom-in regions. Pictures taken in epidermal cells of 5-day-old cotyledons. Scale bars = 10µm.
4.1.2. SYT1 and AtCLB reticulations increase under salt stress

Due to time limitations for a M.Sc. project, I focused my efforts on characterizing AtCLB, the most promising candidate identified in the BLAST search using the consensus SMP sequence (see Chapter 3). AtCLB localization resembles that of SYT1, AtCLB has the highest expression levels among the three NTMC2 putative tethers analyzed (Table 3.2) and co-immunoprecipitation experiments from a collaborator identified AtCLB as a potential SYT1 interactor (appendix D).

SYT1 is an EPCS tether required for NaCl stress tolerance (Pérez-Sancho et al., 2015; Schapire et al., 2008) and our preliminary AtCLB expression analysis indicates that AtCLB is downregulated under salt and osmotic stress (Figure 3.7). Thus, I hypothesized that AtCLB may also be involved in NaCl stress
tolerance and that NaCl stress could cause changes in SYT1-GFP and AtCLB-GFP subcellular localization. To test this hypothesis, I performed localization analyses in Arabidopsis seedlings expressing SYT1-GFP and AtCLB-GFP subjected to 100mM NaCl stress. My results show that after 16-hours of incubation the characteristic “beads and strings” GFP signal pattern shown in mock conditions changes to a well-defined cortical reticulated pattern, indicative of an increase in EPCSs (Figure 4.3). Next, I determined whether this effect was caused by Na$^+$ or Cl$^-$ toxicity or by a more general response to ion toxicity. For this purpose, I treated Arabidopsis seedlings with K$_2$SO$_4$, an ion stress generator that does not contain Na$^+$ or Cl$^-$ ions. The treatment with K$_2$SO$_4$ shows that SYT1-GFP and AtCLB-GFP display a closed reticulated pattern similar to the one obtained after NaCl treatment (Figure 4.3). Further treatments with other salts, including KCl, MgCl$_2$ and Na$_2$SO$_4$, showed closed reticulated patterns similar to the ones observed in response to NaCl and K$_2$SO$_4$ treatments (appendix E).

Since NaCl stress has both ionic and osmotic components (Zhu, 2016), I also tested whether the signal distribution change observed in SYT1-GFP and AtCLB-GFP was specific for ionic stresses. To answer this question, I treated the SYT1-GFP and AtCLB-GFP lines with sorbitol, a chemical that generates

![Figure 4.3: Subcellular localization of SYT1-GFP and AtCLB-GFP under NaCl, K$_2$SO$_4$ and sorbitol stress. White squares show 2x zoom-in regions. Pictures taken in the cortical region of epidermal cells of 5-day-old cotyledons. Scale bars = 10µm.](image_url)
osmotic stress without the ionic component. As result of the treatment, SYT1-GFP and AtCLB-GFP showed a “beads and strings” pattern, that strongly resembles that of the untreated controls (Figure 4.3). To provide quantitative values to the observed phenotypes, I quantified the number of closed reticulations in the cortical regions of SYT1-GFP and AtCLB-GFP epidermal cells after different treatments. The ionic stress generators NaCl and K$_2$SO$_4$ caused statistically significant increases in the number of closed reticulations, compared to the control, while the osmotic stress generator sorbitol showed no significant increase in the number of closed reticulated structures for both SYT1-GFP and AtCLB-GFP markers (Figure 4.4).

Once I established that general ionic stress induces the expansion of the cortical SYT1-GFP and AtCLB-GFP signals, I asked whether the underlying ER morphology was also affected by NaCl stress. To answer this question, I used transgenic plants expressing the luminal ER marker HDEL-GFP and studied changes in localization at the cell cortex using different concentrations of NaCl. The reticulated HDEL-GFP network observed in untreated conditions was expanded upon 100mM NaCl treatment, as indicated by the larger area of its polygonal structures (Figure 4.5). Remarkably, the reticulate structures did not have
Chapter IV: Characterization of the putative tethers

A homogenous expansion in response to NaCl treatment, as a fraction of small area polygons (approximately 30%) were still present in NaCl treated seedlings (Figure 4.5). Together, these results indicate that ionic stress changes both cortical ER morphology and EPCS distribution in Arabidopsis seedlings.

**Figure 4.5: Distribution of HDEL-GFP reticulated structures area under NaCl stress.** Numbers on the x-axis refer to individual reticulated structures out of 285 total measured; the reticulations were organized from widest to narrowest within each treatment. The y-axis refers to the area in µm² of each individual reticulate structure. Pictures shown are representative of the reticulate expansion in different treatments. Scale bars = 2µm.

4.1.3. *atclb1-3* does not show Ca²⁺-mediated NaCl stress phenotypes

In a previous report, it was shown that the loss-of-function mutant *syt1-2* was hypersensitive to NaCl stress under low Ca²⁺ conditions (Schapire et al., 2008). Because both SYT1-GFP and AtCLB-GFP display similar changes in subcellular localization upon NaCl stress (Figure 4.3), I asked whether the loss-of-function of AtCLB also induce NaCl hypersensitive phenotypes.

To address this question, I obtained a T-DNA mutant line, *atclb1-3*, harbouring an insertional mutation in the eighth exon (Figure 4.6a) and used an additional allele, *atclb1-1*, that harbours a T-DNA
Chapter IV: Characterization of the putative tethers

insertion in the tenth intron, which was proposed to confer salt tolerance through an unknown mechanism (Figure 4.6a) (De Silva et al., 2011). First, the AtCLB expression levels in both mutants were measured using quantitative RT-PCR. atclb1-3 displayed a severe reduction in AtCLB mRNA levels (up to 90%), while atclb1-1 showed only a slight reduction in AtCLB mRNA accumulation (10 to 20%) compared to WT plants (Figure 4.7). Based on this result, I selected atclb1-3 as the more suitable mutant allele to perform further phenotypic characterization.

To assess the Ca²⁺-dependent NaCl sensitivity of atclb1-3, mutant seedlings were germinated and grown on 1/10 MS medium for 5 days and then transferred to the same medium supplemented with different concentrations of NaCl, CaCl₂ or NaCl+CaCl₂, and root elongation measurements were performed 4 days after transfer.
after the transfer. In this experiment, Col-0 (WT) and syt1-2 seedlings were used as negative and positive controls, respectively. Figure 4.6b shows no difference in root elongation between WT and mutant seedlings under control conditions. Under NaCl treatments, WT, syt1-2 and atclb1-3 root growth were reduced. As expected, this effect was stronger for syt1-2 but no differences between the root growth of WT and atclb1-3 seedlings were observed. CaCl$_2$ supplementation did not influence the root growth when applied alone, but it alleviated the NaCl-induced growth reduction. Thus, in agreement with Schapire et al., (2008), the addition of CaCl$_2$ rescued the hypersensitivity to salt stress of syt1-2. Altogether, our results show no significant difference between atclb1-3 and WT seedlings in root length, suggesting that redundancy for AtCLB function might exist (Figure 4.6c).

![Figure 4.7: Relative AtCLB mRNA abundance in atclb1-1 and atclb1-3 mutants.](image_url)

10-days-old seedlings, measured by qRT-PCR and analyzed by $2^{-\Delta\Delta C_T}$ method. Abundance relative to Col-0 expression, using ACT2 as housekeeping. Data shows means ± SD of 2 biological replicates with 3 technical replicates each.
4.1.4. Depletion of cytosolic Ca$^{2+}$ increases SYT1 and AtCLB puncta pattern

In mammalian cells, the Extended Synaptotagmin 1 (ESyt1) tether accumulates on EPCS in response to increases in [Ca$^{2+}$]$_{cyt}$ in a process that might be used to coordinate lipid transfer in response to stress (Giordano et al., 2013; Yu et al., 2016). In plants, AtCLB and SYT1 share EPCS localization and both contain putative Ca$^{2+}$-dependent phospholipid binding C2 domains (Figure 3.4) (Pérez-Sancho et al., 2015; Schapire et al., 2008). I hypothesized then that Ca$^{2+}$ could be an important factor in SYT1 and AtCLB distribution at EPCS. To test this hypothesis, I used pharmacological approaches to assess the putative role of Ca$^{2+}$ in the subcellular localization of AtCLB and SYT1 and the subsequent organization of the plant EPCS.

Figure 4.8: Subcellular localization of SYT1-GFP and AtCLB-GFP after depletion of Ca$^{2+}$cyt by EGTA and LaCl$_3$. White squares show 2x zoom-in regions. Pictures taken in the cortical region of epidermal cells of 5-day-old cotyledons. Scale bars = 10µm.
In these experiments, I incubated stable transgenic lines expressing either SYT1-GFP or AtCLB-GFP in liquid media supplemented with either ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, EGTA, a membrane permeable chelator which would sequestrate $[\text{Ca}^{2+}]_{\text{cyt}}$ (Nakagawa et al., 2007), or LaCl$_3$, a plasma membrane Ca$^{2+}$ channel blocker which would prevent the influx of apoplastic Ca$^{2+}$ into the cytosol (Knight, 1996). My results show that, upon EGTA treatment, the SYT1-GFP and AtCLB-GFP subcellular localization in the cell cortex evolves into a denser pattern, with fewer tubules and more punctate structures (Figure 4.8). This effect on SYT1-GFP and AtCLB-GFP localization was even more pronounced when the entry of Ca$^{2+}$ into the cytosol via Ca$^{2+}$ channel was blocked by LaCl$_3$ treatments (Figure 4.8). The number of puncta by unit area at the cell cortex was taken as a proxy for the quantification of SYT1-GFP and AtCLB-GFP changes in subcellular localization under $[\text{Ca}^{2+}]_{\text{cyt}}$ depletion. The quantification and subsequent statistical analyses show significant increases on the cortical SYT1-GFP and AtCLB-GFP signals under EGTA- or LaCl$_3$-induced Ca$^{2+}$ depletion, but no significant differences between SYT1-GFP and AtCLB-GFP distribution were found in any of the treatments tested (Figure 4.9).

Figure 4.9: Quantification of puncta number of SYT1-GFP and AtCLB-GFP in cytosolic Ca$^{2+}$ depletion treatments. Numbers indicate means ± standard deviation. Bars with different letters are significantly different (p<0.001) by two-ways ANOVA followed by LSD test.
4.1.5. SYT1 and AtCLB subcellular pattern expands after increases of PtdIns(4,5)P\(_2\) at the plasma membrane

In Arabidopsis, NaCl stress induces the accumulation of negatively charged lipids, such as PA and PtdIns(4,5)P\(_2\) at the plasma membrane (DeWald et al., 2001; Meijer et al., 2001; Singh et al., 2015; Zhang et al., 2004), and the expansion of SYT1-GFP and AtCLB-GFP labelled EPCS. Since SYT1 and AtCLB binding to the PM is predicted to occur through the C2 phospholipid binding domains, I hypothesized that the increase in negatively charged lipids at the PM upon NaCl stress could be responsible for the SYT1- and AtCLB-mediated EPCS expansion. A prediction derived from this model is that the accumulation of negatively charged phosphoinositides at the PM should mimic the effect of NaCl stress on EPCS expansion. To test this prediction, we used a pharmacological approach and treated plants expressing SYT1-GFP and AtCLB-GFP with a liquid medium containing U73122, an amino steroid that blocks the PLC activity, effectively increasing the amount of PtdIns(4,5)P\(_2\) at the PM (Parre et al., 2007) (Figure 4.10a).

Figure 4.10: Subcellular localization of SYT1-GFP and AtCLB-GFP after PLC inactivation by U73122. a, schematic representation of IP\(_3\) pathway and U713122 inactivation of PLC. b, localization pattern of SYT1-GFP and AtCLB-GFP in the cortical region of epidermal cells of 5-days-old cotyledons after U73122 treatment. White squares show 2x zoom-in regions. Scale bars = 10µm.
Chapter IV: Characterization of the putative tethers

Previous unpublished experiments in our lab used the fluorescent sensor CITRINE-2xPH$_{PLC}$, which specifically binds to PtdIns(4,5)P$_2$ (Simon et al., 2014) to show that PtdIns(4,5)P$_2$ accumulates 3-4 fold in response to NaCl stress and U73122 treatments (appendix F). In terms of subcellular localization, the U73122 treatment induced the expansion of the SYT1-GFP and AtCLB-GFP EPCS, generating a reticulated pattern that closely resembles that obtained in response to NaCl treatment (Figure 4.10b). The pattern changes were quantified, taking into consideration the increase in closed reticulated structures. SYT1-GFP and AtCLB-GFP significantly increase the number of closed reticulated structures in U73122 treatment compared to control conditions. Non-significant differences were found between SYT1-GFP and AtCLB-GFP. Non-significant differences in closed reticulations number were found between NaCl and U73122 treatments (Figure 4.11).

![Figure 4.11: Quantification of closed reticulate structures of SYT1-GFP and AtCLB-GFP under U73122 treatment. NaCl quantification is shown for comparison. Numbers indicate means ± standard deviation. Bars with different letters are significantly different (p<0.001) by two-ways ANOVA followed by LSD test.](image-url)
4.2. Discussion

4.2.1. AtCLB and SYTF subcellular distribution patterns resemble SYT1 and suggest EPCS localization

The analyses of the GFP-tagged putative MCS tethers localization in epidermal cells revealed that the subcellular patterns of AtCLB-GFP and SYTF-GFP resemble the one of SYT1-GFP (Figure 4.1) (Pérez-Sancho et al., 2015). These results suggest that AtCLB and SYTF are EPCS localized tethers. Additionally, co-immunoprecipitation experiments from a collaborator found AtCLB as interactor of SYT1 (appendix D), reinforcing the idea of AtCLB as EPCS tether. Recent research studying reticulons, proteins involved in the ER curvature, found that the plasmodesmata-localized reticulon proteins RTNLB3 and RTNLB6 interact with SYT1 and AtCLB (Kriechbaumer et al., 2015). SYT1 is also present in plasmodesmata-localized EPCS, supporting the presence of AtCLB at EPCSs (Bayer et al., 2017). In contrast, AtCLB was previously reported as a nuclear-localized protein by immunolocalization techniques, in contradiction with our results (De Silva et al., 2011). However, the authors did not identify AtCLB SMP domain and did not discuss the possible presence of a TM domain at the N-terminus region, incompatible with nuclear localization (Figure 3.4).

2TMCLB-GFP shows a reticulated pattern with intense signal in transvacuolar strands, resembling ER-membrane markers (Figure 4.2). This subcellular localization does not support 2TMCLB as EPCS tether. On the other hand, there are reports of MCS between the ER and other organelles in plants (Andersson et al., 2007; Mehrshahi et al., 2013; Mueller and Reski, 2015) and the SMP domain is present in tethers forming contact sites other than EPCS in yeast (Toulmay and Prinz, 2012), suggesting that 2TMCLB, as SMP-containing protein, might be a tether between the ER and other organelles. It is also important to consider that the ER-like, reticulated pattern of 2TMCLB could be due to the high expression caused by the UBQ10 promoter.
Chapter IV: Characterization of the putative tethers

Thus, further studies of the putative tethers driven by their native promoters and co-localization with EPCS and organelle markers are needed to confirm not only AtCLB and SYTF EPCS localization but also 2TMCLB ER-like pattern.

4.2.2. Decreases in cytosolic Ca\(^{2+}\) promote EPCS formation by SYT1 and AtCLB

The study of SYT1-GFP and AtCLB-GFP subcellular localization under depletion of \([\text{Ca}^{2+}]_{\text{cyt}}\) by EGTA or LaCl\(_3\) revealed an increase of GFP signal through the promotion of the puncta pattern, indicating that the decrease in \([\text{Ca}^{2+}]_{\text{cyt}}\) induces the formation of new EPCS (Figure 4.8; Figure 4.9). Previous studies in our lab have shown that ER morphology is greatly affected by LaCl\(_3\), although an obvious effect was not noticed for EGTA (appendix G). This suggests that strong depletion of cytosolic Ca\(^{2+}\) affects not only SYT1 and AtCLB localization but also ER morphology.

EPCS formation by the mammalian ESyt1 is promoted upon increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) (Giordano et al., 2013) and phosphoinositide binding by SYT1 C2 domains is also promoted by the presence of Ca\(^{2+}\) (Pérez-Sancho et al., 2015; Schapire et al., 2008). In sharp contrast, unpublished data from our group did not show an increase in EPCS formation by SYT1-GFP upon increase of \([\text{Ca}^{2+}]_{\text{cyt}}\) by addition of exogenous CaCl\(_2\) or by treatments with thapsigargin, a drug that blocks Ca\(^{2+}\) uptake by the ER and activates SOCE (store-operated Ca\(^{2+}\) entry) (appendix H) (Giordano et al., 2013). Thapsigargin has been successfully used in mammals (Giordano et al., 2013), but plants have no clear homologues of SOCE components (Kudla et al., 2010). Instead, thapsigargin partially blocks plant Ca\(^{2+}\) ATPases and heavy-metal co-transporters but does not affect Ca\(^{2+}\) uptake by ER vesicles (Moreno et al., 2008; Ordenes et al., 2002). This may suggest that the \([\text{Ca}^{2+}]_{\text{cyt}}\) increase caused by thapsigargin in plants may not be sufficient to trigger a response in SYT1 and AtCLB and an increase in EPCS formation by them.

Together, these data suggest that EPCS promotion under depletion of \([\text{Ca}^{2+}]_{\text{cyt}}\) could cause a change in ER morphology, bringing the ER closer to the plasma membrane. It could also suggest that depletion of \([\text{Ca}^{2+}]_{\text{cyt}}\) change ER morphology which in turn promotes the formation of new EPCS. However, the role of
Ca\(^{2+}\) in plant EPCS formation requires further clarification. Experimental controls would be needed in future studies to assure that the addition of exogenous CaCl\(_2\) and the thapsigargin treatment are actually causing an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) in plant cells.

### 4.2.3. AtCLB is not required for plant tolerance to NaCl stress

*syt1-2* loss-of-function insertional mutant is hypersensitive to NaCl stress in low Ca\(^{2+}\) conditions (Schapire et al., 2008). Both *SYT1* and *AtCLB* are ubiquitously expressed and both *AtCLB* and *SYT1* belong to the NTMC2 superfamily and share similar domain distribution. Although *AtCLB* is downregulated by salt stress, our phenotypic analyses did not find any differences between WT seedlings and *atclb1-3* loss-of-function insertional mutant seedlings under NaCl stress treatments supplemented with different Ca\(^{2+}\) concentrations.

Previously, *AtCLB* was reported to be a nuclear-localized C2-containing protein, working as transcription factor of the thalianol synthase gene and acting as negative regulator of root length and NaCl tolerance (De Silva et al., 2011). However, contrary to these previous reports, *atclb1-1* insertional mutation is not located in the tenth exon of *AtCLB* but in the previous intron (Figure 4.6a). As a result, qRT-PCR analyses demonstrated that *atclb1-1* mutant has *AtCLB* expression levels resembling WT plants (Figure 4.7), suggesting that the phenotypes observed by the authors may be due to a second insertion, instead of by reduced amounts of *AtCLB*.

One of the difficulties in the functional characterization of contact site tethers is the redundancy among their sequences (Pérez-Sancho et al., 2016) which may cause functional redundancy. Additionally, contact site tethers seem to be involved in several functions, from lipid and Ca\(^{2+}\) homeostasis to organelle dynamics (Prinz, 2014); specifically, *AtCLB* expression is regulated by abiotic and biotic stresses as well as phytohormones signalling (Figure 3.7), suggesting that *AtCLB* might have more complex functions not only related to NaCl stress.
To further clarify the role of AtCLB in NaCl stress, functional characterization of double mutant plants in *SYT1* and *AtCLB* would be needed. Also, it would be interesting to study the sensitivity to salt stress of triple mutant plants in *SYT1*, *AtCLB* and *SYT5*, since they three are ubiquitously expressed (Table 3.2; appendix A), can interact in vitro (appendix D), and have a similar “beads and string” pattern at the cell cortex (Figure 4.1; data not shown for SYT5).

### 4.2.4. Salt stress and PtdIns(4,5)P₂ increases induce SYT1 and AtCLB EPCS and reduce intermembrane distance

I have demonstrated that salt stress, but not osmotic stress, changes SYT1-GFP and AtCLB-GFP subcellular distribution from a beads and string network into a more closed reticulated network almost lacking puncta (Figure 4.3; Figure 4.4). I have showed that NaCl stress also affects ER morphology, expanding the normal reticulate pattern of the ER-luminal marker GFP-HDEL (Figure 4.5). Interestingly, the partial co-localization of SYT1-RFP and GFP-HDEL in normal conditions increased upon NaCl stress (appendix I). Unpublished experiments in our group used the artificial EPCS marker MAPPER (Chang et al., 2013), made with a ER-luminal GFP, the N-terminal TM domain of the mammalian STIM1 and a cytosolic C-terminal polybasic domain, which binds to phosphoinositides at the plasma membrane, to study the ER-PM intermembrane distance in plants. MAPPER showed a EPCS-like puncta and tubular pattern under normal conditions, but it did not completely co-localize with SYT1, reinforcing the idea that different EPCS exist is plants (appendix J, a) (Siao et al., 2016). MAPPER localization under salt stress resembled those of SYT1 and AtCLB (appendix J, b). Additionally, and likewise SYT1-GFP, MAPPER co-localization with RFP-HDEL increased during NaCl stress (appendix J, c), suggesting that NaCl is promoting EPCS formation by decreasing the distance between the ER and the plasma membrane.

On the other hand, an increase in PtdIns(4,5)P₂ concentration by blockage of PLC changed AtCLB and SYT1 subcellular distribution, mimicking the results obtained in salt stress treatments (Figure 4.10;
Figure 4.11). Similar results were obtained using MAPPER, suggesting that an increase in PtdIns(4,5)P\textsubscript{2} promotes EPCSs formation and reduce the intermembrane distance.

Thus, I hypothesize that salt stress facilitates EPCS formation by promoting the interaction of SYT1 and AtCLB C2 domains and PtdIns(4,5)P\textsubscript{2} located at the plasma membrane. The EPCS promotion could be required for non-vesicular replenishment of PM-localized phosphoinositides such as PtdIns, which are important for PLC signalling, as already discovered for mammalian SMP-EPCS tethers (Lees et al., 2017; Yu et al., 2016). Interestingly, this lipid-transfer function would only be required under ionic stresses, since AtCLB-GFP and SYT1-GFP subcellular distribution is not affected by non-ionic hyperosmotic stress. The specificity of this putative function is supported by a report showing that the accumulation of proline, a well-known compatible osmolyte, under ionic stress requires Ca\textsuperscript{2+} signaling and PLC activity (Parre et al., 2007). However, PLC signaling was not required for proline accumulation in non-ionic hyperosmotic stress, suggesting that the lipid signaling pathway could discriminate between ionic and non-ionic stresses (Parre et al., 2007).

This hypothesis would suggest an exciting model where EPCS formation might be promoted not only by the presence of negatively charged phosphoinositides at the PM, but also by increases in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to external stimuli such as ionic stress. Further studies are required to explore the possibility of non-vesicular lipid-transfer function of SMP-proteins in plants and how this function may be integrated in the EPCS promotion by salt stress.
Chapter V: Conclusions

MCS are subcellular microdomains conserved in all eukaryotes where the membranes of two organelles are in close apposition thanks to protein tethers, which have diverse functions, such as mediating Ca\textsuperscript{2+} homeostasis and non-vesicular lipid transport (Prinz, 2014). Although multiple tethering complexes have been characterized in yeast and mammals, little is known about contact site tethers in plants (Pérez-Sancho et al., 2016). Previous work in our lab identified the Arabidopsis EPCS tether SYT1 as component required for Ca\textsuperscript{2+}-dependent responses to NaCl stress (Pérez-Sancho et al., 2015; Schapire et al., 2008). In this study, I aimed at expanding the knowledge of the molecular components involved in MCS establishment in plants using bioinformatics and cell biology approaches. The research objectives of this thesis are:

1. Identification of novel membrane contact sites tethers in Arabidopsis thaliana.
2. Functional characterization of the putative tethers in salt stress responses mediated by Ca\textsuperscript{2+}.

As a tool to identify contact site tethers in the model organism Arabidopsis thaliana, I used the SMP domain, a lipid-binding domain present in MCS tethers in eukaryotes (Toulmay and Prinz, 2012). I created a SMP consensus sequence by aligning the SMP sequence of experimentally demonstrated MCS tethers and used it as query in BLAST searches against the Arabidopsis database. This approach enabled the identification of AtCLB, an SMP-containing protein and a putative MCS tether. AtCLB belongs to the NTMC2 protein family, which comprises the already known Arabidopsis SYT family and a group of unknown proteins. Two of those proteins, SYTF and 2TMCLB, were also selected as putative tether candidates. The subsequent analyses of the three candidates showed that the SMP consensus approach was too restrictive in the search of MCS tethers as SYTF, a member of the NTMC2 family not identified in the bioinformatic approach, was indeed localized at MCSs. The SMP consensus strategy was useful to identify AtCLB, an EPCS component also identified by unpublished co-immunoprecipitation experiments using
Chapter V: Conclusions

SYT1 as bait (appendix D). All proteins in the NTMC2 family possess a predicted TM domain at the N-terminus, an internal SMP domain and, at least one C-terminal C2 domain. The domain distribution of these proteins, resembling known eukaryotic MCS tethers (Giordano et al., 2013; Pérez-Sancho et al., 2015; Toulmay and Prinz, 2012) and the presence of a SMP domain (Toulmay and Prinz, 2012) suggest that the unknown members of the newly discovered NTMC2 family might be contact site tethers. This would require further study using light and electron microscopy approaches to test whether these new SMP-containing proteins are MCS tethers. The study of the SMP domain across eukaryotes revealed highly conserved amino acid residues. One of these residues, a tryptophan located at the N-terminus of the SMP domain, has been identified as important for SMP interaction and protein dimerization (Jeong et al., 2017). This dimerization is essential for the non-vesicular transfer of lipids between membranes in mammalian and yeast cells and the residue conservation in plants suggest that NTMC2 proteins might also dimerize through SMP domain interactions and could be involved in non-vesicular lipid transfer. Future experiments would need to test the lipid-transfer function of plant EPCS tethers and, using directed mutagenesis, study the importance of the conserved residues in the tethers’ function (Figure 3.9).

In Chapter IV, the study of transgenic plants expressing AtCLB, SYTF and 2TMCLB translationally attached to fluorescent tags revealed that AtCLB and SYTF had a subcellular distribution resembling that of the well-characterized EPCS tether SYT1, while 2TMCLB subcellular distribution was similar to that of the ER marker C4H-GFP. It is important to mention that the AtCLB, SYTF and 2TMCLB GFP fusions were driven by a strong promoter (UB10) that might generate localization artifacts. Therefore, experiments using transgenic plants expressing translational fusions of the putative tethers driven by their native promoter are required to confirm their MCS localization. The subcellular localization of AtCLB, SYTF and 2TMCLB suggests that the SMP consensus sequence, mostly made of yeast and mammalian EPCS tethers, is useful to identify EPCS tethers but might not be a good approach to identify putative tethers involved in the contact of the ER with other organelles.
Concerning these next steps in the project, I have already generated transgenic plants expressing GFP-tagged translational reporters of AtCLB, SYTF and 2TMCLB, driven by their native promoters, to further study the MCS localization of the tethers. I have also generated transgenic plants expressing AtCLB-RFP, SYTF-RFP or 2TMCLB-RFP and SYT1-GFP or GFP-HDEL, as a first step in the co-localization analyses that will further verify the MCS localization of the putative tethers.

In this study, I further showed that depletions of $[\text{Ca}^{2+}]_{\text{cyt}}$ increase the puncta subcellular pattern of SYT1 and AtCLB. Conversely, high cytosolic $\text{Ca}^{2+}$ has been reported to increase EPCSs formation by the mammalian ESyt1 (Giordano et al., 2013) and to promote in vitro phosphoinositide binding of SYT1 C2 domains (Pérez-Sancho et al., 2015; Schapire et al., 2008), although I have been unable to demonstrate an increase in plant EPCS formation in vivo after increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ by exogenous addition of CaCl$_2$ or pharmacological treatments with thapsigargin. Additionally, treatment with LaCl$_3$ also showed strong alterations in ER morphology. The question that remains to be addressed is whether the ER morphology changes are causing the EPCS formation or the sudden increase of ER-PM contacts are modifying ER morphology. Future experiments will reveal how the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ affects EPCS formation. Also, it would be interesting to study how ER morphology is affected in plants lacking EPCS tethers such as AtCLB, SYT1 and SYT5, to shed light on whether depletion of $[\text{Ca}^{2+}]_{\text{cyt}}$ affects ER in the absence of ER-PM tethers.

The results of the phenotypic analysis of the loss-of-function insertional mutant *atclbl-3* suggest that AtCLB is not essential for plant tolerance to salt stress. This can be due to functional redundancy, common in MCS tethers (Pérez-Sancho et al., 2016), or to the involvement of AtCLB in other processes, given AtCLB regulation in different treatments and the multiple roles of MCS tethers (Prinz, 2014). Since SYT1 can interact with SYT5 and AtCLB (appendix D) and these 3 SMP proteins are ubiquitously expressed, I hypothesized that they can form a complex at EPCS, regulating abiotic stress tolerance with SYT1 as the key player. Regarding the next steps in this project, I have generated the double mutants *atclbl-3 syt1* and *atclbl-3 syt5* and the triple mutant *atclbl-3 syt1/syt5*, which will be used in further salt stress
experiments to test the function of SYT5 and AtCLB in absence of SYT1 and how the lack of the three components of our putative EPCS complex affect salt stress tolerance.

Finally, my study shows that salt stress and increases in PtdIns(4,5)P\(_2\) produce changes in the SYT1-GFP and AtCLB-GFP subcellular distribution at the cell cortex that could be linked to EPCS expansions. The EPCS expansions mediated by PtdIns(4,5)P\(_2\) have been previously reported in mammalian cells (Giordano et al., 2013), suggesting that it is a conserved mechanism in eukaryotes. This discovery clarifies the regulation of EPCS formation in plants. However, it does not explain the purpose of EPCS formation. Non-vesicular lipid transfer is an exciting hypothesis, already demonstrated for mammalian SMP-proteins which would require further biochemical studies, mimicking the ones performed in mammals (Lees et al., 2017; Yu et al., 2016).

In summary, in this thesis I successfully identified two members of the NTMC2 family as putative EPCS tethers, demonstrating that the SMP domain can be used as a bioinformatic tool to identify MCS tethers in plants. In addition, I have identified highly conserved residues in the SMP domain of eukaryotes, which may be important for tether dimerization and function. I have also shown that the localization of SYT1 and AtCLB is modified by Ca\(^{2+}\), PtdIns(4,5)P\(_2\) and salt stress, although I have been unable to clarify the specific role of AtCLB in salt stress tolerance and signaling. Further studies are required to shed light on the function of the SMP-containing proteins AtCLB and SYT1 in salt stress tolerance and to identify putative MCS tethers among the newly discovered NTMC2 proteins.
Bibliography


Knight, H. (1996). Cold Calcium Signaling in Arabidopsis Involves Two Cellular Pools and
a Change in Calcium Signature after Acclimation. Plant Cell Online 8, 489–503.


Appendices

Appendix A: Expression level of NTMC2 genes according to RNAseq data

Table A (continue in next page): Expression levels of the NTMC2 family genes in different tissues and developmental stages. Values show absolute read counts. Higher expression values have greener colors while lower values have redder colors; intermediate values have whiter colors.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>SYT1</th>
<th>SYT2</th>
<th>SYT3</th>
<th>SYT4</th>
<th>SYT5</th>
<th>At3g18370 (SYT6/F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthers (Opened)</td>
<td>1976</td>
<td>654</td>
<td>608</td>
<td>11</td>
<td>853</td>
<td>517</td>
</tr>
<tr>
<td>Anthers (Mature flow.)</td>
<td>1214</td>
<td>2986</td>
<td>205</td>
<td>181</td>
<td>910</td>
<td>327</td>
</tr>
<tr>
<td>Carpels (Mature flow.)</td>
<td>3313</td>
<td>34</td>
<td>105</td>
<td>44</td>
<td>1237</td>
<td>803</td>
</tr>
<tr>
<td>Stamens filaments (Mature flow.)</td>
<td>6873</td>
<td>15</td>
<td>46</td>
<td>121</td>
<td>2588</td>
<td>1228</td>
</tr>
<tr>
<td>Petals (Mature flow.)</td>
<td>4678</td>
<td>18</td>
<td>54</td>
<td>71</td>
<td>1952</td>
<td>1759</td>
</tr>
<tr>
<td>Sepals (Mature flow.)</td>
<td>4519</td>
<td>17</td>
<td>176</td>
<td>59</td>
<td>1262</td>
<td>1464</td>
</tr>
<tr>
<td>Flower</td>
<td>3451</td>
<td>632</td>
<td>108</td>
<td>204</td>
<td>1147</td>
<td>1313</td>
</tr>
<tr>
<td>Internode</td>
<td>5050</td>
<td>18</td>
<td>111</td>
<td>174</td>
<td>1528</td>
<td>1324</td>
</tr>
<tr>
<td>Senescent internode</td>
<td>3553</td>
<td>2</td>
<td>1248</td>
<td>42</td>
<td>955</td>
<td>2522</td>
</tr>
<tr>
<td>Whole leaf (Mature)</td>
<td>5029</td>
<td>1</td>
<td>1572</td>
<td>44</td>
<td>998</td>
<td>2051</td>
</tr>
<tr>
<td>SAM at 7-d</td>
<td>4058</td>
<td>0</td>
<td>26</td>
<td>10</td>
<td>2386</td>
<td>992</td>
</tr>
<tr>
<td>Meristem at 10-d</td>
<td>6011</td>
<td>2</td>
<td>123</td>
<td>54</td>
<td>1898</td>
<td>1047</td>
</tr>
<tr>
<td>Inflorescence meristem at 15-d</td>
<td>3839</td>
<td>4</td>
<td>18</td>
<td>15</td>
<td>1125</td>
<td>571</td>
</tr>
<tr>
<td>Pod of the senescent silique</td>
<td>6432</td>
<td>2</td>
<td>647</td>
<td>55</td>
<td>2282</td>
<td>843</td>
</tr>
<tr>
<td>Pedicel</td>
<td>5876</td>
<td>8</td>
<td>63</td>
<td>91</td>
<td>1349</td>
<td>1968</td>
</tr>
<tr>
<td>Root without apex</td>
<td>3152</td>
<td>9</td>
<td>215</td>
<td>157</td>
<td>1137</td>
<td>858</td>
</tr>
<tr>
<td>Seedling cotyledons</td>
<td>6650</td>
<td>6</td>
<td>158</td>
<td>70</td>
<td>1096</td>
<td>1081</td>
</tr>
<tr>
<td>Seedling hypocotyl</td>
<td>6494</td>
<td>9</td>
<td>100</td>
<td>176</td>
<td>1246</td>
<td>2039</td>
</tr>
<tr>
<td>Seedling root</td>
<td>4454</td>
<td>10</td>
<td>83</td>
<td>179</td>
<td>1580</td>
<td>837</td>
</tr>
<tr>
<td>Dry seeds</td>
<td>8251</td>
<td>0</td>
<td>165</td>
<td>1047</td>
<td>2270</td>
<td>1965</td>
</tr>
<tr>
<td>Germinating seeds</td>
<td>7145</td>
<td>1</td>
<td>164</td>
<td>570</td>
<td>1684</td>
<td>3698</td>
</tr>
</tbody>
</table>
Table A: Expression levels of the NTMC2 family genes in different tissues and developmental stages. Values show absolute read counts. Higher expression values have greener colors while lower values have redder colors; intermediate values have whiter colors.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>At3g61050 (AtCLB)</th>
<th>At1g53590 (2TMCLB)</th>
<th>At3g14590</th>
<th>At1g50260</th>
<th>At3g19830</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthers (Opened)</td>
<td>1409</td>
<td>504</td>
<td>1315</td>
<td>908</td>
<td>2685</td>
</tr>
<tr>
<td>Anthers (Mature flow.)</td>
<td>2065</td>
<td>320</td>
<td>318</td>
<td>254</td>
<td>2892</td>
</tr>
<tr>
<td>Carpels (Mature flow.)</td>
<td>2209</td>
<td>661</td>
<td>69</td>
<td>266</td>
<td>402</td>
</tr>
<tr>
<td>Stamen filaments (Mature flow.)</td>
<td>2976</td>
<td>917</td>
<td>153</td>
<td>183</td>
<td>2359</td>
</tr>
<tr>
<td>Petals (Mature flow.)</td>
<td>2330</td>
<td>688</td>
<td>367</td>
<td>258</td>
<td>1288</td>
</tr>
<tr>
<td>Sepals (Mature flow.)</td>
<td>1937</td>
<td>749</td>
<td>304</td>
<td>820</td>
<td>830</td>
</tr>
<tr>
<td>Flower</td>
<td>2020</td>
<td>645</td>
<td>168</td>
<td>304</td>
<td>456</td>
</tr>
<tr>
<td>Internode</td>
<td>1983</td>
<td>1796</td>
<td>462</td>
<td>170</td>
<td>773</td>
</tr>
<tr>
<td>Senescent internode</td>
<td>2436</td>
<td>2900</td>
<td>1680</td>
<td>448</td>
<td>1322</td>
</tr>
<tr>
<td>Whole leaf (Mature)</td>
<td>2081</td>
<td>1574</td>
<td>324</td>
<td>616</td>
<td>618</td>
</tr>
<tr>
<td>SAM at 7-d</td>
<td>2979</td>
<td>878</td>
<td>121</td>
<td>271</td>
<td>138</td>
</tr>
<tr>
<td>Meristem at 10-d</td>
<td>3316</td>
<td>1420</td>
<td>201</td>
<td>400</td>
<td>552</td>
</tr>
<tr>
<td>Inflorescence meristem at 15-d</td>
<td>2169</td>
<td>844</td>
<td>42</td>
<td>196</td>
<td>231</td>
</tr>
<tr>
<td>Pod of the senescent silique</td>
<td>2060</td>
<td>1103</td>
<td>770</td>
<td>2035</td>
<td>643</td>
</tr>
<tr>
<td>Pedicel</td>
<td>2578</td>
<td>972</td>
<td>56</td>
<td>388</td>
<td>814</td>
</tr>
<tr>
<td>Root without apex</td>
<td>1391</td>
<td>988</td>
<td>476</td>
<td>227</td>
<td>312</td>
</tr>
<tr>
<td>Seedling cotyledons</td>
<td>2633</td>
<td>593</td>
<td>51</td>
<td>398</td>
<td>1288</td>
</tr>
<tr>
<td>Seedling hypocotyl</td>
<td>1999</td>
<td>2145</td>
<td>209</td>
<td>337</td>
<td>292</td>
</tr>
<tr>
<td>Seedling root</td>
<td>1745</td>
<td>1094</td>
<td>429</td>
<td>181</td>
<td>341</td>
</tr>
<tr>
<td>Dry seeds</td>
<td>3650</td>
<td>888</td>
<td>6918</td>
<td>3112</td>
<td>1202</td>
</tr>
<tr>
<td>Germinating seeds</td>
<td>3195</td>
<td>1038</td>
<td>5618</td>
<td>2158</td>
<td>1079</td>
</tr>
</tbody>
</table>
Appendix B: Alignment of 2TMCLB model with ESyt2 SMP and C2 domains.

Figure B: Ribbon diagram of the alignment between 2TMCLB model and the SMP and C2 domains of ESyt2. 2TMCLB model: grey; ESyt2 SMP: pink; ESyt2 SMP: light brown; 2TMCLB TM predicted regions: blue; disorder region: green.
Appendix C: Alignment of SYTF model with ESyt2 SMP and C2 domains.

Figure C: Ribbon diagram of the alignment between SYTF model and the SMP and C2 domains of ESyt2. The SMP domain of ESyt2 was aligned 3 times, each one corresponding to a SYTF C2 domain. a, front view. b, top view. SYTF model: grey; ESyt2 SMP: pink; ESyt2 SMP: shades of brown; SYTF TM predicted region: blue; disorder region: green.
Appendix D: SYT1 co-immunoprecipitation analysis shows AtCLB as interactor in vitro.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Gene ID</th>
<th>Alternative names</th>
<th>MW</th>
<th>SYT1-GFP IP</th>
<th>SYT5-GFP IP</th>
<th>GFP control IP</th>
<th>GFP control IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYT1</td>
<td>AT2G20990.1</td>
<td>SYT1</td>
<td>62 kDa</td>
<td>56</td>
<td>52</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>SYT5</td>
<td>AT1G05500.1</td>
<td>SYT5</td>
<td>63 kDa</td>
<td>23</td>
<td>40</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>GFP-HA-Strep</td>
<td></td>
<td>GFP-HA-Strep</td>
<td>30 kDa</td>
<td>18</td>
<td>15</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>NTMC2TYPE4, NTMC2T4 Calcium-dependent lipid-binding (CalB domain)</td>
<td>AT3G61050.1</td>
<td>AtCLB</td>
<td>55 kDa</td>
<td>16</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ECA3, ATECA3 Endoplasmic reticulum-type calcium-transporting ATPase 3</td>
<td>AT1G10130.1</td>
<td>ECA3</td>
<td>109 kDa</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SYT4</td>
<td>AT5G11100.1</td>
<td>SYT4</td>
<td>64 kDa</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PEN3 Plant PDR ABC-type transporter family protein</td>
<td>AT1G59870.1</td>
<td>PEN3</td>
<td>165 kDa</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Early-responsive to dehydration stress protein (ERD4)</td>
<td>AT1G30360.1</td>
<td>ERD4</td>
<td>82 kDa</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure D: SYT1 interacts with SYT1, SYT5 and AtCLB in vitro. Co-immunoprecipitation analysis of SYT1-GFP and SYT5-GFP using anti-GFP antibodies. Numbers represent the amount of peptide fragments obtained that match the given protein. SYT1, SYT5 and AtCLB are labelled in red.
Appendices

Appendix E: SYT1-GFP and AtCLB localization under KCl, MgCl₂ and Na₂SO₄ stresses.

Figure E: Subcellular localization of SYT1-GFP and AtCLB-GFP under KCl, MgCl₂ and Na₂SO₄ treatments. White squares show 2x zoom-in regions. Pictures taken in epidermal cells of 5-days-old cotyledons. Scale bars = 10µm.
Appendix F: PtdIns(4,5)P$_2$ increases under NaCl stress and after blockage of PLC.

Figure F: PtdIns(4,5)P$_2$ increases after NaCl or U73122 treatments. 

a, fluorescence of the CITRINE-2xPH$^{PLC}$ sensor in mock, 100mM NaCl or 50µM U73122 treatments. 

b, quantification of fluorescence intensity of CITRINE-2xPH$^{PLC}$. N represent the numbers of cells measured on each treatment. Values represent means ± SD and are normalized with the mock. **$P<0.01$ by a Student’s t-test. Scale bars = 50µm.
Appendix G: HDEL-GFP subcellular localization under depletion of cytosolic Ca\(^{2+}\).

Figure G: Subcellular localization of HDEL-GFP after depletion of Ca\(^{2+}_{\text{cyt}}\) by EGTA and LaCl\(_3\). 5mM EGTA or 500µM LaCl\(_3\) incubation for 16h. Pictures taken in the cortical region of epidermal cells of 5-days-old cotyledons. Scale bars = 20µm.
Appendix H: SYT1-GFP and HDEL-GFP localization upon increases of cytosolic Ca\textsuperscript{2+}.

Figure H: Subcellular localization of SYT1-GFP and HDEL-GFP upon [Ca\textsuperscript{2+}]\textsubscript{cyt} increases. Pictures taken in the cortical region of 5-days-old shoot epidermal cells. Scale bars = 20µm.
Appendices

Appendix I: SYT1 and HDEL co-localization under NaCl stress.

Figure I: Co-localization analysis of SYT1-RFP and GFP-HDEL in NaCl Stress treatment. Pictures taken in the cortical region of 5-days-old shoot epidermal cells. Scale bars = 15µm.
Appendices

Appendix J: MAPPER shows decrease of intermembrane distance upon salt stress

Figure J: NaCl promotes EPCS expansion. a, co-localization analysis of SYT1-RFP and MAPPER-GFP. b, MAPPER-GFP subcellular localization under NaCl stress and U73122 treatment. c, co-localization analysis of MAPPER-GFP and RFP-HDEL upon NaCl stress. Pictures taken in the cortical region of 5-days-old shoot epidermal cells. Scale bars a, b = 20µm; c = 15 µm.