

Anchoring mechanism of the plant protein remorin to membrane nanodomains

Anthony Legrand

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Anthony Legrand. Anchoring mechanism of the plant protein remorin to membrane nanodomains. Molecular biology. Université de Bordeaux, 2020. English. NNT: 2020BORD0285. tel-03118537

HAL Id: tel-03118537 https://theses.hal.science/tel-03118537

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THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

DOCTEUR DE L'UNIVERSITÉ DE BORDEAUX

PAR

Anthony Thomas Pascal LEGRAND

Anchoring mechanism of the plant protein remorin to membrane nanodomains

Sous la direction de Dr Sébastien MONGRAND Dr Birgit HABENSTEIN

Soutenue le 16 décembre 2020

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Résumé

La rémorine du groupe 1 isoforme 3 de Solanum tuberosum (StREM1.3) est une protéine membranaire de la famille multigénique de protéines de plante appelée rémorines (REMs), impliquées dans l'immunité des plantes, la symbiose, la résistance aux stress abiotiques et la signalisation hormonale. La caractéristique la plus connue des REMs est leur capacité à se ségréger en nanodomaines au feuillet interne de la membrane plasmique (MP). Pour StREM1.3, ceci se fait via une interaction entre deux lysines de l'ancre C-terminale de la rémorine (RemCA) et le phosphatidylinositol 4phosphate (PI4P) négativement chargé. Ainsi, RemCA modifie sa conformation et s'enfonce partiellement dans la MP, résultant en un accrochage membranaire intrinsèque. Capitalisant sur les données structurales déjà disponibles concernant cet isoforme, nous investiguons StREM1.3 davantage quant à ses propriétés d'interaction membranaire, en utilisant un large éventail de techniques, allant de la microscopie de fluorescence et de la RMN à l'état solide (ssNMR) à la microscopie de force atomique (AFM), la cryo-microscopie électronique (cryoEM) et la modélisation informatique. Nous souhaitons découvrir l'impact de l'oligomérisation et de la phosphorylation de StREM1.3 sur ses interactions membranaires et son activité biologique, ainsi que d'examiner son influence sur la dynamique des lipides et les lipides requis pour l'accrochage à la membrane et le regroupement en nanodomaines. Enfin, forts de toutes les données structurales disponibles, nous entreprendrons la reconstruction in vitro et la caractérisation de nanodomaines minimaux de StREM1.3.

Abstract

Group 1 isoform 3 remorin from Solanum tuberosum (StREM1.3) is a membrane protein belonging to the multigenic family of plant proteins called removins (REMs), involved in plant immunity, symbiosis, abiotic stress resistance and hormone signalling. REMs' most well-known feature is their ability to segregate into nanodomains at the plasma membrane's (PM) inner leaflet. For StREM1.3, this is achieved by an interaction between two lysines of the remorin C-terminal anchor (RemCA) and negatively charged phosphatidylinositol 4-phosphate (PI4P). Thus, RemCA undergoes conformational changes and partially buries itself in the PM, resulting in an intrinsic membrane anchoring. Capitalising on pre-existing structural data about this isoform, we investigate StREM1.3's membrane-interacting properties further, using a wide array of techniques, ranging from fluorescence microscopy and solid-state nuclear magnetic resonance (ssNMR) to atomic force microscopy (AFM), cryo-electron microscopy (cryoEM) and computational modelling. We aim to discover the impact of StREM1.3's oligomerisation and phosphorylation on its membrane interactions and biological activity, and to assess its influence on lipid dynamics as well as its lipid requirements for membrane binding and nanoclustering. Finally, based on all available structural data, we will undertake the in vitro reconstruction and characterisation of minimal nanodomains of StREM1.3.

Acknowledgments

Dr Birgit Habenstein welcomed me as a master 2 intern to work with her on the project that would, in time, become the subject of my PhD thesis. Soon after, I met with Dr Sébastien Mongrand, and both helped me prepare for the doctoral school's ranked exam. Throughout the entirety of my internship-then-doctorate, I could count on their thoughtful insights, their patience and their nice words of encouragement. To their kindness I must extend my gratitude.

I should also acknowledge Dr Denis Martinez, Dr Paul Gouguet and Dr Ahmad Saad for their counselling, their support and, perhaps unknowingly, teaching me how to dedramatise failure and performance anxiety. Special thanks also go to Mélanie Berbon for teaching me sample preparation and Axelle Grélard and Estelle Morvan for teaching me NMR spectroscopy – all the while bearing with my very clumsy self.

At last, I must also thank my office neighbours Gaëlle Lamon and Dr Arpita Tawani, as well as Mathilde Bertoni both for our scientific discussions and the pleasant atmosphere we created in IECB. My encouragements go to Marie-Dominique Jolivet who started working on the remorin about a year ago as a PhD student and to whom I wish her a happy and productive thesis. The working mood in LBM, which has always been exceptionally pleasant, has vastly improved with your arrival along with Marguerite Batsale, Marion Rocher, Delphine Bahammou and Julie Castets.

Working with all of you made me grow immensely, both as a scientist and as a person.

I am grateful to the jury for the time spent reviewing this manuscript.

Table of contents

LIST OF ABREVIATIONS	9
INTRODUCTION	. 11
I. Biological membranes	. 11
A. The discovery of cell membranes	. 12
B. Towards the fluid mosaic model	. 12
C. The composition of lipid bilayers	. 14
1. Phospholipids	. 14
a. Generalities	. 14
b. Phosphoinositides (PIPs)	. 15
2. Glycolipids	. 16
3. Sterols	. 17
4. Lipid phase behaviour and sterols	. 19
5. Sphingolipids	. 21
6. Lipid composition and asymmetry of PMs	. 22
a. Of animal cells	. 22
b. Of plant cells	. 22
c. Of yeasts	. 22
d. Of <i>Bacillus</i>	. 23
D. Membrane proteins	. 23
1. Hydrophobic interactions	. 23
2. Hydrogen bonding	. 23
3. Electrostatic interactions	. 24
a. Polar residues within a membrane	. 24
b. Protein-lipid electrostatic interactions at the membrane's surface	. 24
4. Possible folds of membrane proteins	. 26
E. Membrane domains	. 28
1. Early observations	. 28
a. Coexistence of lipid phases	. 28
b. Caveolae, a visible heterogeneity	. 28

c. Detergent-insoluble membranes (DIM)	29
d. Cell polarity	29
2. From lipid rafts to nanodomains	29
a. Birth of the lipid raft hypothesis	29
b. Restatements	31
c. Interdigitation and domain registration	31
d. Pinning	31
e. A clarification of terminology	33
II. Models of membrane nanodomains across Nature's clades	36
A. In animals	36
1. Ras	36
2. GM1	37
3. Caveolae	37
B. In yeasts: on different nanodomain distributions of many systems	38
C. In prokaryotes: flotillins	39
D. In plants: Rho of plants (ROP)	40
III. Remorins	41
A. Phylogeny	41
B. Biological implications	43
1. Immunity	44
a. Against viruses	44
b. Against bacterial and fungal infections	44
2. Symbiosis	45
3. Stress resistance	45
4. Cell-to-cell communication	45
C. Biophysics of membrane anchoring and nanodomain formation, with a spattention to <i>St</i> REM1.3	ecial 45
1. Remorin C-terminal anchor (RemCA)	45
2. Oligomerisation domain	48
3. IDD	49
4. Towards a model of StREM1.3 nanoclustering	49
a. Remorin/lipid-lipid interactions: PIPs cluster on their own	49
b. Remorin-remorin interactions: remorins tether with their peers	50

c. Remorin-cytoskeleton interactions: the cytoskeleton directs nanc	clustering
IV Objectives	
1. What is the nanoclustering mechanism of StREM1.3?	
2. What is the minimal set of partners required to make	StREM1.3
nanodomains?	
3. How interactors of <i>St</i> REM1.3 may regulate its relationship with m and biological functions?	embranes 52
4. Professional context	52
V. Biophysical tools to study membrane nanodomains	52
A. Detergent insoluble membranes	52
B. Fluorescence microscopy	54
1. The fluorescence phenomenon	54
2. Confocal microscopy	54
3. Super-resolution microscopy	57
a. Airy scan	57
b. Stimulated emission depletion (STED)	57
c. Single-particle tracking photoactivated localisation microscopy (s	spt-PALM)
	58
C. Electron microscopy (EM)	58
1. Negative staining TEM	61
2. Cryo-TEM	62
D. Atomic force microscopy (AFM)	62
E. Computational modelling	63
F. Nuclear magnetic resonance (NMR)	64
1. Spin	64
2. NMR spectrometer	65
3. Energy levels	66
4. The simplest NMR experiment	67
5. NMR spectrum	69
6. Liquid-state vs. solid-state NMR	69
7. ssNMR to study membrane nanodomains	70
8. ssNMR to elucidate protein structures	71
a. Cross polarisation (CP)	71

b. Proton Driven Spin Diffusion (PDSD)	72
c. Secondary chemical shifts	74
9. IsNMR to study soluble intrinsically disordered proteins	74
a. Generalities	74
b. On intrinsically disordered proteins (IDPs)	74
c. On folded proteins	75
10. ssNMR to monitor lipid dynamics: ² H ssNMR	75
11. ssNMR to monitor lipid dynamics: ³¹ P ssNMR	77
Article I	80
Article I: addendum	95
I. REM ₈₆₋₁₉₈ in liposomes by ssNMR	95
II. Structure of RemCA in micelles	96
A. Material and methods	96
B. Results	97
III. Structural analysis of RemCA in native-like conditions by ssNMR 1	00
IV. On a putative role of the N-terminal IDD of StREM1.3 on the structure of	its
filaments1	03
V. Conclusion 1	03
Article II 1	05
Article II: addendum 1	47
Article III 1	50
Article IV 1	61
Article IV: addendum 1	87
Article V 1	89
I. Introduction 1	90
II. Material and methods 1	90
A. Protein purification 1	90
B. Preparation and observation of giant vesicles (GVs) 1	91
C. Nanodomain reconstitution for cryoEM 1	91
D. Agroninfiltration in <i>Nicotiana benthamiana</i> 1	91
E. Confocal microscopy 1	91
III. Results 1	91
A. StREM1.3 specifically binds to PA and PIPs, but not PS 1	91
B. StREM1.3 must be oligomerised to ensure a tight membrane binding 1	93

C. Close visual of synthetic remorin nanodomains	194
IV. Discussion	196
V. Conclusion	196
Conclusion	199
Annex: an up-to-date review on remorins	203
References	245

LIST OF ABREVIATIONS

Bold symbols represent vectors. Regular symbols represent their modulus. ASG: acylated steryl glucoside AFM: atomic force microscopy **B**₀: magnetic field induced by the spectrometer's superconducting magnet CL: cardiolipin CP: cross-polarisation CS, σ : chemical shielding CTF: contrast transfer function CTX: Vibrio cholerae's toxin δ: chemical shift DAG: diacylglycerol DIM: detergent insoluble membrane DLPC: di-linoleoyl-PC DMPC: di-myristoyl-PC DOPC: di-oleoyl-PC POPC: palmitoyl-oleoyl-PC DPC: dodecyl-PC DPPC: di-palmitoyl-PC DRM: detergent resistant membrane DSM: detergent soluble membrane EE: early endosome EM: electron microscopy ER: endoplasmic reticulum FID: free induction decay y: gyromagnetic ratio GIPC: glycosyl inositol phosphoryl ceramide GluCer: glucosyl cermaide GTP: guanosine triphosphate HIV: human immunodeficiency virus HMQC: homonuclear multiple quantum correlation IDD: intrinsically disordered domain IDP: intrinsically disordered protein Ins: inositol InsP: inositol phosphate KO: knocked-out L_α: liquid disordered phase L_β: gel phase LCB: long chain base

- <u>LE:</u> late endosomes
- Lo: liquid-ordered phase

IsNMR: liquid-state NMR

LUV: large unilamellar vesicle

M: magnetisation

<u>MVB:</u> multivesicular bodies

NA: numerical aperture

NMR: nuclear magnetic resonance

NOESY: nuclear overhauser spectroscopy

 $\underline{\omega}$: rotational frequency of a spin

 $\underline{\omega_{0:}}$ Larmor frequency

PA: phosphatidic acid

PALM: photoactivated luminescence microscopy

PC: phosphatidylcholine

PD: plasmodesmata

PDSD: proton-driven spin diffusion

PE: phosphatidylethanolamine

PG: phosphoatidylglycerol

PH: pleckstrin homology

PI: phosphatidylinositol

PI(3/4/5)P: phosphoatidylinsitol (3/4/5)-phosphate

PI(3,4/3,5/4,5)P2: phosphatidylinositol (3,4/3,5/4,5)-bisphosphate

PI3,4,5P₃: phosphatidylinositol 3,4,5-trisphosphate

PIP: phosphoinositide

PLC: phospholipase C

PM: plasma membrane

<u>PP-IPPs:</u> pyrophosphorylated (PP) inositol polyphosphates (IPPs)

PS: phosphatidylserine

PtdIns, PIP: phosphoinositide

<u>REM:</u> remorin

RemCA: remorin C-terminal anchor

ROP: Rho of plants

 σ , CS: chemical shielding

SE: steryl ester

SG: steryl glucoside

spt: single-article tracking

SNR: signal-to-noise ratio

STED: stimulated emission-depletion

SUV: small unilamellar vesicle

SV40: simian virus 40

ssNMR: solid-state NMR

TGN: trans-Golgi network

<u>Tm:</u> gel-liquid phase transition temperature

TOCSY: total correlated spectroscopy

INTRODUCTION

I. Biological membranes

The word membrane refers to a thin semi-permeable material separating two liquid compartments. In the context of biology, compartmentation using membranes is very common (van Meer et al., 2008): from the plasma membrane (PM), delimiting a cell's inside from its outside, the nuclear membrane, delimiting the nucleus from the cytoplasm, the mitochondrial double membrane, the chloroplast double membrane and thylakoids, to the endoplasmic reticulum (ER) membrane. Some viruses even envelope themselves in the plasma membrane of the infected cell they budded from (Chazal and Gerlier, 2003). A cell may or may not have all the organelles cited above but, at the very least, it has a PM: this one is ubiquitous. Indeed, encapsulation of some self-replicating RNA by a primitive membrane could have been at the origin of the first cell ever (Kamat et al., 2015).

Although the PM acts as a physical barrier between the cell and its environment, it must also, in terms of signalling and metabolism (i.e. signal transduction and exchange of molecules), take from the outside and release from the inside (Grecco et al., 2011; Groves and Kuriyan, 2010; Ray et al., 2016). This implies a great complexity in the PM's organisation, far from being a homogeneous and impenetrable wall.

In this section, we shall rewind the history of membrane discovery, starting from the first observation of a cell in the 17th century (Lombard, 2014). We shall see how the mere existence of the PM was debated, what was thought of its composition and how its structural organisation was depicted. Once the fluid mosaic model (Singer and Nicolson, 1972) will be introduced, we shall discuss about membrane composition and spatial heterogeneity, such as microdomains and nanodomains.



1st observation of a cell Later observations of cells Figure 1

Trombinoscope of the pioneers of cell and organelle discovery.

A. The discovery of cell membranes

The emergence of the microscope unlocked the key to the microscopic realm for biologists of the 17th century and beyond. The first published observation of a cell dates back to 1665 when Robert Hooke (Figure 1) cut a thin slice of cork and observed, under a microscope (Hooke, 1665), an ensemble of cavities separated by a thick, solid and continuous structure. He named these cavities cells, likely as a reference to cells within a monastery, and referred to the cell wall as a wall. Later observations enforced the existence of cells as a basic unit of biological matter (Grew, 1672; Leeuwenhoek and Hoole, 1800; Malpighii, 1675). But if the cell exists, what about its frontier?

As shocking as it may be from our modern point of view, the very existence of membranes as physical barriers was questioned: some argued it was the mere optical manifestation of a liquid-liquid phase separation between two immiscible liquids, for example, in the case of the PM, a membrane-less cell and its environment. By the late 19th century, seminal work on cell osmosis, diffusion, electrophysiology and cytolysis managed to engrave the existence of cell membranes in the scientific discourse (Lombard, 2014). Meanwhile, the first report on the observation of an organelle, the nucleus, dates back to 1833 (Brown, 1833). Other organelles soon followed, as reviewed in (Mullock and Luzio, 2013).

B. Towards the fluid mosaic model

By the end of the 19th century, it was known that living matter was made of proteins, lipids (called fats back then), carbohydrates, nucleic acids and salts (Dahm, 2008; Loeb, 1906). Charles Ernest Overton showed PMs to be apolar, by virtue of the fact that they allowed the diffusion of apolar molecules yet blocked the diffusion of polar ones (Lombard, 2014). Therefore, the best candidates to be membrane components were "lecithin and cholesterin" or, in modern terms, phospholipids and sterols (section I.C). But in which manner are they organised? The emergence of Langmuir trough, first developed by Agnes Pockels (Pockels, 1891), then popularised by Irving Langmuir (Langmuir, 1917), allowed in vitro surface measurements. Ratios between monolayer surface and cell surface at the PM ranged greatly: Gorter and Grendel found 2 (Gorter and Grendel, 1925), then Bar et al. found 1.3 (Bar et al., 1966). The use of different cell types, with variable protein content, and different purification protocol are obvious pitfalls that could explain such discrepancies. It should be noted that the last value, 1.3, is the most truthful for PMs, if we remind ourselves of the high amount of proteins within (Cacas et al., 2016). Nonetheless, electron microscopy of PMs showed a "railroad"-like structure: two thin and dense layers encasing a broader and lighter one, with a total thickness below 10 nm (Robertson, 1957), reinforcing the hypothesis of a phospholipid bilayer with hydrophilic parts exposed to water and hydrophobic parts buried.

By the 1960s, mainly three models subsisted (Singer, 2004): the Davson-Danielli-Robertson model (Danielli and Davson, 1935), where a phospholipid bilayer was sandwiched between two layers of unfolded proteins, the Benson model (Benson, 1966), where membranes were a juxtaposition of lipoproteins, and the lipid-protein mosaic model (Lenard and Singer, 1966), where folded membrane proteins would bury

their hydrophobic parts in a phospholipid bilayer while keeping their hydrophilic parts in contact with solvent water (Figure 2). Further refinements on the fold of membrane proteins led to the achievement of the fluid mosaic model (Figure 3) (Singer and Nicolson, 1972), which is, to this day, still the basis for the depiction of biological membranes in virtually any biology textbook.



Figure 2

Before the fluid mosaic model (Singer and Nicolson, 1972), three models of membrane structure prevailed. The Davson-Danielli-Robertson model (Danielli and Davson, 1935) proposed a phospholipid bilayer with hydrophobic tails groups buried and hydrophilic ones exposed to solvent water, sandwiched between two layers of unfolded proteins. The Benson model pictured membranes as a juxtaposition of lipoproteins (Benson, 1966). The lipid-protein mosaic model (Lenard and Singer, 1966) is a prototype version of the fluid mosaic model with a less detailed description of membrane protein folding, although the schematic displayed here was used to illustrate both models. Adapted from (Singer, 2004).



Figure 3

The fluid mosaic model. Biological membranes can be envisioned as twodimensional bilayers of phospholipids and proteins with hydrophobic parts buried and hydrophilic parts exposed to solvent water. Membrane proteins must adopt particular conformations in order for their insertion to be thermodynamically stable. From (Singer and Nicolson, 1972).

C. The composition of lipid bilayers

The composition of biological membranes displays tremendous diversity. To remain concise, we shall restrain our description to the three major lipid classes: phospholipids, sterols and sphingolipids and the distribution of all three at the PM, with a special focus on plant membranes. Membrane proteins will be discussed in section I.D.

1. Phospholipids

a. Generalities

Phospholipids, whose full name are glycerophospholipids, share a common chemical structure: two acyl chains esterified in sn-1 and sn-2 of a glycerol moiety, and a polar head etherified in sn-3. The polar head starts with an inorganic phosphate. It is eventually followed by other moieties, the nature or absence of which defining the polarity and shape of the molecule (Figure 4). Depending on the organism, acyl chains may be 16 to 24 carbons long (Cacas et al., 2016; Sassa and Kihara, 2014; Villasmil et al., 2017), though longer chains exist (see sphingolipids), and may be desaturated (i.e. bearing C=C double bonds) (Nakamura, 2017; Wisnieski et al., 1973).



Figure 4

General description of phospholipids. (A) A phospholipid is made of two acyl chains, whether saturated or unsaturated, esterified in sn-1 and sn-2 and an inorganic phosphate etherified in sn-3 of a glycerol moiety. The inorganic phosphate may, or not, be grafted with an additional group, the nature of which defines the phospholipid class it belongs to. An oleoyl-linoleoyl-phosphatidylcholine is drawn as an example. (B) Chemical structure of the various additional groups and their abbreviations and (C) overall shape of the entire phospholipid (circle: polar head, sticks: acyl chains, not to scale). Inorganic phosphates may be added to PI, introducing new negative charges on the polar head and making it bigger.

b. Phosphoinositides (PIPs)

One class of phospholipids will be of particular interest to us: phosphoinositides (PIPs). They are characterised by their inositol sugar moiety to which inorganic phosphates are added (figure 5). They represent a minor class of all eukaryotic phospholipids, whose biological implications range from endo- and exo-cytosis to signal transduction and membrane targeting by proteins (figure 6) (Di Paolo and De Camilli, 2006; Heilmann, 2016). Prokaryotes do not bear PIPs but possess PIP-specific phosphatases as virulence factors (Heinz et al., 1998). Positions 3, 4 and 5 of the *myo*-inositol ring can be specifically phosphorylated or dephosphorylated, leading to a multitude of PIP species, whose localisations could be assigned as follows (figure 7) (Noack and Jaillais, 2017), leading to the concept that PIPs are signature lipids of a given cellular structure.

This manuscript will focus on plant PMs, where PI4P and PI4,5P₂ are the main PIPs (Noack and Jaillais, 2017; Simon et al., 2014). They are also found enriched in detergent insoluble membranes (DIMs, see section V.A) (Furt et al., 2010) and have a tendency to cluster with one another at the PM (Bilkova et al., 2017; van den Bogaart et al., 2011; Ji et al., 2015).



Figure 5

On the diversity of phosphoinositide polar heads. A phosphoinositide is a phospholipid with a terminal *myo*-inositol upon which one, two or three inorganic phosphates are grafted. Positions 2 and 6 cannot be phosphorylated.



Figure 6

On the biological involvements of PIPs. (A) In a membrane, PIPs may serve as specific anchor for proteins. As the distribution of PIPs in all membranes is heterogeneous, this allows a fine addressing of organelles by specific membrane proteins. (B) Once hydrolysed, e.g. by phospholipase C (PLC), their products are substrates of major cell signalling pathways. Both products of hydrolysis may be phosphorylated further, up to all 6 positions in the case of the nascent inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃), yielding InsP₆. PtdIns: PIPs; DAG: di-acyl glycerol; PtOH: PA; DGPP: DAG pyrophosphate; InsP₅: inositol pentakisphosphate; InsP₆: inositol hexakisphosphate; PP-IPPs: pyrophosphorylated (PP) inositol polyphosphates (IPPs). From (Heilmann, 2016).

2. Glycolipids

Monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulfoquinovosyldiacylglycerol are galactolipids synthesised in chloroplasts and major constituents of their photosynthetic membranes, *in lieu* of phospholipids (Nakamura, 2017).



Distribution of PIPs in plant cells. There are two pools of PIPs: those phosphorylated at position 3 for late endosomes (LE), multivesicular bodies (MVB), tonoplasts, autophagosomes and vacuoles (*left*); those phosphorylated at position 4 for early endosomes (EE), the *trans*-Golgi network (TGN) and the PM (*top and right*). An increasing gradient of PI4P exists from the Golgi to the PM (*right*), and a similar increasing gradient of PI3P exists from tonoplasts to late endosomes (Simon et al., 2014). The PM is enriched in PI4P and PI4,5P₂. The nucleus is enriched in PI5P (not shown). Modified from (Noack and Jaillais, 2017).

3. Sterols

Unlike animals and yeasts that rely on cholesterol and ergosterol, respectively, plants possess a plethora of phytosterols. Sterol profiles may strongly vary between species. Later, we will be interested in *Solanum tuberosum* (potato), whose major sterols are sitosterol and stigmasterol, in an approximate 11/1 molar ratio (Figure 8) (Nyström et al., 2012; Wewer et al., 2011). Most chemical differences between phytosterols rely on their aliphatic part or substitution at position 4 (Moreau et al., 2018). Cholesterol may exist in plants but remains in minor quantities (Diener et al., 2000). Sterols may be conjugated via their hydroxyl group into steryl glycosides (SG), steryl esters (SE) and acylated steryl glycosides (ASG) (Figure 9) (Wewer et al., 2011). In lipid membranes, sterols are oriented perpendicular to the membrane plane, with their hydroxyl group at the height of the phospholipid glycerol backbones. This has been proved only for free cholesterol but is likely true for other sterols (Dufourc, 2008; Léonard et al., 2001).

Besides being crucial membrane components, phytosterols are involved in the biosynthesis of phytoecdysteroid, an anti-parasitic agent (Speranza, 2010) and

brassinosteroids, a plant growth hormone (Moreau et al., 2018). Similarly, cholesterol is at the basis of the steroid hormone metabolism. Evidence of biological function for sterol conjugates are scare, though ASG are involved in thermal shock response in plants (Grille et al., 2010).



11/1 ratio

Figure 8

Chemical structures of common sterols. Animals and yeasts use only one sterol (cholesterol and ergosterol, respectively) while plants employ a multitude of them, among which cholesterol may be found in small quantities. In this manuscript, we will interested in PMs of *Solanum tuberosum* (potato), whose main phytosterols (in blue) are sitosterol and stigmasterol in an 11/1 ratio (Nyström et al., 2012).



Display of the various types of phytosterol conjugates. Here, a simplified sitosterol is used as example. All conjugation rely on attaching an ester (sterol ester, SE), a sugar (steryl glycoside, SG) or an esterified sugar (acetylated steryl glycoside, ASG) to the sterol's hydroxyl function. Adapted from (Moreau et al., 2002).

Prokaryotes possess no sterol. Instead they produce hopanoids, pentacyclic sterol analogues (Bramkamp and Lopez, 2015). Although they are thought to behave similarly to sterols, the absence of hydroxyl group on the A ring implies that (1) hopanoids cannot be conjugated and (2) the polar and apolar regions are inverted compared to sterols, with rings being apolar and the tail being polar (Dufourc, 2008).

4. Lipid phase behaviour and sterols

Depending on their nature, phospholipids, glycolipids and sphingolipids may exhibit a variety of phase behaviours (Koynova and Tenchov, 2013). Yet, in the context of the fluid mosaic model, we are referring to a lipid bilayer, which is a lamellar phase (Figure 10). In addition, depending on lipid composition, temperature and hydration, the rate of diffusion of molecules within, the thickness and the acyl chain packing (hereafter called order) will vary (Feigenson, 2006; Koynova and Tenchov, 2013). Here, we will be interested in the phase behaviour of mixtures of phospholipids, sphingolipids and sterols at physiological temperature (i.e. 0-40°C). Here, phospholipids and sphingolipids (section I.C.4) have a phase transition temperature (Tm) above which they make a liquid-disordered (L α) phase and below which they enter into a gel phase $(L\beta \text{ or } L\beta)$, depending on the lipids). Tm can reach rather extreme values, so that the concerned lipids are present almost exclusively in a L α - (e.g. unsaturated acyl chains) or a L β - or L β '-phase (saturated acyl chains). Briefly, lipids in a L α phase diffuse fast and are loosely ordered, while lipids in a L β or L β ^t phase diffuse slowly and are much more ordered. Another important phase is the sterol-enriched liquid-ordered phase (Lo) where lipids still diffuse fast but are more ordered.

How do free sterols compare to each other in terms of function? Cholesterol is known to fluidify membranes in the gel phase at low temperature and to rigidify them in the liquid phase at higher temperature. This latter effect is lessened for sitosterol and even inverted for stigmasterol, depending on the membrane composition, while campesterol retains properties similar to cholesterol (Beck et al., 2007; Grosjean et al., 2015). Conjugated sterols can order membranes further, with an increased efficiency in the case where free sterols are already present (Grosjean et al., 2015).



Figure 10

Representation of lipid phases. (A) Biological membranes are in lamellar phase. At the lowest temperature, a given phospholipid is in a subgel (L_c) phase with slow lateral diffusion and high acyl chain order. Upon temperature increase, it will transition to an untilted (L_β), then tilted (L_β[']), then rippled (P_β[']) gel phase, depending on the nature of the lipid. Once a certain temperature Tm is reached, a gel-liquid-disordered (L_α) phase transition occurs. In this latter phase, lipids diffuse quickly and acyl chain order is lower. *In lieu* of the L_α phase, the sterol-enriched liquid-ordered phase (L_o) (not shown) may be reached: it retains a higher acyl chain order than the L_α phase. Lipids in water may spontaneously aggregate into spherical (B) and non-spherical structures (C). Adapted from (Koynova and Tenchov, 2013).

5. Sphingolipids

Plant sphingolipids are made of a long (acyl) chain base (LCB) amide linked to a FA to create a ceramide, with the eventual addition of phosphate and sugar moieties. In plants, four classes may be distinguished: free LCBs, ceramides, glucosylceramides (GluCers) and glycosyl inositol phospho-ceramides (GIPCs) (Figure 11). The first three classes are entirely synthesised in the ER while GIPC synthesis is pursued with ceramides in the Golgi apparatus. Animals cells contain no GIPCs but they possess sphingomyelin (Futerman and Hannun, 2004).



Figure 11

Display of plant sphingolipids. An FA, possibly hydroxylated, is grafted to an LCB. The primary hydroxyl can be functionalised with a head group, defining its sphingolipid class. From (Pata et al., 2010).

GIPCs are the most abundant sphingolipids in *Arabidospsis thaliana* leaves (Markham et al., 2006). GIPCs make up a wide class of molecules whose diversity relies on: (1) the LCB, its double bonds and hydroxylations, mostly t18:0 or t18:1 (where t means trihydroxylated); (2) the FA length, its double bonds and hydroxylations, most commonly a very long chain FA (VLCFA) or a 2-hydroxylated VLCFA up to 30 carbons ; (3) the glycans, their number, their nature and their glycosidic links (Buré et al., 2014; Cacas et al., 2016; Pata et al., 2010). The polar head composition defines the series to which a GIPC belongs (Mamode Cassim et al., 2019).

In synergy with phytosterols, sphingolipids can modulate membrane rigidity: GluCers fluidify membranes in presence of stigmasterol where GIPC would rigidify, while a 1/1 GluCer/GIPC mixture would strongly rigidify membranes in presence of sitosterol or campesterol (Grosjean et al., 2015). Beside their structural role, GIPCs were also found to act as toxin receptors: necrosis and ethylene-inducing peptide 1-like (NLP) protein may bind GIPCs from series A, predominant in eudicotyledons and bearing two hexose moieties, but not those from series B, predominant in monocotyledons and bearing three hexose moieties. This explains why eudicotyledons plants are sensitive to NLPs while monocotyledons are not (Lenarčič et al., 2017).

Yeast sphingolipids have sphingolipids similar to plants', with the exception that GIPCs are replaced by mannosyl inositol phospho-ceramides (Marquês et al., 2018). Prokaryotes, with very few exceptions, bear no sphingolipids (Hannich et al., 2011).

6. Lipid composition and asymmetry of PMs

Understanding membrane organisation implies quantifying and situating all its compounds. This section will provide the necessary data about the PMs of the organisms discussed in this manuscript. The description of endomembranes (e.g. nuclear membranes, mitochondrial membranes, plastid membranes...) is out of the scope of this manuscript; the interested reader is referred to (Cheesbrough and Moore, 1980; Rolland et al., 2009; Schwertner and Biale, 1973; Zachowski, 1993; Zhendre et al., 2011).

a. Of animal cells

The erythrocyte, lacking a nucleus, is commonly used to study PM composition and asymmetry as the PM is the only membrane it possesses. Cholesterol represents about 40% of the whole PM (Steck and Lange, 2018), sphingolipids around 25% (Virtanen et al., 1998) while the remainder is made of phospholipids. Sphingolipids are almost exclusively in the outer leaflet along with PC while other phospholipids, particularly anionic ones, are in the inner leaflet (Zachowski, 1993). The repartition of cholesterol between both leaflets is still up to debate (Steck and Lange, 2018).

b. Of plant cells

Phospholipids account for 30-40% of the plant PM, sterols make up 20-30% of it while sphingolipids make up the remainder (Cacas et al., 2016). Although the PM's lipid distribution is well studied in animal cells (Zachowski, 1993), data in plant cells are scarce (Cacas et al., 2016; Tjellström et al., 2010). Recently, building upon these few articles, a model of PM's lipid asymmetry was proposed (Mamode Cassim et al., 2019): (1) GIPCs are exclusive to the outer leaflet, (2) GluCers and sterols, both free and conjugated, are enriched in the outer leaflet, (3) phospholipids, including glycolipids, are enriched in the inner leaflet and (4) PS, PA and PIPs are exclusive to the inner leaflet. We must remind ourselves it is only a model that remains to be tested.

c. Of yeasts

According to (Patton and Lester, 1991), the PM of *Saccharomyces cerevisiae* is made up of phospholipids for 34%, sphingolipids for 16%, cardiolipin for 2% and ergosterol for 48%. There again, aminophospholipids and PIPs remain mostly in the

inner leaflet while sphingolipids, ergosterol and PC are mostly in the outer leaflet (Mioka et al., 2014; Santos and Riezman, 2012; Solanko et al., 2018).

d. Of Bacillus

Bacteria of the *Bacillus* clade are Gram-positive bacteria, with one PM coated by a thick layer of polysaccharides. *Bacillus subtilis* was shown to contain PG/PE/CL in a 70/12/4 molar ratio (Clejan et al., 1986), while a study on the PM asymmetry in *Bacillus amyloliquefaciens* hints at an enrichment of 70% of all phospholipids in the outer leaflet while CL would remain in the inner leaflet. The amount and distribution of hopanoids at the PM remains an open question.

Overall, anionic- and amino-phospholipids remain in the inner leaflet while sphingolipids and PC remain in the outer leaflet. Sterol distribution may vary and is generally very disputed. Proteins of both leaflets are completely different, being either cytoplasmic, apoplasmic or transmembranous, in ways we will see in the next section.

D. Membrane proteins

(Singer and Nicolson, 1972) make a distinction between peripheral membrane proteins, which superficially interact with membranes, and integral ones, which bind tightly and may even span the entirety of the bilayer. Examples of the former would be pleckstrin domains (Lenoir et al., 2015a) while examples of the latter would be GPCRs (Katritch et al., 2013), ATP-synthases (Zhou et al., 2015), voltage-dependent ion channels (Hosaka et al., 2017) and remorins (section III). For an integral protein to interact with a membrane, thermodynamics tell us there are three types of interactions to consider: (1) hydrophobic interactions, (2) hydrogen bonding and (3) electrostatic interactions. Basing ourselves upon the lecture of (Singer, 2004), we shall comment all three then conclude on the kind of protein fold we may expect for membrane-interacting proteins.

1. Hydrophobic interactions

Let us consider the solubility of a typically hydrophobic molecule such as methane (CH₄) in both water and benzene:

CH_4 (water) \rightleftharpoons CH_4 (benzene)

with $\Delta G = -11.5$ kJ/mol (Kauzmann, 1959). The dielectric constant, i.e. the electric permeability of a material over that of the void, for a protein is D_{protein} = 4 and D_{water} = 78.5 (Dwyer et al., 2000), so D_{protein} < D_{water}, so proteins may bury their hydrophobic residues in their core. Alternatively, such hydrophobic residues may be buried in the core of the hydrophobic membrane whose D_{membrane} = 2-3 (Gramse et al., 2013; Huang and Levitt, 1977).

2. Hydrogen bonding

In addition to some residues' lateral chains, the peptide backbone itself is involved in hydrogen bonding. Solvent water may bond with such groups as well as other protein groups, but what if they are within the hydrophobic core of a bilayer? Absence of hydrogen bonding costs about $\Delta G = + 18.5$ kJ/mol (Klotz and Franzen, 1962). The only H-acceptors there would be the protein's H-donors. This implies the integral part of a protein must be meticulously folded to be both thermodynamically stable and functional.

3. Electrostatic interactions

a. Polar residues within a membrane

Charges from polar residues (i.e. aspartate, glutamate, histidine, lysine and arginine) in the polar environment of solvent water (e.g. at $pH\approx7$ in the cytosol) may be balanced by it or nearby polar solutes. Can polar residues find themselves stably inside a bilayer? All the above-mentioned residues are in exchange between a charged state and a discharged state. As an example, for glutamate:

 $-COO^{-} + H_3O^{+} \rightleftharpoons -COOH + H_2O$

with pK = 4.25 in 0.1 M NaCl water at 25°C (Thurlkill et al., 2006). The interior of a protein is itself much less polar than water, as attested by their respective dielectric constants of $D_{protein} = 4$ and $D_{water} = 78.5$ (Dwyer et al., 2000). The thermodynamic cost to keep a glutamate in the discharged state at pH = 7 is $\Delta G = +15.7$ kJ/mol in solvent water, making it unfavourable. Yet we must keep in mind that pK constants for aminoacids inside a protein may be heavily shifted, as exemplified in (Dwyer et al., 2000) where a buried glutamate had a pK = 8.8 with an unusually high $D_{protein} = 12$, in which case a substantial proportion of glutamates are discharged. Thus, we should remind ourselves that the peculiar environment of a protein's inside may impose behaviours on residues much different from solvent water. Yet, discharging a polar residue to bury it in a lipid bilayer seems unfavourable.

Counterbalancing a charged residue with another charged residue or an ion of opposite sign would be another mechanism to embed a polar residue in a bilayer. This mechanism finds its limit illustrated with the poor solubility of a zwitterionic glycine in acetone, whose $D_{acetone} = 21.0$, compared to water whose $D_{water} = 78.5$ (Lide, 2010). A lipid bilayer core has $D_{membrane} = 2-3$ (Gramse et al., 2013; Huang and Levitt, 1977). Since, as D decreases, the solubility of glycine decreases, it shows that counterbalancing a charge does not improve its solubility in an apolar environment.

The free energy cost of burying a residue was systematically assessed in (MacCallum et al., 2007). It strengthens the conclusion that, for a part of a protein to be embedded in a lipid membrane, polar residues should be avoided.

b. Protein-lipid electrostatic interactions at the membrane's surface

However, lipid polar head groups may favour the presence of polar residues at the membrane-water interface. In (MacCallum et al., 2007), free energy costs to bury a residue in a DOPC membrane were compared with the costs of keeping these residues at such an interface (Table 1). Arginine, asparagine, glutamine, threonine, and tyrosine are very costly to bury but are prone to stay at the membrane-water interface.

Residue	At interface	Buried
Leu	-14.1	-15.2
lle	-20.6	-22.1
Val	-12.2	-13.8
Phe	-14.9	-12.8
Ala	-6.8	-8.4
Trp	-21.6	-4.9
Met	-10.5	-4.4
Cys	-6.6	-3.4
Tyr	-14	6.6
Thr	-4.2	13.9
Ser	-0.7	15.8
Gln	-8.9	20.2
Lys ¹	-18.6	19.9
Asn	-6.5	23.9
Glu ²	-1.68	21.1
Asp ²	1.6	31
Arg ³	-21.2	58.1

¹ Charged at interface, neutral if buried

² Neutral in both cases

³ Charged at interface, both states are equiprobable if buried

Table 1

Free energy cost (in kJ/mol) to bury a residue in a lipid bilayer or to keep it at the membrane-water interface. Values from (MacCallum et al., 2007).

This leads to the question: how much do electrostatic interactions with lipid polar groups contribute to the stabilisation of a membrane protein? We will consider the case of the peripheral pleckstrin homology (PH) protein domain, which is a well-known marker of phosphoinositides both *in vivo* and *in vitro* (Kavran et al., 1998). These domains merely touch the membrane to bind their target partner (Lenoir et al., 2015b, 2015a). In (Figure 12), we visualise the loop responsible for FAPP1-PH's membrane-interaction docked *in silico* to a micelle of dodecylphosphorylcholine (DPC) / phosphatidylinositol 4-phosphate (PI4P). It is only a few angstroms deep inside the micelle. Recalling (Table 1), the residues' positions are not surprising: L12 may favourably be buried deep within the micelle while other residues are more stable at the interface. This explains why mutations of W8 to a less hydrophobic residue (i.e. less prone to be at the interface) nullifies binding. A similar result is obtained for the mutant N10T, highlighting the necessity of a basic residue that is likely to interact with the negatively charged 4-phosphate moiety of the PI4P polar head group (Lenoir et al., 2015b).

The structural analysis of FAPP1-PH demonstrates that electrostatic interactions at the membrane-water interface can ensure a protein's tethering to a membrane.



Figure 12

In silico structure of FAPP1-PH's loop (grey) with a micelle (orange surface) of dodecylphosphorylcholine (DPC) / phosphatidylinositol 4-phosphate (PI4P). From (Lenoir et al., 2015a).

4. Possible folds of membrane proteins

In the example above, the interplay between hydrophobic and electrostatic interactions is blatant. Now that we are aware of the different types of interactions between a membrane protein and its membrane, we may wonder what they imply in terms of membrane protein fold.

A first consideration is that proteins will keep their polar residues in water and away from the apolar membrane core. For apolar residues, the opposite is true. In addition, we just saw that some residues, whether polar or not, may be found at the membrane-water interface and play a crucial role in membrane binding.

FAPP1-PH's membrane anchoring is shallow. What about integral protein domains? Most do fold in one of the following manners: (1) into helices spanning part of or all the membrane, lying in its plane or tilted (Katritch et al., 2013; Lins et al., 2008; Zickermann et al., 2015a). (2) Into β -strands, granted they are long enough to span the membrane and numerous enough to make a complete barrel, as in porins, forming a β -barrel (Figure 13) (Kefala et al., 2010).

Less canonical folds exist for peripheral membrane proteins. We already discussed FAPP1-PH. Another example would be the fusion protein gp41 from the human immunodeficiency virus (HIV), folding into a β -sheet in the host membrane, allowing membrane hemi-fusion (Figure 14) (Lee et al., 2019). We shall discuss, in section III.C.1, a less canonical fold of an integral membrane anchoring domain, in which a horizontal α -helix is only partially embedded in the membrane while one hydrophobic extended β -strand is deeply buried.



Figure 13

Display of the most common protein fold in membranes. *Left:* the mitochondrial complex I is embedded in the inner mitochondrial membrane via many helices. The majority of them are transmembrane (i.e. perpendicular to the membrane plane), some are tilted and a few are amphiphatic, thus lying in the membrane plane (Zickermann et al., 2015b). *Right:* OmpF is a pore granting passage of small molecules (< 600 Da) through *Escherichia* coli's outer membrane. Each monomer is made of 16 β-strands folded in a β-barrel (Kefala et al., 2010).



Figure 14

Schematic structure of gp41, a subunit of the HIV's envelope. The

transmembrane domain (TMD, red) is made of helices spanning the viral membrane while the fusion peptide (FP) and the FP proximal region (FPPR) fold into β -strand in the targeted cell membrane. This fold promotes membrane fusion and viral entry into the cell. From (Lee et al., 2019).

E. Membrane domains

1. Early observations

We discussed the complex composition of membranes (sections I.C and I.D). The amount of possible interactions between all its components is gigantic. We also discussed that both leaflets of the PM possess different lipids (section I.C.6) and proteins (section I.D), creating a strong heterogeneity. Could there be heterogeneities within one leaflet of the PM?

a. Coexistence of lipid phases

We are already familiar with the concept of lipid phases (section I.C.4). Soon after the publishing of the fluid mosaic model, evidence of *in vitro* lipid phase separation in membranes were brought in (Stier and Sackmann, 1973) monitored, using paramagnetic electron resonance, the activity of the peripheral P450 cytochrome reductase on both a soluble spin label and a fatty acid one, embedded in liver microsomal membranes, at various temperatures. The Arrhenius plot showed a drop-in energy activation above 32°C only for the fatty acid label. Stier and Sackmann interpreted this as proof of the presence, below 32°C, of rigid membrane domains created by the interaction between the peripheral P450 cytochrome reductase and the otherwise fluid bulk membrane (**Figure 15**).



Figure 15

The earliest proof of coexistence of lipid phases. P450 cytochrome reductase is a peripheral protein. It was hypothesised to order the lipids in its vicinity (fine stripes), creating a rigid membrane domain in the otherwise fluid bulk membrane (large stripes). From (Stier and Sackmann, 1973).

b. Caveolae, a visible heterogeneity

In 1955, electron microscopy of mouse gall bladder epithelium cells showed small invaginations of about 50 to 90 nm (Figure 16) (Yamada, 1955). This is a visual example of spatial heterogeneity in biological membranes. Since these structures resemble cavities within the PM, they were named caveolae. Caveolae will be discussed in greater lengths in section II.A.3.

For now, we need only to remember that one could see, as early as 1955, that biological membranes were not homogeneous.



Figure 16

The earliest observation of a caveolae. Thin slice of a mouse's gall bladder epithelium by negative staining electron microscopy with osmium tetroxide. Scale bar: 1 µm. S: synaptic vesicle, M: mitochondria, C: caveolae. From (Yamada, 1955).

c. Detergent-insoluble membranes (DIM)

In parallel, lipids and membrane proteins of human erythrocytes treated by Triton X-100, a mild non-ionic detergent, at cold, followed by ultracentrifugation on a sucrose gradient, showed variable solubilities (Yu et al., 1973): detergent-solubilised material was pelleted while DIM remained in the supernatant. We may note that phospholipids were mostly soluble while sphingomyelin, an animal sphingolipid, was not. It was yet another hint that biological membranes may not be homogeneous.

Twenty years later, (Sargiacomo et al., 1993) purified DIM enriched in proteins bearing glycosyl-phosphatidyl-inositol (GPI) anchors and caveolin, the main protein component of caveolae (section II.A.3). The enrichment in such GPI-bearing proteins was a commonly explored feature of DIMs (Brown and Rose, 1992; Hooper and Turner, 1988). (Schroeder et al., 1994) pushed the lipidomic analysis of DIMs further by reconstituting minimal DIMs. Cholesterol and lipids with high Tm, such as sphingolipids and saturated phospholipids, were shown to promote the formation of DIMs, in the absence of any protein.

d. Cell polarity

Meanwhile, investigations on animal cell polarity, more specifically on animal epithelial cells, revealed differential lipid and protein composition between apical and basolateral membrane domains (Simons and Van Meer, 1988). This lead to the formulation of putative lipid and protein sorting mechanisms for the specialisation of these membranes (Simons and Wandinger-Ness, 1990)

2. From lipid rafts to nanodomains

a. Birth of the lipid raft hypothesis

Evidence stated above led to the formulation of the raft hypothesis: the existence of (1) small membrane domains, the size of a caveolae (50-90nm), (2) enriched in cholesterol, high-Tm lipids and GPI-anchored proteins (Figure 17) (Simons and Ikonen, 1997). It was proposed that lipid rafts could be purified in DIM fractions: they would float at the top of a sucrose gradient, hence their designation as rafts (Dupree et al., 1993). In this initial description, lipid rafts were regarded as sorters of membrane trafficking (Simons and Ikonen, 1997).



Figure 17

First hypothetical model of lipid rafts and caveolae at the animal PM, in 1997. Rafts (red) segregate from the bulk PM (blue). Caveolae are proposed as a peculiar kind of raft-like structure. (a) A lipid raft enriched in cholesterol bearing some membrane proteins attached to one leaflet, a GPI-anchored protein (red) to the outer leaflet and the Src-family kinase Yes (blue) to the inner leaflet, or spanning the entire bilayer, here hemagglutinin (HA, yellow). (b) Asymmetry of the animal PM, as described in section I.C.6.a: sphingolipids and PC are in the outer leaflet while other lipids, particularly anionic ones, are the inner leaflet. (c) Caveolae are formed by selfassociating caveolin proteins making a hairpin loop in the membrane. Interactions with raft lipids may be mediated by binding to cholesterol and by acylation of Cterminal cysteines. From (Simons and Ikonen, 1997).

b. Restatements

Many more observations of lipid rafts occurred, which did not relate on caveolae and GPI-anchored proteins (de Laurentiis et al., 2007; Mongrand et al., 2004; Yuan and Johnston, 2001), rending the initial description of lipid rafts obsolete. In 2006, at the Keystone Symposium on Lipid Rafts and Cell Function, an updated, much broader definition was proposed (Pike, 2006). "Membrane rafts", for it was accepted that proteins could also be involved in their formation, "are small (10-200nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalise cellular processes. Small rafts can sometimes be stabilised to form larger platforms through protein-protein and protein-lipid interactions." Major limitations of DIM purifications as indicators of membrane rafts were pointed out (Munro, 2003; Shogomori and Brown, 2003) and a clear distinction was made between simple in vitro system, where a distinction between phases may apply, and *in vivo* systems, where the multiplicity of interactors makes lipid phases harder to assess. The question of biological functions was deliberately left unanswered beyond the statement that "[membrane] rafts are involved in the compartmentalisation of cellular processes", whatever they may be.

This led to a restatement, by one of the author of the initial lipid raft hypothesis, of what a raft should be (Lingwood and Simons, 2010) (Figure 18). In this view, lipid rafts (used as a synonym of membrane rafts) are also thicker, L₀ phase membrane domains, something that was previously deemed too specific of simple *in vitro* systems (Pike, 2006).

c. Interdigitation and domain registration

This last redefinition also addressed the concept of interdigitation: if a lipid raft is in the outer leaflet, would a raft be recruited (registration) or chased away (antiregistration) from the inner leaflet (Figure 19)? Theoretical calculations oppose antiregistration to minimise membrane thickness variations to registration via interdigitation of aliphatic chains (Williamson and Olmsted, 2015). Works on planar (Collins and Keller, 2008) and supported (Lin et al., 2006; Rinia et al., 2001) model membranes, favour registration.

d. Pinning

The concept of interdigitation is often linked to the notion of pinning, as in pinning and keeping these registered membrane domains together. Such phenomena were observed *in vivo* between GPI-anchored proteins of lipid rafts of the PM's outer leaflet and actin bound to phosphoinositides of lipid rafts of the PM's inner leaflet (Dinic et al., 2013; Raghupathy et al., 2015).

(Tsuji and Ohnishi, 1986) were the first to demonstrate a pinning of the anion transporter Band 3 by the actin cytoskeleton in erythrocytes in a spectrin-dependent manner. A model of pinning was thus proposed (Figure 20), often referred to as the fence and picket model (Kusumi et al., 2005). Similar findings were obtained on transferrin and α_2 -macroglobulin upon disruption of actin filaments and microtubules (Sako and Kusumi, 1994).



Figure 18

Restatement of the lipid raft hypothesis in 2010. Lipid rafts are liquid-ordered L₀ membrane domains enriched in sterols, sphingolipids, some particular membrane proteins and among which some may interact with actin (green). The may exist in various membrane systems, even though most examples within relate to the PM and animals. (A) Lipid rafts are dynamic structures with particular lifetimes. They may fuse or dissociate. (B) Lipid rafts may have heterogeneous contents. Their formation may be trigger by lipid-lipid segregation, protein-lipid multivalent binding and protein-protein oligomerisation. Protein-actin interaction as a mechanism to initiate lipid raft formation by pinning (see section I.E.2.d) its component in place is also suggested. (C) Casting the concept of actin pinning aside, lipids and proteins may coalesce, or not, into lipid rafts by their sheer preference for a certain degree of membrane order, liquid-ordered L₀ for lipid rafts or liquid-disordered L_d for the bulk membrane, and biochemical interactions. GPL: glycerophospholipid; GSL/SM: glycerosphingolipid/sphingomyelin; TM: transmembrane. From (Lingwood and Simons, 2010).



Figure 19

The two leading behaviours of inter-leaflet membrane domain coupling. Interleaflet interactions between acyl chains favour domains in both leaflets to be registered (A) while hydrophobic mismatch favours domain anti-registration (B) to even membrane thickness. From (Fujimoto and Parmryd, 2017).



Figure 20

Pinning of membrane proteins. Pinning of band 3 (white circles) by the actin cytoskeleton, later termed the "membrane skeleton fence model" (Sako and Kusumi, 1994). Black dots represents actin and its associated band 4.1 protein, connected to six spectrins (white lines) at the PM's inner leaflet. Each spectrin is either in a tetrameric state (full lines) or a dissociated dimeric state (broken lines), forming a mesh. The fate of three pinned band 3 molecules a, b and c trying to escape the membrane region of interest represented here is drawn. a diffuses through the mesh and escapes quickly. b encountered a dead-end in the mesh, so it escapes slowly. c is blocked and will never escape: it is pinned down until a neighbouring spectrin tetramer dissociates. From (Tsuji and Ohnishi, 1986).

e. A clarification of terminology

We must have noted that the nomenclature of membrane domains is quite heterogeneous: between DIMs (also synonymously called detergent resistant membranes, DRMs), lipid rafts, membrane rafts, microdomains and nanodomains. Now, we shall define each term with precision and decide on which to commit to.
DIMs are the insoluble fraction of a biological membrane preparation treated with a mild detergent at cold temperature. They are separated from their soluble counterparts by ultracentrifugation on a sucrose gradient.

Lipid rafts and membrane rafts are synonymous, although the former is the historical and most remembered word. The term raft was a reference to DIMs that would float at the top of a sucrose gradient (Dupree et al., 1993). Although it is still used in scientific parlance, we prefer the broader designation of nanodomain (Gronnier et al., 2018).

Nanodomains are small (10-200 nm) membrane domains of specific and independent compositions. This broad definition welcomes a wide variety of phenomena, some of which will be described in section II. Common features of nanodomains may include an enrichment in sterols and lipids with high Tm, such as sphingolipids and saturated PL, a higher acyl chain order parameter and, therefore, a slightly greater membrane thickness. This definition emphasises the spatial segregation of membrane components and avoids confusion with DIMs, whose physiological relevance can be questioned (see section V.A).

Microdomains are micrometric ($\geq 1\mu$ m) membrane domains related to cell polarisation, fabricated through polarised secretion or endocytosis. A large patchwork of nanodomains may also be considered a microdomain. This distinction in size between nano- and micro-domains is capital to emphasise that the former is a sub-membrane specialisation while the latter concerns a whole portion of it (Figure 21). This implies that microdomains can be resolved by classic confocal microscopy while nanodomains cannot.

(A) Pollen tube



Figure 21

Examples of membrane microdomains in plants cells. (A) The four microdomain types in a pollen tube along with some respective components and characteristics: shank, subapical, flank and apical. (B) Schematic of a leaf epidermal cells highlighting three proteins with polar repartitions: Breaking of Asymmetry in the Stomatal Lineage (BASL, red) in planar microdomains, desperado (DSO, purple) in apical microdomains and the boron transporter 1 (BOR1, blue) in basal microdomains. (C) Schematic of endodermal root cells. There are planar, equatorial, inner and outer polar microdomains. Some typical components within and general characteristics are given. From (Gronnier et al., 2018).

II. Models of membrane nanodomains across Nature's clades

We wish to understand the structure, the formation and the dynamics of membrane nanodomains. This requires the study of a wide variety of membrane nanodomain systems before achieving a more general model. This section will provide details on a few well studied membrane nanodomain systems.

A. In animals

1. Ras

Ras proteins form a family of three proto-oncogene GTPases: H-Ras, K-Ras and N-Ras. They are ubiquitously expressed. Mutations of K-Ras and N-Ras are often found in human cancers (Prior et al., 2012). Ras are involved in two signalling pathways related to cell proliferation and survival: the mitogen-activated protein kinases (MAPK) pathway and the P13K/Akt pathway (Braicu et al., 2019). Their G-domains, responsible for the binding of guanine nucleotides and effectors, are highly similar while their C-termini vary greatly. Ras anchors include a carboxymethyl S-farnesylated cysteine for all isoforms, palmitoylations for H- and N-Ras, and a cluster of lysines for K-Ras (Zhou and Hancock, 2015).

It is through this extremity that Ras attaches to the inner leaflet of the PM (Hancock, 2003). A detailed structural and functional analysis of membrane anchor mutants of K-Ras4B highlighted the mechanisms behind its interactions with membranes what it entails in terms of cell signalling (Zhou et al., 2017). Nanoclustering of K-Ras4B with asymmetric PS species (one saturated acyl chain and one unsaturated acyl chain) relies on a delicate balance of H-bonds, hydrophobic and electrostatic interactions that can easily be shattered by a single mutation. Indeed, a membrane anchor mutant was shown to interact and cluster with PI4,5P₂ rather than PS, resulting in altered cell signalling. K-Ras4B is an example of an animal oncoprotein where just one mutation can impact nanoclustering and subsequent interactions with its protein partners (Figure 22).



Figure 22

Atomic force microscopy (AFM) on K-Ras4B nanodomains. Bilayers of 20/5/45/5/25 DOPC/DOPG/DPPC/DPPG/cholesterol and K-Ras4B in a GDP-bound (left) or a GTP-bound state (right). K-Ras4B clusters in nanodomains ranging from 500 nm to 1µm only in the liquid-disordered (l_d) phase, never in the liquid-ordered (L_o)

phase. Nanodomains appear thicker when K-Ras4B is loaded with GTP. Scale bar: 2 μ m. Adapted from (Weise et al., 2011).

2. GM1

GM1 gangliosides (Figure 23) are sphingolipids critically involved in many metabolic and signalling pathways in animals cells, such as ion transport, neuronal differentiation, GPCRs, immune system reactions, neuroprotection, toxin and viral susceptibility (Ledeen and Wu, 2015). It is these latter aspects (toxin and viral susceptibility) that we will discuss now. Indeed, atomic force microscopy (AFM) reveals the formation of GM1 nanodomains in simple monolayer systems. Moreover, incubation with cholera toxin induces the formation of larger nanodomains, hinting at a direct interaction between GM1 and the cholera toxin (Yuan and Johnston, 2001). Similarly, the simian virus 40 (SV40) can target giant unilamellar vesicles (GUV) enriched in GM1 and produce invaginations, demonstrating the mean of entry of SV40 in animal cells exhibiting GM1 at their surface (Ewers et al., 2010).



Figure 23



3. Caveolae

Caveolae are invaginations of the PM. They were first observed in 1955, making it the first nanodomain system to be ever discovered (Yamada, 1955). Caveolae are formed by proteins called caveolins that bind to the PM's inner leaflet (figure 24). They are mainly involved in endocytosis, cell signalling (Lisanti et al., 1995; Williams and Lisanti, 2005) and cellular entry of pathogens such as SV40 (section II.A.2) and toxins like *Vibrio cholerae*'s toxin (CTX). Indeed, internalisation of SV40 utilises both GM1 gangliosides and caveolae nanodomain systems (Norkin and Kuksin, 2005; Pang et al., 2004; Parton, 1994). Drug-induced removal of cholesterol from the PM makes these initially relatively immobile structures quite mobile (Thomsen et al., 2002). Thus,

cholesterol seems not involved in their formation but may impact their biological activity. Lastly, caveolae are rigid structures: neighbouring caveolae tagged with different fluorophores do not mix, unless cholesterol is depleted (Tagawa et al., 2005).



Figure 24

A general description of caveolae. (A-B) Electron micrographs of adipocytes showing invaginations at the PM: caveolae. Notice how some caveola appear to be interconnected to each other. (C) Schematic of a caveola. Insertion of caveolins (blue) in the PM provoke the formation of a caveola. Their N-termini scaffolding domains are putatively involved in interactions with cholesterol while their central part is embedded in the PM. In addition, their C-termini domains bear palmitoylation sites. Modified from (Parton and Simons, 2007).

B. In yeasts: on different nanodomain distributions of many systems

(Spira et al., 2012) described an ensemble of nanodomain systems in yeast with distinct localisations. A distinction was made between patch-like systems of punctate domains (i.e. nanodomains) and network-like systems displaying varying levels of connectivity, excluded from nanodomains (Figure 25). Some nanodomains appeared larger than for animals (up to 1 μ m). This was attributed to a slower diffusion of membrane components compared to animal cells favouring their aggregation (Valdez-Taubas and Pelham, 2003). Knockoffs of ergosterol or sphingosine biosynthesis switched patch-like systems to network-like systems, indicating a loss of patch-like membrane repartition capability upon suppression of typical membrane nanodomain components (see section I.E.2.d), a proof that these patches could be nanodomains.



Screening the subcellular localisation of yeast proteins. Some cluster in patch-like domains while others adopt a network-like reparation, as if they were excluded from some membrane nanodomains. From (Spira et al., 2012).

C. In prokaryotes: flotillins

Flotillins and flotillin-like proteins are ubiquitous, appearing by convergent evolution in all clades of Nature (Rivera-Milla et al., 2006). YuaG is involved in the sporulation of *Bacillus subtilis*, localises at the PM's inner leaflet into nanodomains of size below 500 nm and is purified in DIMs enriched in PG and cardiolipin (Donovan and Bramkamp, 2009). Fluorescence microscopy with the lipid order-sensitive probe Laurdan demonstrated YuaG's ability to regulate membrane fluidity. Indeed, a knockoff of YuaG resulted in the coalescence of initially punctate ordered membrane domains into a cell-wide increase of lipid ordering. A similar phenotype was observed for a knockoff of squalene synthase, responsible for the synthesis of hopanoids, the bacterial equivalents of sterols (Bach and Bramkamp, 2013). This dependence on molecules with similar functions as sterols reinforces the idea of the existence of membrane nanodomains in bacteria. Finally, many of YuaG's interacting partners were found to co-cluster leading to propose a role for YuaG as maker of membrane hubs in various cell processes (Bramkamp and Lopez, 2015) (Figure 26).



Figure 26

Epifluorescence microscopy of *Bacillus subtilis* expressing CFP and FloT-GFP. Images use false-colours. From (Bramkamp and Lopez, 2015).

D. In plants: Rho of plants (ROP)

Ras proteins in animals and Rho proteins in yeast have a plant vegetal equivalent with Rho of plants (ROP), which exist and cluster into nanodomains. The molecular basis of its membrane-interacting properties are similar to animal Ras proteins (see section II.A.1) (Nagawa et al., 2010; Platre et al., 2019; Zhou et al., 2017) and will not be re-discussed here. Rather, the involvement of membrane nanodomains in signal transduction will be detailed. Auxin, a plant hormone involved in growth and development (Leyser, 2018), can remodel cellular distribution of PS, activate ROP6 and promote its PS-dependent nanoclustering (Platre et al., 2019). Conversely, variations in PS levels at the PM define ROP6-mediated auxin response. As a result, co-clustering of ROP6 and PS is an obligatory step in auxin signalling, as part of an analogue-digital-analogue signal converter (Figure 27) (Jaillais and Ott, 2020; Tian et al., 2007): (1) a certain amount of auxin interacts with its cognate receptors; (2) resulting in a given level of ROP6 nanoclustering; (3) defining a particular signal output level. This model, which was first hypothesised for Ras proteins (Tian et al., 2007), was further expanded to ROP in a recent preprint (Smokvarska et al., 2020).

ROP11 is pinned by microtubules forming a fence preventing the free diffusion of the protein. These ROP11 microdomains were shown to define cell shapes (Oda and Fukuda, 2012).



Figure 27

A model for the role of Ras and ROP nanodomains in cell signalling. (A) Let us assume that hormone receptors of the PM bind increasing concentrations of their ligand and subsequently recruit and activate ROP, which clusters in PM nanodomains, such as the more ligand is bound, the more ROP is clustered in nanodomains and the more of these nanodomains there are (analogue-to-digital conversion). Nanoclustering allows signal concentration, making the signalling pathway more efficient, such as the more ROP nanoclusters, the higher the signalling output (digital-to-analogue conversion). (B) ROP binds the PM via PS, the quantity of which, at the PM, positively regulates ROP signalling. From (Jaillais and Ott, 2020). Another plant membrane nanodomain system will be the subject of this manuscript: the removins.

III. Remorins

The multigenic, plant-specific, family of remorin proteins (Raffaele et al., 2007a) is a well-known marker of such nanodomains in plants (Demir et al., 2013; Gronnier et al., 2017; Mongrand et al., 2004; Raffaele et al., 2009a). Remorins were first discovered as soluble DNA-, oligogalacturonide- and pectin-binding phosphorylated receptors (Alliotte et al., 1989; Farmer et al., 1989; Jacinto et al., 1993) in tomato and potato plants. In the latter, this protein, having an apparent molecular weight of 34 kDa by SDS-PAGE, was named pp34 (Jacinto et al., 1993). It is only after it was cloned and turned out to be totally hydrophilic (albeit membrane bound) that it was renamed remorin, as a reference to the remora fish hanging up to boats or other fishes from *El Libro De Los Seres Imaginarios* (Borges and Guerrero, 1969), just like remorin is bound to the plasma membrane (Reymond et al., 1996).

To avoid spoiling this manuscript, contributions of articles in which I was involved were removed and replaced with their related interrogations and hypotheses as they were at the beginning of this PhD thesis.

A. Phylogeny

Remorins are split into six phylogenetic groups (Raffaele et al., 2007a). All bear a predicted C-terminal coiled-coil domain ended by a remorin C-terminal anchor (Perraki et al., 2012; Raffaele et al., 2013) and an N-terminal intrinsically disordered domain (IDD), where lie most differences between isoforms (Figure 28). It should be noted that group 3 remorins lack an IDD. In comparison, C-terminal domains have a well conserved size of about 110 residues. A brief overview of each group will be given.

Group 1 contains remorins from ancestral plants such as gymnosperms (i.e. naked seed plants such as conifers), ferns and early angiosperms (i.e. floral plants), from monocotyledons to eudicotyledons. These remorins possess proline-enriched IDDs (8.9% for group 1a and 14.4% for group 1b) of about 81 ± 11 residues with a high homology (Raffaele et al., 2007a). *In silico* analysis of group 1 IDDs reveals a propensity for phosphorylation, hinting at a putative regulatory mechanism and a functional diversity of these domains, as phosphorylation is a common mean to control and tune an IDD's function (Khan et al., 2017; Uversky, 2013). Group 1a remorins' gene expression is correlated with genes involved in cell wall biology and extracellular functions, while group 1b remorins' gene expression seems more related to genes involved with the Golgi apparatus or the PM (Raffaele et al., 2007a).

Group 2 contains isoforms coming mostly from legumes and poplars. Their IDDs are about 86 ± 7 residues long and bear no significant homology with group 1's IDDs. They are mainly expressed in eurosids, even though we should note that a REM2.1 has recently been evidenced in *Arabidopsis thaliana* (Gouguet et al., 2020).

Group 3 gathers remorins without IDDs. As such, it could be labelled as the shortest remorin group. This absence of N-terminal domain could indicate a more restricted set of functions performed by remorins from this group, with all the biological activity originating from the coiled-coil C-terminal domain and its membrane anchor. They are expressed in rosids.

Group 4 removins have IDDs about 160 ± 31 residues long, which are notoriously longer and different from group 1 IDDs, and these domains are enriched in both prolines and serines, highlighting a different set of biological functions. They are expressed in both monocotyledons and eudicotyledons.

Group 5 removes have more divergent C-terminal domains in addition to a 91 \pm 45 residues long IDD that is poor in prolines. This absence of conformation-restrictive aminoacids could be a hint at a higher order parameter of these N-terminal domains and a greater ability to form, upon interaction with a ligand, a complex structure.

Group 6 remorins are the longest with highly variable, 279 ± 89 residues long IDDs, for total lengths between 240 and 522 residues. Such discrepancies may indicate the existence of many yet uncharacterised subgroups.



Figure 28

Phylogenetics of the remorin family. All remorins share a common C-terminal coiled-coil signature. Their classification relies on their distinct N-terminal domains. Organisms are colour-coded as described in the inlet. Abbreviations: *Ac, Adiantum capillus-veneris; Ak, Amborella trichopoda; Ap, Allium cepa; At, Arabidopsis thaliana* (thale cress); *Cr, Ceratopteris richardii; Mc, Mesembryanthemum crystallinum; Mt,*

Medicago truncatula (barrelclover); Na, Nuphar advena; Nt, Nicotiana tabacum (common tobacco); Os, Oryza sativa (rice); Pa, Persea americana; Pd, Pinus taeda; Pi, Pinus pinaster, Pp, Physcomitrella patens; Ps, Picea sitchensis; Pt, Populus trichocarpa; SI, Solanum lycopersicum (tomato); St, Solanum tuberosum (potato); Wm, Welwitschia mirabilis; Zm, Zea mays (corn). From (Raffaele et al., 2007b).

B. Biological implications

The functions of remorins remain partly enigmatic although it is attributed some recurring roles such as in the regulation of cell-to-cell communication, plant immunity, symbiosis and stress resistance. Sections III.B.1 to III.B.4 are heavily inspired from the comprehensive bibliographic review of (Gouguet et al., 2020), currently in positive revision in Plant Physiology and of which I am co-author (see Annex). Thematics associated with remorins are summarised in (Figure 29), some of which will be discussed below. A complete list of known biological involvements is given at the end of Annex.



Summary of remorins biology. (A) Main biological roles of remorins (Gouguet et al., 2020). (B) Localisation (blue) or anti-colocalisation (red) of some remorins in nanodomains observed by spt-PALM (Jarsch et al., 2014).

1. Immunity

a. Against viruses

Plasmodesmata are membranous channels, about 20-40 nm large, connecting neighbouring cells, across PMs and cell wall. They are partially filled with ER from one side to another (Nicolas et al., 2017). Thus, they link the cytosols of virtually every plant cell, allowing the exchange of molecules. Consequently, PD are capital elements of plant development (Otero et al., 2016; Wu et al., 2016), metabolism (Ross-Elliott et al., 2017) and immunity (Benitez-Alfonso et al., 2010). On this last point, we should emphasise that, if metabolites and signalling molecules can travel from one cell-to-another, so can plant viruses by hijacking the PD to increase its size exclusion limit. It is on such a basis that we can grasp the role of remorins in viral immunity.

Overexpression of StREM1.3, slowed down the cell-to-cell propagation of the plant virus model Potato Virus X (PVX), as could be assessed by a decrease in PVX infection foci area. Conversely, underexpression of StREM1.3 increased the size of infection foci (Perraki et al., 2014; Raffaele et al., 2009a). The closure of PD is correlated with the deposition at their neck region of a $1,3-\beta$ -glucan named callose that seems to clog the pore. Conversely, the degradation of callose open the PD pore. The passage of the PVX through PD is made possible by its viral movement protein TGBp1 (Howard et al., 2004). Coincidentally, StREM1.3 interacts with TGBp1 and can be found at PD (Gronnier et al., 2017; Raffaele et al., 2009a). There are two non-exclusive hypothetical mechanism to explain these phenomenon: (1) StREM1.3 interacts with TGBp1 (Raffaele et al., 2009a) and prevents the passage of PVX components through PD; (2) StREM1.3 promotes callose deposition at PD, thus confining the PVX to the cells it already infected. This last mechanism is supported by the fact that co-expression of lone viral movement proteins TGBp1 from the PVX, or 30K from the Tobacco Mosaic Virus (TomV) or Hc-Pro from the Potato Virus Y, along with StREM1.3, restricted the cell-to-cell diffusion of free GFP, indicating PD closure (Perraki et al., 2014).

b. Against bacterial and fungal infections

Arabidopsis thaliana treated with flagellin 22, a short segment of a bacterial flagellin a known immune response elicitor, display a more phosphorylated *At*REM1.3, on residues S14 and T58, but not *At*REM1.2, which does not have these phosphorylation sites (Benschop et al., 2007). Yet, both *At*REMs co-localise to the same nanodomains (Jarsch et al., 2014). Similarly, upon overexpression of the bacterial effector gene AvrRPM1 from *Pseudomonas syringae*, AtREM1.2's expression increases (Widjaja et al., 2010). In the fight between plants and pathogens, extracellular vesicles (EVs) are excreted from both sides, containing proteins, metabolites and interfering RNAs. Among these, we find again *At*REM1.2, *At*REM1.3 and RIN4, a known interactor of *At*REM1.2 related to Pathogen-Associated Molecular Pattern (PAMP)-triggered immunity (PTI) against *Pseudomonas syringae* (Meyer et al., 2009; Micali et al., 2011). *At*REM1.3 is also known to bind ergosterol, which happens to be toxic for plants (Khoza et al., 2019).

StREM1.4 is up-regulated against Ralstonia solanacerum, a pathogen of potato (Kong et al., 2016), while StREM1.3 is enriched in PMs near haustoria of Phytophtora

infestans, i.e. fungal protrusions into plant cells (Bozkurt et al., 2014; Lu et al., 2012). In maize, *Zm*REM6.3 is genetically correlated to the resistance against *Steosphaeria turcica*, responsible for the northern leaf blight plant disease, and *Zm*REM1.3 infers resistance against *Puccinia polyspora* (Bilska-Kos et al., 2016; Jamann et al., 2014).

2. Symbiosis

If remorins are involved in protecting the plant from harmful pathogens, it is equally involved in symbiotic relationship with bacteria. Indeed, *Mt*REM2.2 is expressed in roots, especially in nodules as a necessary response to nodulation factors sent by symbionts during nodulation initiation. It acts as a scaffold for receptor-like kinases NFP, DMI2 and LYK3, as well as flotillin 4, all involved in nodulation (Lefebvre et al., 2010; Liang et al., 2018). Overexpression of *Lj*SYMREM1, a group 2 remorin, increases the number of nodules per root (Tóth et al., 2012). Finally, *Qr*REM4.1 is upregulated in case of symbiosis with *Piloderma croceum* (Tarkka et al., 2013).

3. Stress resistance

We described remorins as organisers of symbiosis and biotic stress (i.e. defence against pathogens). They are also involved in abiotic stress. A first hint would the ability of group 1 remorins to bind poly-galacturonic acid, a product of cell wall degradation (Reymond et al., 1996). A large bibliography records remorins from groups 1, 4 and 6 as inferring resistance to drought and salt-stress. Treatment with various plant hormones whose levels increase during biotic and abiotic stress, including abscisic acid, auxin, brassinosteroids, cytokinins, jasmonates and salicylic acid, also upregulates these remorins, confirming a general link between remorins and stress resistance (Gouguet et al., 2020).

4. Cell-to-cell communication

In rice, *Os*REM6.6 Grain Setting Defect 1 (GSD-1) mutant provokes an accumulation of starch and free sugars in leaf blades and a lack of starch in grains. This implies a correlation between this remorin and metabolite transfer across tissues. This can be corroborated to the presence of a subset of *St*REM1.3 at PD (Gronnier et al., 2017; Raffaele et al., 2009a).

C. Biophysics of membrane anchoring and nanodomain formation, with a special attention to *St*REM1.3

The remorin family is vast, yet one isoform received much attention not only for its biological implications but also as a model to study its membrane anchoring and nanodomain formation mechanism: *St*REM1.3. Many structural data are already available, making this isoform a candidate of choice to further our understanding of remorins' biophysics. However, other remorins will be discussed in order keep in mind the larger picture of learning about the biophysics of remorins.

1. Remorin C-terminal anchor (RemCA)

Removing the last 28 aminoacids of *St*REM1.3 turns it from an integral, tightly membrane-bound protein into a fully soluble protein (Perraki et al., 2012). This C-terminal feature, RemCA, is common to all remorins and has convergent analogues outside of plants sharing the same overall fold and aminoacid bias: a long helix lying at the membrane interface and a shorter C-terminal helix buried in the membrane, both linked by a small loop (Figure 29) (Raffaele et al., 2013). A comparison of RemCA domains between remorins of *Arabidopsis thaliana* and *Mt*REM2.2 displays the extent of their diversity, some bearing many S-acylated cysteines while others none, most showing a strong pattern of hydrophobic residues at their C-terminal and basic residues towards their N-terminal, with the notable exceptions of At4g36970, At1g53860, At4g67590 and *St*REM1.3 (Konrad et al., 2014). However, all of them are detected in PM nanodomains, but not necessarily the same nanodomains: some do colocalise while others exclude one another (Jarsch et al., 2014). This could imply that: (1) remorins may bind different and specific target at the PM or (2) actively exclude one another on a yet unknown basis (section III.C.4).



Figure 29

First structural studies on RemCA. *Left:* aminoacid composition bias of 117 RemCA from 26 species, compared to randomly selected 28 aminoacids long peptides from Uniprot. From (Raffaele et al., 2013). *Right:* first structural model of *St*REM1.3-RemCA from (Perraki et al., 2012).

A detailed biophysical analysis of *St*REM1.3's RemCA (*St*REM1.3-RemCA) and 16 mutants provided the structural basis for its PM nanodomain targeting (Gronnier et al., 2017). Lowering the PI4P content in the PM's inner leaflet of *Nicotiana benthamiana* by expressing the myristoylated/palmitoylated-phosphatidylinositol 4-phosphatase SAC1p enzyme, *St*REM1.3's PM targeting was largely abolished. Using a combination of solid-state nuclear magnetic resonance (ssNMR), Langmuir trough and infrared spectroscopy, *St*REM1.3-RemCA's fold was re-evaluated: the short C-terminal helix was replaced by an extended β -sheet (Figure 30). In this model, K192 and K193 (of the extended β -sheet) are positively charged and interact with PI4P through an electrostatic interaction with the inorganic phosphate in position 4 of the inositol.

Attempts at deciphering the aminoacid code responsible for *St*REM1.3-RemCA's membrane interacting properties, by mutant analysis followed confocal microscopy or super-resolution microscopy, revealed the anchor's net charge as an interesting parameter to follow: a shift from a non-charged WT anchor to negatively charge mutated anchor is correlated with a decrease in PM targeting. Surprisingly, a fine monitoring of some WT and mutated *St*REM1.3-RemCA, by single-particle tracking photoactivated localisation microscopy (spt-PALM), revealed that all variants were still able to make nanodomains, although with significantly different sizes, diffusion rates, relative areas and nanodomain localisation (Figure 31).



Figure 30

Structural model of *St*REM1.3's RemCA by molecular dynamics. Comparing to figure remca_2012_2013, the second helix of the latter is now an extended β-sheet in the former. In this model, PI4P specificity is explained by an electrostatic interaction between K192, K193 and the last inorganic phosphate of the polar head. From (Gronnier et al., 2017).

Critical mutations of StREM1.3-RemCA shown to abolish PM targeting decreased, if not cancelled, *St*REM1.3's ability to slow down cell-to-cell viral propagation (Perraki et al., 2014), proving the need of an optimal repartition of StREM1.3 at the PM localisation to ensure its anti-viral activity.



Spt-PALM analysis of EOS-tagged WT and mutated StREM1.3 in Nicotiana
benthamiana leaves (EOS is a fluorphore suited for spt-PALM). (A) Representative PALM image analysis for each protein with tessellation-based automatic image segmentation (images are automatically fragmented so that only one remorin occupies a given tile). Nanodomains are circled in red. (B) Diameter distributions of nanodomains for each protein. (C) Percentage of the PM occupied by nanodomains. (D) Percentage of EOS-tagged StREM1.3 in nanodomains. (E) Number of nanodomains per μm². From (Gronnier et al., 2017).

2. Oligomerisation domain

Early on, some group 1 remorins, including *St*REM1.3, were shown to spontaneously assemble into filaments *in vitro* (Figure 32) (Bariola et al., 2004). Cross-linking experiments and analytical centrifugation (although the latter was labelled as "data not shown") indicated a continuum of oligomeric states from degree 2, 4 and above. In opposition, size exclusion chromatography on *St*REM1.3 would indicate a homo-trimeric state (Perraki et al., 2012), but this experiment suffered from a poor choice of column and corresponding separation range. The ability of remorins to spontaneously make filaments certainly hinders the measurement of intermediate oligomeric states.

Beyond *in silico* analyses (Raffaele et al., 2007a; Reymond et al., 1996), the putative coiled-coil fold of the oligomerisation domain has yet to be proven.



Negative-staining electron microscopy of *St***REM1.3** (from potato) and *SIREM1.2* (from tomato) filaments, *in vitro*. Adapted from (Bariola et al., 2004).

3. IDD

Bioinformatic tools predict remorins' N-termini as IDD (Raffaele et al., 2007a). Although no experimental structural investigation can confirm this yet, if it is taken for granted, the study of this domain in interactions with partners of remorins would allow to grasp the structural implications of such associations.

Phosphorylation is a recurrent mean to change an IDD's properties, or to switch it from an unfolded inactive state to a folded active state, or *vice versa* (Khan et al., 2017; Nishi et al., 2013; Uversky, 2013). Remorins' IDDs are no exception (Marín and Ott, 2012), so if a non-phosphorylated IDD is indeed unfolded, what about its phosphorylated state? There again, no structural study of a remorin's IDD is available beyond sequence analysis.

4. Towards a model of StREM1.3 nanoclustering

The study of (Gronnier et al., 2017) was the first to formulate a hypothetical membrane nanoclustering mechanism of a remorin: (1) cytosolic, homo-trimeric *St*REM1.3 would bind to PI4P of the PM's inner leaflet and (2) cluster with one another together with sterols. The bundling of three RemCA domains within a single trimer would confer the protein a higher avidity for PI4P, and maybe other PIPs, than a lone *St*REM1.3-RemCA.

Although the first step, the PM binding, is easy to accept, given the evidence at our disposal (section III.C.1), the second step remains elusive. What promotes nanoclustering?

a. Remorin/lipid-lipid interactions: PIPs cluster on their own

Due to their big negatively charged polar heads conferring an inverted conical shape, PIPs have the interesting property to promote a negative membrane curvature and cluster (Bilkova et al., 2017; van den Bogaart et al., 2011; Ji et al., 2015; Picas et al., 2016). This nano-environment is reminiscent of our definition of nanodomains. The

presence of sterols could be explain by the presence of a pool of saturated PIPs in DIMs of plants (Furt et al., 2010).

In this aspect, the mere binding of *St*REM1.3 would be enough to ensure its nanoclustering. However, *St*REM1.3 binds tightly to the PM, as salt- and high pH shocks cannot alter its binding (Perraki et al., 2012). In this regard, it is an integral protein. We may expect protein insertion to modify if not shatter this pre-existing organisation.

At last, this mechanism alone cannot explain the coexistence of many different remorin-enriched nanodomains (Jarsch et al., 2014).

b. Remorin-remorin interactions: remorins tether with their peers

Remorins are likely to make oligomers through their putative coiled-coil domains (Raffaele et al., 2007a; Reymond et al., 1996), and they can make filaments *in vitro* (Bariola et al., 2004). Although these have never been unambiguously observed *in vivo*, the underlying self-assembling behaviour could persist. This would explain why remorins with high homology, such as those within a given phylogenetic group, could cluster together while divergent remorins could not. The structural basis of such discrimination is unknown. It could originate either from the oligomerisation domain or the IDD. It could also be directed by pinning, as we will see in the next section.

c. Remorin-cytoskeleton interactions: the cytoskeleton directs nanoclustering

Disruption of microfilaments or microtubules changed the localisations of *At*REM1.2 and *At*REM1.3 from DIMs to detergent-soluble membranes (DSMs) (Figure 33) (Szymanski et al., 2015). Coincidentally, for AtREM1.2, the characteristic dot-like pattern of fluorescently-tagged remorins observed by confocal microscopy turned into a more homogeneous PM distribution upon disruption of microfilaments but not microtubules. We should remind ourselves that confocal microscopy cannot resolve remorin nanodomains of about ≈80 nm in diameter (section III) (Gronnier et al., 2017; Raffaele et al., 2009a), so we can only conclude that the overall PM organisation of this remorin has been modified. In any case, these observations indicate an interaction between remorins and cytoskeletal components, namely actin and microtubules, to pin remorins (Dinic et al., 2013; Raghupathy et al., 2015; Tsuji and Ohnishi, 1986). Disruption of the cytoskeletal network would unleash these remorins from their nanodomains and grant them freer movement.



Relationship between the cytoskeleton and *At*REM1.2 or *At*REM1.3. (A) Mass spectrometry analysis of *At*REM1.2 content of *Arabidospsis thaliana* seedlings. (B) Principle of DRM (or DIM) purification. (C) Analysis of DRM purification by western blot on untreated (control), disrupted for actin (cyt-d) or disrupted for microtubule (oryzalin). (D) Confocal microscopy of *Arabidopsis thaliana* seedlings expressing YFP-*At*REM1.2 of untreated or treated with cyt-d or oryzalin. Scale bars: 20 µm. Adapted from (Szymanski et al., 2015).

IV. Objectives

As can be gauged from the previous section, a lot of unknowns hover in the realm of remorins. To work upon pre-existing knowledge, we will concern ourselves with the biophysics of *St*REM1.3 in membranes. We will be interested in answering the following questions:

1. What is the nanoclustering mechanism of *St*REM1.3?

What is *St*REM1.3's impact on membrane dynamics? How does it oligomerise? Is oligomerisation relevant in understanding its membrane interactions? How does it make filaments? The seminal work of (Gronnier et al., 2017) must be pursued to test and further our model of *St*REM1.3's nanoclustering.

2. What is the minimal set of partners required to make *St*REM1.3 nanodomains?

Can we reconstruct such domains *in vitro*? If we achieved it, it would mean that: (1) we understood how *St*REM1.3 nanodomains are formed and (2) we would have a powerful *in vitro* tool to study the formation and the behaviour of such nanodomains.

3. How interactors of *St*REM1.3 may regulate its relationship with membranes and biological functions?

The interactors and the precise mechanism by which *St*REM1.3 can block the cell-to-cell propagation of the PVX remain to be elucidated.

4. Professional context

This doctoral work is the fruit of a joint effort by a team of biologists, biochemists and remorin specialists at the *Laboratoire de Biogenèse Membranaire*, UMR 5200, CNRS led by Dr Sébastien Mongrand and a team of biophysicists and NMR spectroscopists at the Institute of Chemistry and Biology of Membranes and Nanoobjects, UMR 5248, CNRS led by Dr Birgit Habenstein. About a quarter of the work was performed in the "biology team" and the rest was done in the "NMR team", including protein production and purification. As such, and due to my student cursus, my outlook on the project is inherently focused on structural biology rather than plant biology. Nonetheless, I was trained to perform experiments belonging in either of these categories.

To achieve the aforementioned objectives, we disposed of many biophysical tools, whose description will be the subject of the next section.

V. Biophysical tools to study membrane nanodomains

The aim of this section is to list the biophysical tools of interest for the comprehension of this manuscript. Principles will be explained and relevance to our field of study indicated. It is not intended to be a full-length description of these tools.

A. Detergent insoluble membranes

Although no DIM purification will be performed in this manuscript, given the importance of this technique in membrane nanodomain research, it deserves to be discussed in more details.

Density gradient separation is a powerful biochemical tool to separate components of varying densities. Proteins have densities around 1.4 g/cm³ (Fischer et al., 2004; Quillin and Matthews, 2000), lipid membranes have densities \approx 1.05 g/cm³, water's density is 0.997 g/cm³ and a 60% (w/v) sucrose solution, a common maximum in such experiments, is 1.29 g/cm³. Here, proteoliposomes tend to behave, in terms of density, like pure liposomes. The membrane preparation is incubated at 4°C overnight in presence of 1% (v/v) Triton X-100. It is adjusted to 60% sucrose, covered with 40%, 35% then 30% sucrose and submitted to an ultracentrifugation. A short run of 2-3 h will maintain gradient discontinuity, while a longer run of 16h will turn the density gradient continuous. Solubilised proteins will be located at the bottom of the gradient while membrane proteins anchored to DIMs will float near the top of it (Figure 34) (Brown and Rose, 1992; Carter and Hakomori, 1981; Mongrand et al., 2004; Raffaele et al., 2009a; Yu et al., 1973).



Figure 34

Schematic of a typical DIM purification. Purified membranes are incubated with 1% Triton X-100 (w/v) at 4°C overnight. The sample is then adjusted to 52% sucrose and covered with layers of sucrose of decreasing concentrations. After ultracentrifugation (200000 g for 16h), DIMs will float at one interface near the gradient's top while soluble material will be pelleted. Protocol based on (Raffaele et al., 2009b).

The formation of DIMs is correlated to the enrichment in high-Tm lipids, such as sphingolipids and saturated phospholipids, and sterols (Schroeder et al., 1994), which is, as we saw in section I.E.2.d, an expected feature of many membrane nanodomain systems. Moreover, DIM purification is the only technique amenable to lipidomic investigations (Furt et al., 2010; Schroeder et al., 1994). However, it does not inform on spatial segregation: just because two proteins are enriched in DIMs does not imply they are segregated in the same membrane nanodomains: we saw, in the case of remorins, that many remorins seem to exclude each other while being enriched in DIMs (Jarsch et al., 2014). Also, some membrane nanodomain systems are not particularly enriched in lipids suitable to behave as DIMs, such as the Ras system that relies on

PS in the fluid phase (section II.A.1). Finally, the appearance of DIMs depends on the choice of detergent (Schuck et al., 2003), casting doubt on the results this purification technique may provide. This is the main reason to explain its decline.

B. Fluorescence microscopy

Recent definitions of membrane nanodomains increasingly emphasize spatial segregation, which may only be observed by some kind of microscopy. Light microscopy allows the study of both *in* vivo and *in vitro* samples in native or native-like conditions. Since sample fixation is not mandatory, one can also observe time-dependent phenomena.

1. The fluorescence phenomenon

Suppose a fluorophore F, whose size is nanometric, (1) receives an incident light carrying an energy E = h.c. λ^{-1} where h = 6.626.10⁻³⁴ m².kg.s⁻¹ is the Planck constant and c = 299792458m/s the celerity of light (Bureau International des Poids et Mesures, 2006). λ is such that an electron may jump from a ground electronic state S₀ to an excited electronic state S₁. Note the multiple vibrational energy states for each electronic state. Here, F is at the highest electronic and vibrational states S_{1,3} of our diagram. (2) Following a process called interconversion, F will relax to the lowest vibrational state S_{1,0} in about 10⁻¹²s. (3) F will relax further by returning to S₀ by emitting a photon. Yet, F carries less energy in (3) compared to (1), so the photon's energy will be less: E_{excitation} > E_{emission} so $\lambda_{excitation} < \lambda_{emission}$ (Figure 35) (Lakowicz, 2006). The whole process takes about 10⁻⁸s and although excitation is done at a specific $\lambda_{excitation}$ via a laser, fluorescence emission covers a range of $\lambda_{emission}$.



Figure 35

An explanation of the fluorescence phenomenon. A fluorophore F is in an electronic and vibrational state S_{0,1} of lowest energy. Following an excitation with a photon of energy h.v_{excitation}, F is excited to the highest electronic and vibrational state S_{1,2}. F will lose some energy through a process called internal conversion, reaching the lowest vibrational state S_{1,0}. Finally, F will relax to S₀ by emitting a photon of energy h.v_{emission}, such that h.v_{excitation} > h.v_{emission}.

2. Confocal microscopy

Epifluorescence microscopy differs from confocal microscopy in that the former illuminates the whole sample at once and recovers the fluorescence signal across all of it. The latter illuminates one point of the sample at a time and recovers the fluorescence signal from that point only (Figure 36). Due to a detection pinhole, photons will not bleed from nearby areas towards the detector, so the resolutions both in the (x,y) plane and along z are much higher. Assuming one can graft a fluorescent label without modifying the properties of a protein of interest, its membrane interacting properties can be studied. Lipid-binding fluorescent proteins can also be used to inquire on lipid repartition. Altogether, confocal microscopy allows the study of protein and lipid repartition at the cellular and sub-cellular scales. We already saw many examples of what this technique can do. We also saw it being replaced by techniques with higher resolutions. Indeed, let us discuss the resolution of confocal microscopy in the context of nanodomains.



Figure 36

General schematic of a confocal microscope. Right: lasers send photons to the specimen. *Bottom*: the scanner ensures only one point of the sample is imaged at any given time. *Top*: the emitted fluorescence signal is directed to detectors. Note the use of pinholes the lasers and before the detectors: these ensure out-of-focus photons are not detected, effectively increasing the resolution. From (Reichman, 2013).

First, we shall differentiate the act of detecting from that of resolving (Figure 37). Presented with a clear sky, our eyes may detect Mars: all we would see is a brighterthan-usual spot. Yet, our eyes cannot resolve Mars: we cannot distinguish any of its many features, as we can do for the Moon which appears, not as a spot, but as a sphere of a certain diameter, littered with craters and various shades of grey. To resolve Mars, a telescope is needed: an apparatus with a higher resolution. It is the same issue with light microscopy and nanodomains.



On the difference between detecting and resolving. (A) Moon and Mars seen from Earth. From (https://earthsky.org/todays-image/photos-videos-mars-brightestclosest-2018). (B) Mars seen 2500 km away from its surface by Viking 1 Orbiter, NASA, on the 22nd of February 1980 (MG07S078-334SP, NASA). (C) PM repartition of GFP-*St*REM1.3 by confocal microscopy in *Nicotiana benthamiana* leaves. (D) Spt-PALM of an EOS-*St*REM1.3 nanodomain microscopy in *Nicotiana benthamiana* leaves. (C) and (D) are adapted from (Gronnier et al., 2017).

Visible light, when meeting an obstacle or when passing through a hole of similar or smaller size than the light's wavelength, makes a diffraction pattern (Figure 38). This implies that even a perfectly focused beam of light will produce a diffraction pattern instead of a point. Moreover, no matter the fineness of the apparatus, its centre cannot be made smaller. John William Strutt Rayleigh found that this pattern's diameter d is defined by:

$$d = 0.61 \frac{\lambda}{NA}$$
(Eq.1)

where λ is the wavelength, NA = n.sin(θ) is the numerical aperture, with n the immersion medium's refractive index, θ the half-angle from the image to one half of the cone of light reaching the photomultiplier, and 0.61 = 3.83/2 π where 3.83 is the first minimum of a diffraction pattern behaving as a Bessel function J₀:

$$J_0(x) = \frac{1}{\pi} \int_0^{\pi} \cos(x \cdot \cos(\theta)) d\theta$$
 (Eq.2)



Illustrating the importance of Airy discs in light microscopy. *Top*: Airy discs are diffraction patterns of a perfectly focused beam of light through a given optical system. *Bottom*: 3D projection with intensity as height. (a-c) Increasing the microscope's numerical aperture (e.g. by switching from an air immersion objective of NA = 1 to an oil immersion objective of NA = 1.4). (d-e) When multiple spots (here two) are in proximity, it becomes difficult to assess the centre of each spot, making sample resolution harder. Adapted from (<u>http://zeiss-</u> campus.magnet.fsu.edu/print/basics/imageformation-print.html).

For example, one GFP, which is among the fluorophores with the smallest $\lambda_{emission}$ with $\lambda_{emission}$ (GFP) = 509 nm, observed with an oil-immersion objective whose NA = 1.40, yields a spot of size d = 222 nm. d is also called the Airy unit (AU) for the GFP channel.

In the case of remorins, nanodomains are smaller than 100 nm (Demir et al., 2013; Gronnier et al., 2017; Raffaele et al., 2009a), so it would not be possible to resolve a GFP-REM nanodomain with confocal microscopy. Although confocal microscopy can be used to assess overall PM organisation (Spira et al., 2012; Szymanski et al., 2015), its resolution is too poor to resolve many nanodomain systems. Alternative methodologies must be employed, regrouped in what is called super-resolution microscopy.

3. Super-resolution microscopy

a. Airy scan

The Airy scan relies on the idea that, although a diffraction pattern has an immutable size, the area it covers can be finely scanned then de-convolved to break the resolution limit up to 120nm in (x,y) and 340nm in z (Huff et al., 2017). This is done by using a 32-channel photomultiplier array detector *in lieu* of a single photomultiplier detector.

b. Stimulated emission depletion (STED)

STED uses the concept that the electronic states of a Jablonski diagram can be manipulated in both directions. Thus, photons of a given energy are sent until every illuminated fluorophore reaches the excited state S_1 just before a third photon of slightly smaller energy returns fluorophores from a small central area to S_0 , all before fluorescence occurs, effectively depleting fluorescence from an area smaller than 1 AU, thus breaking the resolution limit (Hell and Wichmann, 1994). It is the oldest super-resolution methodology, reaching a maximal resolution of 40 nm (Vicidomini et al., 2018).

c. Single-particle tracking photoactivated localisation microscopy (spt-PALM)

Spt-PALM relies on the fact that if only a single fluorophore emits fluorescence at a given time, one could locate the centre of its diffraction pattern and call it the position of the fluorophore. This requires that (1) only a subset of fluorophores is excited during each acquisition, so any diffraction pattern is due to exactly one fluorophore, and (2) this subset of excited fluorophores must change over many acquisitions so all fluorophores are imaged (Betzig et al., 2006; Liu et al., 2015). Classical fluorophores, such as GFP, cannot be used: some of them must remain "dark" and not fluorescent while others are excited and fluoresce (Dempsey et al., 2011; Rust et al., 2006). This requires the use of photoactivable (PA) fluorophores, such as PA-GFP (Patterson and Lippincott-Schwartz, 2002), that can be switched, by irradiation at a specific wavelength, from an active to an inactive state. First, a laser activates a subset of all targeted fluorophores. Then, a second laser excites the activated fluorophores. As this cycle is repeated, new fluorophores will be activated while a fraction of already activated fluorophores will be de-activated (Betzig et al., 2006). A resolution around 20 nm can be reached, and time series can be used to track single particles and reconstitute their trajectories, granting unique insights into the dynamics of live cells (Gronnier et al., 2017; Liu et al., 2015).

Super-resolution microscopy is becoming increasingly common. Yet, other kinds of microscopies, whose methodologies are older, more commonly available and easier to perform exist.

C. Electron microscopy (EM)

Recalling (Eq.1), one way to improve resolution would be to decrease λ . Yet, lenses do not exist for every wavelength of the light spectrum: none for ultraviolets and x-rays. De Broglie states the wave-particle duality:

$$\lambda = \frac{h}{p} = \frac{h}{mv}$$
(Eq.3)

The kinetic energy E_k of an electron is:

$$E_k = \frac{1}{2} m v^2$$
 (Eq.4)

where $m = 9.1.10^{-31}$ kg is the rest mass of an electron (Bureau International des Poids et Mesures, 2006). Combining (Eq.4) and (Eq.5):

$$\lambda = \frac{h}{\sqrt{2mE_k}} = \frac{h}{\sqrt{2meU}}$$
(Eq.5)

where $e = 1.6.10^{-19}$ C is the elementary electric charge (Bureau International des Poids et Mesures, 2006) and U the acceleration voltage applied to an electron. If we are to send an electron beam on a sample at acceleration voltage U, we will do so at a non-negligible proportion of the speed of light c = 299792458 m/s in vacuum. Thus, the special theory of relativity (Einstein, 1920) applies and eU must be corrected:

$$\lambda = \frac{h}{\sqrt{2meU(1 + \frac{eU}{2mc^2})}}$$
 (Eq.6)

If U = 200 kV then λ = 2.51 pm. Thus, electrons allow us to reach wavelengths in the picometre range, much below the length of a C-C bond (154 pm), and far below the few hundred nanometres of fluorescence microscopy, therefore providing a very high theoretical resolution.



Figure 39

Schematic of transmission electron microscope. An electron gun shoots an electron beam accelerated to a high voltage (100-300 keV). A series of condenser lenses (electromagnetic lenses) and diaphragms focus the electron beam onto the sample. Electrons passing through will encounter the objective lens to magnify the electron beam. A fluorescent screen may be employed for direct visualisation (by the

eye), or a CCD camera to record electron microscopy clichés. From Dr Graham Beards, published on Wikipedia (<u>https://en.wikipedia.org/wiki/Electron_microscope</u>).

However, the sample's nature, the quality of preparation and the instrumentation will be the most determining factors of the final resolution. The details of sample preparation will be discussed later. The sample is loaded onto a grid and inserted in an electron microscope, in vacuum, and bombarded with electrons (Figure 39). Note that the glass lenses of a fluorescent microscope are replaced here by electromagnetic

lenses. The possible fates of electrons when reaching the sample stage are many (Figure 40). Here, we deal with transmission electron microscopy (TEM), which makes use of transmitted and scattered electrons, even though we must acknowledge the existence of scanning electron microscopy (SEM), using back-scattered and Auger electrons and whose resolution is poorer. Electrons will reach a charge-coupled device (CCD) camera that will produce fluorescence upon excitation by transmitted electrons. This fluorescence will be recorded as a grayscale image, the brightness of a pixel being positively correlated to the amount of electrons received. It follows that a dark spot indicates electron absorption by the sample and thus the presence of a material of some kind blocking the electron's path.



Illustrating the possible fates of electrons in an electron microscope. *Top*: An incident electron may elastically (without losing energy) back-scatter (yellow), non-elastically (with energy loss) be reflected back from the sample's surface (orange), excite an atom that will return to its ground by emitting X-rays (violet) or Auger electrons (cyan) to the incident side. These electrons are captured in scanning EM (surface observation, low resolution). The sample will heat upon beam exposure. *Bottom*: an incident electron may pass, undisturbed, through the sample (black) or be scattered elastically (red) or not (blue). Adapted from Prof. Alain Brisson's lecture on EM.

Biological matter is primarily made of light atoms such as hydrogens, carbons, nitrogens, oxygens, sulphurs and phosphorus, along with a handful of heavier atoms. These light atoms do not block much electrons, so we say that biological matter does not yield much amplitude contrast: most incident electrons are either transmitted (black arrows) or scattered (red and blue arrows). Scattered electrons are phase-shifted upon interaction with the sample, experience a longer path towards the detector compared to transmitted electrons and are finally refocused by the objective lens and sent to the CCD camera for detection. Phase interference between all transmitted and scattered electrons in the image plane increases contrast: this is phase contrast. It is defined by the contrast transfer function (CTF) (Erickson and Klug, 1970; Thuman-Commike and Chiu, 2000):

CTF = sin
$$[-\pi \Delta z \lambda k^{2} + \frac{\pi C_{s} \lambda^{3} k^{4}}{2}]$$
 (Eq.7)

where C_s represents spherical aberration (waves passing through the edges of the lens being refocused too far away from the focal plane), Δz is the defocus, λ is the electron's wavelength and k is the spatial frequency (1/Å). CTF are plotted as functions of k (figure 41).



Simulation of the CTF for 400 kV electrons with an LaB6 gun and a Cs = 4.1 mm. The first zero of the contrast transfer function, denoted by the arrows, occurs at 1/12.5 Å⁻¹ for a defocus Δz of (a) 0.95 µm and at 1/21 Å⁻¹ for a defocus of (b) 2.7 µm. From (Thuman-Commike and Chiu, 2000).

If CTF (k) = 0, no contrast is yielded for features of spatial frequency k. As CTF (k) approaches 1 (or -1), k is imaged with a higher contrast (or a higher negative contrast). Thus, not all values of k may be represented, much less equally, in an EM image. If the defocus Δz is 0, phase contrast is minimal and there is not so much contrast at all in the final image. By increasing the defocus Δz , the CTF can be modified to gain contrast at the expense of resolution. Here, a higher Δz allows for an easier detection of the objects of interest, yet the resolution is lowered as many k values now have poor contrast. Hence one of the main issues of biological TEM: getting enough contrast.

In this manuscript, two methodologies will be employed: negative staining TEM and cryo-TEM.

1. Negative staining TEM

The idea is that, if the sample itself does not yield much contrast, an addition of heavy atoms to fill the empty spaces left by the sample on the grid will provide contrast instead. Therefore, electron absorption (i.e. dark spots) will be caused by the stain while the sample, which does not absorb much electrons, will remain light, hence the term of negative staining TEM: the object of interest will be light, not dark.

A sample, which may or may not be fixated (e.g. with glutaraldehyde), is blotted onto an electrically charged carbon-coated copper grid. Excess sample is removed by blotting against an absorbent paper then stained, typically with a uranyl acetate solution, and dried by blotting. The resolution, though heavily dependent on the sample's nature and the quality of the stain, can reach up to a few nanometres in the best cases (Scarff et al., 2018). As the sample is dehydrated and littered with heavy atoms, its shape may be deformed by the non-physiological conditions. Typically, liposomes observed by negative staining TEM can display a characteristic flat balloon shape (see Article IV).

2. Cryo-TEM

A way to maintain the physiological state of a biological object of interest and to reach atomic resolution exists: freezing the sample fast enough so it does not have time to react to the change of environment. This is cryo-TEM. Since there is no stain and biological matter is made of light atoms, contrast is lower compared to negative staining TEM. Nevertheless, the native-like state of the object of interest combined with the absence of stain to interact or hinder resolution made this technique famous over the past decades. By building image classes of an object of interest, which can be considered as 2D projections of such an object, a 3D model of it can be reconstructed. For a protein, a resolution as high as 1.8 Å can be reached (Merk et al., 2016).

In this manuscript, our use of cryo-TEM will be more modest: it will provide highresolution clichés of protein assemblies and proteoliposomes in native-like conditions at a nanometric scale.

D. Atomic force microscopy (AFM)

The first technique to ever be able to provide an image of individual atoms was atomic force microscopy (AFM) (Figure 42). Here, the tip of a cantilever will touch or gently scrap the surface of a sample, yielding a height map (Figure 43). In the field of biology, spreading vesicles of chosen composition on a mica surface and measuring their height map upon various treatments is common (Frederix et al., 2009; Garcia-Manyes and Sanz, 2010; Jung et al., 2010; Whited and Park, 2014; Yuan and Johnston, 2001). More specific measurements concern the force needed to rupture a bilayer (Garcia-Manyes and Sanz, 2010) or that which is required to unfold a protein domain (Pimenta-Lopes et al., 2019), the latter being sometimes referred to as force spectroscopy.

In this manuscript, we will attempt to reconstitute supported bilayers of just enough components and remorin to form nanodomains.



Principle of AFM. The sample (yellow) is placed atop a scanner that will move with high precision. A cantilever with a tip culminating with a single atom will tap or gently scratch the sample's surface. The tip's position is monitored by a laser pointed at the cantilever, which will reflect light onto another mirror then a photodiode. The cantilever will adjust its position to aim at the photodiode's centre. Thus, the sample's height map is generated. Adapted from (Zeng et al., 2012).



Figure 43 Xenon atoms (blue) on a mica surface (grey) manipulated to form the name IBM. From (<u>https://www.ibm.com</u>).

E. Computational modelling

Computational methods are well suited to apprehend protein-lipid nano-objects, assuming one can correctly describe each interaction using the right software and parameters (Marrink et al., 2019). Atoms are considered as balls and interactions are described as springs linking two atoms together. In this classical picture, since quantum mechanics refutes the "balls and springs" analogy, every energy level of every interaction between two atoms has the following shape as a function of distance (Figure 44). The entire system evolves to minimise its energy and maximise its entropy (Smith, 2014).



Lennard-Jones potential Φ as a function of distance r between two atoms. σ is the length scale, i.e. atoms' diameter. ϵ is the energy scale, i.e. interaction strength. r_{cut} is the value of r above which $\Phi = 0$.

The resolution issue is inverted: the bigger the system and the longer the simulation, the more computational power is required. A way to circumvent this issue is to lower the resolution of the modelled system: to replace a group of neighbouring atoms with similar properties by one single object. This methodology is called coarse grain (Chavent et al., 2016; Friedman et al., 2018). It allows the increase in size and time length of the modelled system. It has been thoroughly used to model lipid phase behaviour and nanoclustering (Ackerman and Feigenson, 2015; van den Bogaart et al., 2011; Gronnier et al., 2017; Javanainen et al., 2017; Koldsø et al., 2014). Moreover, it is becoming ever more successful at predicting protein structures (Baker, 2019).

F. Nuclear magnetic resonance (NMR)

Having performed many NMR experiments in the making of this work, I had the opportunity to delve further into the theoretical details of this technique. As a result, this subsection is a vulgarisation effort: an introductory lecture to NMR for beginners and particularly biologists, who may be interested in what this technique has to offer yet do not understand its functioning.

1. Spin

NMR deals with a fundamental property of particles called spin I, which is either positive or null. It denotes, if I > 0, an intrinsic angular momentum **L** linked to a magnetic momentum μ by:

$$\boldsymbol{\mu} = \boldsymbol{\gamma} \, \boldsymbol{\mathsf{L}} \tag{Eq.8}$$

where γ is a constant called the gyromagnetic ratio. If I = 0, the particle has no spin and it follows that **L** and **µ** are null vectors. As its name suggests, NMR deals with magnetic nuclei whose spin I > 0, a non-exhaustive list of which is provided (Table 3). The value of I describes the nucleus' behaviour in NMR in a way that will be explained later. The absolute value of γ gives the nucleus' sensitivity, its sign gives the spinning direction (trigonometric if positive, clockwise if negative). The natural abundance describes the occurrence of this isotope in nature. Thus, we may note, for example, that a proton (¹H) has a greater sensitivity and is highly abundant compared to ¹⁵N, which is much less sensitive and represents only a third of a hundredth of all nitrogens. As we discussed for EM, biological matter, particularly proteins and lipids, is mainly made of hydrogens, carbons, nitrogens, oxygens, sulfurs and phosphorus along with a couple of heavier elements. Aside from ¹H, performing ¹³C-, ¹⁵N- or ²H-NMR will require isotopic enrichment. We will make use of ¹³C-, ¹⁵N-labelled proteins produced by *Escherichia coli* BL21-DE3 on a minimal medium enriched in isotopically labelled metabolites, such as ¹³C-glucose and ¹⁵NH₄Cl, although more complex labelling schemes can be achieved by replacing these fully labelled metabolites with specifically labelled ones and labelled or unlabelled aminoacids (Habenstein and Loquet, 2016). Deuterated lipids, here PC with at least one deuterated acyl chain, are synthesised by means of organic chemistry (Bragina and Chupin, 1997).

Nucleus	¹ H	¹³ C	¹⁵ N	² H	³¹ P
I	1/2	1/2	1/2	1	1/2
γ (10 ⁷ .rad.s ⁻¹ .T ⁻¹)	26.75	6.73	-2.71	4.11	10.84
Natural abundance (%)	99.99	1.07	0.364	0.0115	100

Table 3

Nuclei of interest in biological NMR. I is the spin umber and γ is the gyromagnetic ratio (or sensitivity). Values from Bruker.

2. NMR spectrometer

To perform an NMR experiment, the sample must be placed in a strong magnetic field called **B**₀, whose value is expressed in tesla T (or N.m⁻¹.A⁻¹ or kg.s⁻².A⁻¹) (Bureau International des Poids et Mesures, 2006), typically produced by a superconducting magnet. It is held in place by a probe, whose main function is to send radiofrequency photons and record an electromagnetic induction of an electric current, all via a small coil surrounding the sample. This signal will be amplified, converted to lower frequencies to be digitalised then sent to the desk computer from which the experiment is controlled (Figure 45).



Simplistic schematic of an NMR spectrometer. It has two main components: a superconducting magnet cooled with liquid helium then liquid nitrogen and a probe, to hold the sample, send radiofrequency photons and record an electromagnetic induction of an electric current. From Dr Erick Dufourc's lecture on NMR.

3. Energy levels

Outside of the NMR spectrometer, with the approximation that the Earth's magnetic field is negligible, there is no significant magnetic field and magnetic momenta μ are randomly oriented, so the sum of all μ that is the net magnetisation **M** is null. Once inserted in the NMR spectrometer, the sample is submitted to a strong magnetic field **B**₀ along **z** from the superconducting magnet. Then, magnetic momenta μ will rearrange so $M_x = M_y = 0$ but $M_z \neq 0$. It is important to emphasise that all μ are not necessarily parallel to **B**₀ and **z** but their sum is null along both **x** and **y** (Figure 46).



Figure 46 Behaviour of 12 nuclei of spin I = 1/2 exposed to a magnetic field B₀.

Individual magnetic momenta μ will cancel each other along x and y but not along z: 7 μ_z are aligned with **B**₀ while 5 μ_z are aligned against it. This small discrepancy creates a net magnetisation **M** (red arrow) such that, at equilibrium, **M** = **M**_z. From Dr Erick Dufourc's lecture on NMR. An energy diagram may be drawn with a number of energy levels 2I+1 (Figure 47): it is called a Zeeman splitting. States of lower energies are slightly more populated, giving rise to a non-zero net magnetisation along **z**. In NMR, the populations of each energy level are immovable: do not try to make an analogy with fluorescence! NMR spectroscopy is about manipulating **M**, not modifying energy state populations *per se*.



Figure 47

Energy levels for a population of spins I = 1/2. Two states are possible: the α state, in which μ_z are aligned with B_0 , and the β state, in which μ_z are aligned against B_0 . As the α state has the lowest energy, it is more populated than β so $N_{\alpha} > N_{\beta}$. The energy gap is ΔE_m . For I > 1/2, there is no general rule to determine which state has the lowest energy: consider it a property of the nuclei of interest.

4. The simplest NMR experiment

We will consider the case of a population of nuclei whose spin I = 1/2, as this is the simplest case. Let us perform the simplest NMR experiment (also called a pulse sequence) possible: to move **M** away from **z** into the **xy** plane and to record an NMR signal (Figure 48).



Figure 48

The simplest NMR experiment: 90° - pulse acquisition. The probe sends a radiofrequency wave tuned at the nucleus' NMR frequency, with a power and for a duration required to flip **M** by 90° from **z** to **xy**. The pulse is a few microseconds long. Then, the probe switches, in a few microseconds, to receiver mode and acquires the electromagnetic induction of an electric current: the NMR signal. After a period of time T₁ (a few seconds), **M** is considered to have relaxed and is back along **z**, so another scan can begin.

This requires to move **M** by exactly 90°. We are confronted to a matter of size: how can we apply a magnetic field strong enough to compete and win over B_0 with the probe's tiny coil? Cranking up the power is bound to burn the probe long before it reaches the necessary power to do so. Instead, we will use a geometrical trick. Our nuclei are at equilibrium and are submitted to B_0 thanks to a superconducting magnet

that cannot be turned off, so they rotate on themselves at the exact Larmor frequency ω_0 :

$$\omega_0 = \gamma B_0 \tag{Eq.9}$$

In practice, not all nuclei may rotate at ω_0 exactly for reasons we will discuss soon. Until now, we were thinking in the fixed laboratory frame. We shall switch to a rotating frame of frequency ω_0 . Here, nuclei are immobile and the apparent magnetic field will be null, so we only need to apply a radiofrequency field of ΔE on the sample. Translated back into the fixed frame, this means applying an oscillating radiofrequency field of frequency ω_0 and energy ΔE :

$$\Delta E = \gamma \frac{h}{2\pi} B_0 = \frac{h}{2\pi} \omega_0$$
 (Eq.10)

Applying a radiofrequency field is called a pulse: it is generally short enough so nothing of interest happens in that period (generally a few μ s). The power, in watts, and the length of the pulse must be tweaked so **M** is flipped by exactly 90°. Once in the **xy** plane, **M** will rotate around and slowly flip back to **z**. After a period of time T₁, equilibrium is reached again: it is said that the spins have relaxed (Figure 49A). The precession of **M** is recorded by the probe as the electromagnetic induction of a current: it is the free induction decay (FID), the NMR signal.



Figure 49

From signal collection to the NMR spectrum. (A) Trajectory of **M** in the xy plane. After a 90° pulse, **M** is flipped from z to xy and starts precessing around and slowly flip back to z after a period of time T₁. (B) Recorded signal in y (black). Note the exponential decrease in signal (red) as **M** flips back to z. (C) A Fourier transform of (B) with subsequent phase correction yields an NMR spectrum with a single peak of frequency ω .

Nuclei have slightly different Larmor frequencies ω . This implies that every magnetisation of every nucleus with a particular ω will, in time, drift away from the others. The result is a rapid collapse of the NMR signal after a period of time T₂. The reason for such discrepancies in ω is chemical shielding σ (sometimes abbreviated CS):

$$\sigma = \gamma B_{\text{eff}} = \gamma (B_0 - \sigma B_0) = \omega_0 (1 - \sigma)$$
(Eq.11)

 σ arises from the influence of the atom's electrons on the nucleus' magnetic behaviour, which depend on the atom's chemical environment (e.g. nearby presence

of an electronegative atom, hybridisation states, aromaticity...). It follows that if $\sigma = 0$ then $\omega = \omega_0$.

We already discussed that a small difference in spin populations yields a non-trivial **M** that is responsible for the NMR signal. Consequently, the signal-to-noise ratio (SNR) is small too. Thus, most NMR experiments must be repeated enough, up to tens of thousands of scans for some of them, to yield an NMR spectrum with acceptable SNR.

5. NMR spectrum

We are interested in the relative abundance of each nuclei with a given Larmor frequency ω . However, the FID gives an amplitude as a function of time (Figure 49B). Fitting the FID with a decreasing exponential (which is the overall shape of any FID) and applying a Fourier transform (to switch from a time-domain to a frequency domain) with phase correction (the Fourier transform does not give phase values, it is computed by the software or found manually based on spectral line shapes) will yield an NMR spectrum: NMR intensity as a function of frequency. (Figure 49C) NMR intensities are defined such as the integral of a peak gives the relative amount of nuclei with a given ω . Most spectra employ normalised frequencies in parts per million (ppm):

$$\delta = \frac{\omega_{\text{sample}} - \omega_0}{\omega_0} \cdot 10^6$$
 (Eq.12)

This choice of unit makes comparison between spectra easier (e.g. ¹H resonates between 0 and 10 ppm no matter the spectrometer and its exact **B**₀). It is because ω may deviate from ω_0 due to σ that we can have peaks at δ values other than 0.

We just discussed the simplest NMR experiment: 90° pulse – acquisition. Most NMR spectroscopy requires pulse sequences significantly more complex than this, whose derivations require a basic knowledge of quantum mechanics that we successfully avoided above by making use of the simpler vector model, its classical analogue. Derivations of such experiments is not the subject of this chapter: the interested reader is redirected to (Keeler, 2016). However, we will discuss what these NMR experiments may teach us.

6. Liquid-state vs. solid-state NMR

A liquid sample has all molecules rotating rapidly and no preferential orientation: the sample is said to be isotropic and this results in sharp NMR peaks. In a solid sample, molecules do have preferential orientations and rotate slowly, if at all: the sample is said to be anisotropic and yields a poorly resolved spectrum with an important full width at half-height. If one is interested in orientation parameters, as in the case of ²H static ssNMR, such spectra can be satisfactory. If not, one needs to "remove" this anisotropy. Some light quantum mechanics are required.

Let H be the Hamiltonian operator (the quantum mechanical equivalent of the total energy function in classical mechanics) and let us consider some nuclei of spin I = 1/2. We are concerned only with spin states so any other contribution can be neglected (Levitt, 2008):

$$H = H_Z + H_{CS} + H_D^{IS} + H_D^{II} + H_Q$$
 (Eq.13)
where H_Z is the Zeeman contribution (from spin populations being split between energy levels), H_{CS} is the chemical shielding contribution (arising from chemical shielding), H_D^{IS} and H_D^{II} are the heteronuclear and homonuclear dipolar coupling contributions (accounting for the magnetic fields induced by the nuclei themselves) and H_Q is the quadrupolar coupling (due to the non-sphericity of the nucleus; it is null for non-quadrupolar nuclei, i.e. all those whose I = 1/2). The anisotropic contributions of each component are (Alia et al., 2009; Smith et al., 1992a, 1992b):

$$\begin{aligned} H_{CS} &= \frac{h}{2\pi} \gamma B_0 I_z \sigma_{zz} (\frac{3\cos^2\beta - 1}{2} + \frac{\eta}{2}\cos(2\alpha)\sin^2\beta) \\ H_D^{IS} &= \frac{h}{4\pi} \frac{\gamma_A \gamma_B}{r_{AB}^3} I_z^A I_z^B D_{zz} (\frac{3\cos^2\beta - 1}{2}) \\ H_D^{II} &= \frac{h}{4\pi} \frac{\gamma^2}{r^3} (3 I_z^A I_z^B - I^A I^B) D_{zz} (\frac{3\cos^2\beta - 1}{2}) \\ H_Q &= \frac{eQ}{4I(2I-1)} (3 I_z^2 - I^2) V_{zz} (\frac{3\cos^2\beta - 1}{2} + \frac{\eta}{2}\cos(2\alpha)\sin^2\beta) \end{aligned}$$
(Eq.14)

where α is a rotation along **z** (in the **xy** plane), β is a rotation along **y** (in the **zx** plane), η is the asymmetry parameter for a given parameter A such as $\eta = \frac{A_y - A_x}{A_z}$, I, σ , D and V are the spin, chemical shielding, dipolar coupling and quadrupolar coupling tensors, respectively, and eQ is the quadrupolar constant. Hz has no anisotropic contribution.

We wish that all the terms in (Eq.13) equal 0. In practice, we assume $\eta = 0$, so the sample's anisotropy depends on $3 \cos^2\beta - 1$:

$$3 \cos^2\beta - 1 = 0$$

$$\beta = 54.7^\circ = \text{magic angle}$$
(Eq.15)

The value of this magic angle happens to be the same than between a cube's diagonal and one of its consecutive edges. By tilting the sample at 54.7° from z, and therefore B_0 , and spinning it, to average the orientations of all molecules within the sample, one can remove anisotropic contributions: this is magic angle spinning (MAS) ssNMR. The spinning rate remains an instrumental limitation: it must surpass the value, in hertz, of the contributions it means to suppress. Thus, NMR rotors (Figure 50), in which samples are held, and their associated probes are designed for spinning at a dozen of kHz at least, over 100 kHz for a 0.7 mm (outer diameter) rotor.

7. ssNMR to study membrane nanodomains

As we saw in section I.E, membrane nanodomains are big, insoluble and noncrystalline objects. Therefore, X-ray crystallography and liquid-state NMR are disqualified. Remorins being rather small, about 23 kDa, cryoEM will have difficulties to analyse anything smaller than high-order stable oligomeric complexes. ssNMR is limited by spectral overlap, i.e. the presence of too many peaks in a small spectral region hindering or even prohibiting analysis. Overcrowded spectra may result from sample heterogeneity, i.e. more than one possible structural conformation of the protein under investigation, increasing the number of peaks or broadening them until analysis becomes difficult if not impossible. ssNMR can provide unique data on lipid dynamics using the lineshapes of 2H and 31P static ssNMR spectra – on a sample using 2H-doped lipids, and protein structure, using ¹³C- and ¹⁵N- MAS ssNMR on isotopically labelled proteins, where each peak will represent the protein's local structure at a given nucleus whose magnetic behaviour is modulated by its chemical environment, namely solvent and surrounding residues. Thus, a great part of our study will rely on ssNMR.



Figure 50

Image of ssNMR rotors. From left to right: 7 mm, 4 mm, 3.2 mm, 2.5 mm and 1.3 mm (of outer diameter) MAS ssNMR rotors from Bruker. These are zirconium oxide cylinders, closed at the top by a plastic cap with small wings. To achieve MAS, air is blown onto them. 2.5 mm rotors and below must also be closed at the bottom with a specific cap. A dark semi-circle is drawn to ensure a correct optical measurement of MAS frequency. A Canadian cent is given for size reference (19.05 mm in diameter, according to the Royal Canadian Mint, https://www.mint.ca). From Glenn Facey's blog on NMR, University of Ottawa, Canada (https://u-of-o-nmr-facility.blogspot.com/2008/04/how-much-sample-do-i-need-to-get-solid.html).

8. ssNMR to elucidate protein structures

Unlike liquid state NMR, which relies mostly on ¹H detection, ssNMR is mainly limited by dipolar coupling anisotropy (Thureau et al., 2014). As a result, ¹H detection is very tasking and requires very specific and expensive gear. Instead, we used simpler methodologies, where the loss of ¹H detection is be compensated by ¹³C-detected experiments – the nucleus with the second highest sensitivity γ between ¹H and ¹⁵N.

a. Cross polarisation (CP)

To enhance the signal-to-noise ratio (SNR), the higher gyromagnetic ratio γ of ¹H is used by transferring the magnetisation **M** to ¹³C via a technique called cross polarisation (CP). While ¹³C is on **z**, ¹H is brought into **xy**, then both nuclei are irradiated: ¹³C with a hard pulse and ¹H with a shaped pulse of increasing power and whose duration is termed "contact time". At a given power of the shaped pulse, the effective magnetic fields on both nuclei are equal, and magnetisation **M** is transferred from ¹H to nearby ¹³C. Acquisition is performed on the ¹³C channel while decoupling, i.e. a rapid succession of pulses, occurs on ¹H, to destroy dipolar couplings between

both nuclei. Note that CP works for any two nuclei, granted one is significantly more sensitive than the other.

It follows that a typical ssNMR analysis of a protein starts by recording a onedimensional ¹H MAS spectrum, which is not very informative besides determining the sample's temperature from the water's chemical shift (Wishart et al., 1995), defining the "0 ppm" using the peak provided by a molecule containing a single ¹H, such as DSS (sodium trimethylsilylpropanesulfonate), and detecting contaminants. Then, a ¹H-¹³C CP gives a one-dimensional ¹³C spectrum (Figure 51). Chemical shift references for all nuclei of all residues can be found in the literature (Merutka et al., 1995; Wang, 2002; Wishart et al., 1992; chemical shifts statistics of the Biological Magnetic Resonance Bank (BMRB)). One can discern C α (50-75 ppm) and aliphatic carbons (10-50 ppm), aromatic moieties (100-160 ppm) and amide carbons (Co, 160-180 ppm). When dealing with proteins, such a spectrum is often very crowded, and gives only little structural information: peak overlap hinders spectral assignment, i.e. matching peaks with their corresponding nuclei.



Figure 51

A typical ¹H-¹³C CP spectrum of a ¹³C-labelled protein sample. *Inlet:* pulse sequence. ACQ: acquisition, DEC: decoupling.

b. Proton Driven Spin Diffusion (PDSD)

We will perform a ¹³C-¹³C proton driven spin diffusion (PDSD) experiment to spread all peaks in a two-dimensional (2D) spectrum. This experiment also starts with a ¹H-¹³C CP. To get a second axis, the delay labelled as t1 in the pulse sequence (Figure 52A) is incremented and transformed into a frequency by Fourier transform, thus providing another spectral dimension. It follows that a 2D spectrum is a stack of 1D spectra with an incremented delay. The chemical shift coming from this incremented delay is F1 (vertical axis) and the one coming from the FID is F2 (horizontal axis). Since peaks are spread along one additional axis, peak overlap is reduced. Moreover, the intensity of cross-peaks (off-diagonal peaks) is positively correlated to the proximity of the nuclei they are assigned to, the rigidity of the protein and the length of the mixing time tm: increasing tm allows obtaining cross-peaks between nuclei that are further apart, at the cost of SNR. As an example (Figure 52B): let us consider an anisotropic sample composed of one hypothetical kind of molecule with three ¹³C. We are studying

it using ssNMR. Here is its fictional ¹³C-¹³C PDSD spectrum. We can neglect diagonal peaks, as they correspond to those of a ¹H-¹³C CP spectrum. Ideally, the spectrum is symmetric along its diagonal, so any peak on one side should also be on the other side (dashed lines). As a rule of thumb, if cross-peaks appear at chemical shifts corresponding to two different nuclei, then these nuclei are close in space, and the bigger the peaks, the closer they are. Here, 1 and 2 seem close from each other while 3 seems further away from 1 and 2. Thus, this experiment offers the possibility to derive distance constraints, which will prove useful in protein structure determination. The ¹³C-¹³C PDSD experiment has one drawback: peaks will only appear if the sample is rigid enough. This is not an issue for an amyloid protein core or a bacterial needle (Daskalov et al., 2015, 2016; Demers et al., 2014; Habenstein et al., 2012). However, flexible regions of a protein may disappear and proteins in a less rigid structural regime may lead to weak SNR, or lack thereof.



Figure 52

On how to read a ¹³C-¹³C PDSD spectrum. (A) Pulse sequence. t1 is an incremented delay: the experiment will be repeated at various t1 values, turned into frequencies reported on the vertical axis in (B). tm is the mixing time: increasing it allows to collect longer distance restraints between two nuclei, however it decreases SNR. (B) Schematic example of a ¹³C-¹³C PDSD spectrum of a molecule with three

¹³C, numbered from 1 to 3. In this experiment, cross-peaks (off-diagonal peaks) will appear if nuclei are close in space. Here, 1 and 2 seem close from one another while 3 seems far away from both. Ideally, the spectrum is symmetrical to its diagonal, as indicated by the dashed lines: "if 1 sees 2 then 2 sees 1".

c. Secondary chemical shifts

By calculating the difference between a measured chemical shift and the expected value for a random coil conformation, one can assess whether a given part of a protein is in a helical (α), if positive, or strand (β) conformation, if negative: this is called the secondary chemical shift or the chemical shift index (Wang, 2002; Wishart et al., 1992). Even if spectral assignment happens to be very tedious, this method can potentially already yield robust structural data.

9. IsNMR to study soluble intrinsically disordered proteins a. Generalities

Thanks to its high gyromagnetic ratio and abundance, IsNMR makes an extensive use of ¹H. Unlike ssNMR, a protein's structural fingerprint will not be a ¹³C-¹³C correlated spectrum but a ¹H-¹⁵N Heteronuclear Multiple Quantum Coherence (HMQC). Here, one peak will appear for every amide function. In practice, that is: one peak for every residue's backbone amide with the exceptions of prolines and the N-terminal residue (they have none), one peak per amide of a tryptophan sidechain and one doublet (two peaks in ¹H, one peak in ¹⁵N) per amide of an asparagine or a glutamine sidechain (Merutka et al., 1995) (chemical shift statistics of the BMRB).

b. On intrinsically disordered proteins (IDPs)

*St*REM1.3 has an N-terminal intrinsically disordered domain, which we will study here: the freshly purified protein will have no particular structure, making nuclei of identical residues appear in the same crowded spectral region, with severe spectral overlap as a consequence. Thus, spreading the peaks with 3D spectra is imperative. A plethora of NMR experiments exist to establish connectivities between residues, i.e. to identify a residue i by assessing which residues could be i-1 and i+1 (Figure 53).

A disordered protein is in exchange between a variety of conformations (Uversky, 2013). It can be said to "scan a conformational space". Upon interaction with a ligand or post-translational modifications, such as phosphorylation, this conformational space is modified. Thus, an otherwise disordered protein may fold, at least partially, upon such events. Mapping of phosphorylated residues or interaction surfaces can therefore be achieved by following the evolution of a protein's ¹H-¹⁵N HMQC spectrum over the course of phosphorylation or interaction with a ligand (Theillet et al., 2013; Kragelund and Skriver, 2020).



Some experiments to establish connectivities between residues in IsNMR. The ¹H and ¹⁵N chemical shifts (δ) for a backbone amide of a residue i are generally obtained by a ¹H-¹⁵N HMQC spectrum. The first set of 3D experiments (*top*) links i to i-1 by their carbonyls: e.g. the Co of i-1 yields a blue peak and a purple peak at the δ of 1H and 15N for i, but it yields only a blue peak at the chemical shifts of ¹H and ¹⁵N for i-1. The second set of 3D experiments (*bottom*) links i to i-1 by their C α : e.g. the C α of i-1 yields a cyan peak and a green peak at the δ of ¹H and ¹⁵N for i, but it yields only a green peak at the δ of ¹H and ¹⁵N for i-1. In addition, the HN(Co)CaCb gives the δ of the C β of i-1, allowing residue identification based on their chemical shifts.

c. On folded proteins

If our previously disordered protein is now folded, how do we determine its structure? Where ssNMR makes use of ¹³C-¹³C distance constraints obtained from ¹³C-¹³C PDSD spectra, IsNMR will employ ¹H-¹H Nuclear Overhauser Effect SpectroscopY (NOESY) to obtain distance constraints between neighbouring protons. Software such as ARIA2 and the CCPNMR Grid interface (Nilges et al., 2008; Rieping et al., 2005, 2007; Vranken et al., 2005) can perform computational modelling based on distance restraints obtained by IsNMR and ssNMR as well as secondary chemical shifts (section V.E.9).

10. ssNMR to monitor lipid dynamics: ²H ssNMR

ssNMR permits the study of membrane biophysics under native conditions. The nuclei of interest are ³¹P (section V.F.11), which allows easy characterisation of a sample's phase behaviour (Huster, 2014) and shape of liposomes (Pott and Dufourc, 1995), and ²H which, which informs us about lipid order (Seelig, 1977) and membrane thickness (Grélard et al., 2013). ²H, unlike ³¹P, is a poorly abundant isotope. To perform ²H NMR, one must use a deuterated reporter lipid, whose physical properties may vary slightly from its naturally abundant counterpart (e.g. DMPC has a phase transition temperature of 295K while DMPC-d54's, a DMPC deuterated on both acyl chains, is 293K).

Liposomes, bearing a deuterated reporter lipid, must be big enough to be anisotropic (>100 nm at least). We commonly employ multilamellar vesicles for their ease of production: hydration of a lipid film or a lipid powder followed by a few freeze-thaw-vortex cycles to ensure homogeneity in size and composition. The sample is then fitted into an ssNMR rotor.

Experiments are performed under static conditions. To enhance the signal-to-noise ratio (SNR), the quadrupolar interaction of ²H must be refocused by employing a quadrupolar spin echo pulse sequence (90°- τ -90°- τ -acquisition) *in lieu* of the classical Hahn echo pulse sequence (90°- τ -180°- τ -acquisition). This interaction will dominate the spectrum (Seelig, 1977). Spectral lineshapes being capital for data analysis, signal acquisition must be started slightly before the top of the echo so signal processing can be performed exactly at the top of it. Powder spectra thus obtained can rapidly give us a global order parameter <S_{CD}>_{chain} for C-D bonds of aliphatic chains by computing first order spectral moments M₁:

$$M_{1} = \frac{\int_{-\infty}^{+\infty} (\omega - \omega_{0}) g(\omega - \omega_{0}) d\omega}{\int_{-\infty}^{+\infty} g(\omega - \omega_{0}) d\omega}$$
(Eq.16)
$$< S_{CD} >_{chain} = \frac{\sqrt{3}}{\pi A_{Q}} M_{1}$$
(Eq.17)

where ω is the NMR frequency, ω_0 is the Larmor frequency, $g(\omega-\omega_0)$ is the spectral lineshape and A_Q = 167kHz (Burnett and Muller, 1971).

Plotting M₁ or $(S_{CD})_{chain}$ as a function of temperature is a powerful mean to assess the phase transition temperature of a sample or to detect any major difference in global order between samples. However, a finer spectral analysis is possible. Powder spectra are a sum of Pake doublets: one per kind of ²H in a sample; and an optional isotropic peak centred around 0 ppm originating from small liposomes (about < 100 nm in diameter) and deuteriums from water (one usually uses deuterium-depleted water to avoid this unwanted signal). In a DMPC-d54 sample, there are as many Pake doublets as carbon positions along the acyl chains. As the sample is anisotropic, if θ is the angle between the local membrane plane and B₀, a ²H NMR spectrum is a sum of ²H NMR subspectra for each value of θ . This forbids direct measurement of quadrupolar splittings. One must construct a ²H NMR spectrum for only one value of θ : a de-paked spectrum. De-Pake-ing (McCabe and Wassail, 1997) is a calculation routine made specifically to compute such a depaked spectrum. Quadrupolar splittings Δv_Q measured herein can be translated into local order parameters:

$$\Delta v_{Q}(\theta) = \frac{3}{2} A_{Q} \left| \frac{3\cos^{2}(\theta) - 1}{2} \right| S_{CD}$$
 (Eq.18)

Measurements of Δv_Q are refined by spectral simulation, along with half-height widths, interpreted as 1/T₂, and magnetically induced liposome deformation (Pott and Dufourc, 1995; Seelig, 1977). In our case, we are simply interested in getting S_{CD} values. The general procedure of ²H static ssNMR analysis is described in (Figure 54).

Plotting $|2^*S_{CD}|$ as a function of carbon positions along deuterated acyl chains informs us about lipid dynamics at the atomic level. (Grélard et al., 2013) describes a convenient way to compute membrane thicknesses from S_{CD} .



On data analysis of a ²**H static ssNMR experiment.** *Left*: the experimental spectrum (black) is recorded. A depaked spectrum at angle θ is calculated (blue), allowing the measurement of quadrupolar splittings Δv_Q as the difference, in Hz, between both peaks of a given Pake doublet. These Δv_Q are refined by spectrum simulation (red). *Top right*: Δv_Q are translated into local order parameters of C-D bonds S_{CD} as a function of carbon position along a deuterated acyl chain. From the here-obtained S_{CD}, a membrane thickness can be computed. *Bottom right*: computation of first spectral moments M₁ from experimental spectra over a range of temperatures T allows the monitoring of phase behaviour and thermotropism.

11. ssNMR to monitor lipid dynamics: ³¹P ssNMR

²H static ssNMR unlocks a wealth of structural information about the behaviour of lipids in membranes. Yet, another nucleus is also of interest: ³¹P, for which data interpretation is significantly easier. The experiment consists of a Hahn echo on ³¹P with ¹H decoupling. The resulting spectrum can be described by (Huster, 2014):

$$\omega = \omega_{iso} + \omega_{aniso} = \sigma_{iso} \gamma B_0 + \frac{\Delta}{2} (3\cos^2\theta - 1 - \eta \cos(2\alpha) \sin^2\beta) \qquad (Eq.19)$$

where α is a rotation along z (in the xy plane), β is a rotation along y (in the zx plane), η is the asymmetry parameter for a given parameter A such as $\eta = \frac{A_y - A_x}{A_z}$ and Δ is the anisotropy parameter. In practice, $\eta = 0$. The spectral lineshape informs on the orientation and the phase behaviour of the lipid bilayers (Figure 55). Spectral simulation allows to compute Δ , half-height widths, and magnetically-induced liposome deformation as described in (Pott and Dufourc, 1995).



On the different lineshapes of a phospholipid by ³¹**P NMR.** (A) In solution, there will be one single peak at δ_{isotropic}. (B, C) In oriented bilayers, due to the sample's anisotropy, this peak will shift to higher values if the normal **n** is parallel to **B**₀ (B) or to lower values if **n** is perpendicular to **B**₀ (C). (D) In large vesicles, the angle θ between **n** and **B**₀ may take any value. The probability for θ to take a given value defines the line shape (Pott and Dufourc, 1995), which is characteristic of a lamellar phase: with some imagination, one could picture a chair with its back to the right side. (E) For hexagonal phases (here an inverted hexagonal lipid phase made of POPE at 20°C), a mirrored line shape is obtained: a chair with its back to the left side. The width of this line shape is halved compared to its lamellar counterpart (obtained at 5°C) (Cullis and de Kruyff, 1976). *Inlet*: phospholipids in an inverted hexagonal phases for more details. (A-D) is modified from (Huster, 2014) and E is modified from (Cullis and de Kruyff, 1976).

In our case, we want to confirm that our samples are indeed in a lamellar phase: that we are indeed studying lipid bilayers. A minor isotropic contribution is also expected from small liposomes (roughly < 100 nm in diameter).

Article I

Coiled-coil oligomerization controls localization of the plasma membrane REMORINs

Denis Martinez, **Anthony Legrand**, Julien Gronnier, Marion Decossas, Paul Gouguet, Olivier Lambert, Mélanie Berbon, Loris Verron, Axelle Grélard, Véronique Germain, Antoine Loquet, Sébastien Mongrand, Birgit Habenstein

> Journal of Structural Biology 2018 206:1, p12-19

This first article reports on a work that began before my master 2 internship. One of the main focus of this internship was to set up the production and purification of WT and truncated StREM1.3. The work of (Gronnier et al., 2017) had just been published, consecrating the first biophysical analysis of RemCA, and we wondered how the, then putative, coiled-coil oligomerisation (Raffaele et al., 2007a) would come into play regarding membrane interactions. Initial protein constructs had very low production yield: a few milligrams at best. We focused our efforts on the best candidate of all, REM₈₆₋₁₉₈, and, after a few optimisations, managed to produce and purify about 20 mg per litre of LB medium – which became 40 mg per litre later on. When we dialysed the eluted REM₈₆₋₁₉₈ to remove imidazole, we noticed the sample had turned turbid. Negative staining electron microscopy revealed a branched filament architecture. If such ordered structures were made possible by joined assembly of oligomerisation domains, we expected to observe a strong ¹³C-CP ssNMR signal originating from it. Therefore, we undertook a structural analysis of these filaments using ssNMR, cryoEM and molecular modelling. Taking into account previous data on StREM1.3's oligomeric state, we built a structural model of homotrimeric coiled-coil domains of REM₈₆₋₁₉₈ in filaments, in which the oligomerisation's driving force seemed to be hydrophobic contacts between conserved residues. Disruption of the coiled-coil in vivo yielded proteins with diminished PM targeting.

With the help of Mélanie Berbon and Dr Denis Martinez, and the participation of Loris Verron, I set up and performed the production and purification of REM₈₆₋₁₉₈, as well as the sample preparation and observation for electron microscopy. I also participated to the writing of the revised article.

ARTICLE IN PRESS

Journal of Structural Biology xxx (xxxx) xxx-xxx



Contents lists available at ScienceDirect

Journal of Structural Biology



journal homepage: www.elsevier.com/locate/yjsbi

Coiled-coil oligomerization controls localization of the plasma membrane REMORINs

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ARTICLE INFO

Keywords: REMORIN Nanodomain Membrane protein Solid-state NMR Protein filament

ABSTRACT

REMORINS are nanodomain-organized proteins located in the plasma membrane and involved in cellular responses in plants. The dynamic assembly of the membrane nanodomains represents an essential tool of the versatile membrane barriers to control and modulate cellular functions. Nevertheless, the assembly mechanisms and protein organization strategies of nanodomains are poorly understood and many structural aspects are difficult to visualize. Using an ensemble of biophysical approaches, including solid-state nuclear magnetic resonance, cryo-electron microscopy and *in vivo* confocal imaging, we provide first insights on the role and the structural mechanisms of REMORIN trimerization. Our results suggest that the formation of REMORIN coiledcoil trimers is essential for membrane recruitment and promotes REMORIN assembly *in vitro* into long filaments by trimer-trimer interactions that might participate in nanoclustering into membrane domains *in vivo*.

1. Introduction

Membrane nanodomains are dynamic cellular signalling platforms in the plasma membrane of a size of up to a several hundred nanometers that execute essential messaging events between cells and their surroundings (Lingwood and Simons, 2010). Selective dynamic recruitment of specific proteins mediates the formation of functional membrane sub-compartments, related to signal transduction (Grecco et al., 2011) and membrane trafficking (Simons and Sampaio, 2011). Clustering of proteins into membrane compartments is notably driven by protein-lipid and protein-protein interactions (Lingwood and Simons, 2010). A significant number of membrane proteins are involved in protein-protein interactions leading to homo- or hetero-oligomers (McBride et al., 2017). Protein homo- or hetero-oligomerization has been proposed to mediate protein clustering (Kasai and Kusumi, 2014; Suzuki et al., 2012). Moreover, specific nanoclustering of proteins can drive functional compartmentalization, i.e. distinct nanodomains can be enriched in distinct nanodomain-associated proteins (Bucherl et al., 2017; Jarsch et al., 2014). As a consequence of the complexity of the system, i.e. proteins associated to membrane domains, insights into the structural and mechanistic organization of these proteins are sparse.

The REMORIN family consists of six phylogenetically distinguishable groups (Raffaele et al., 2007) of plant-specific nanodomain-organized proteins of the inner-leaflet of the plasma membrane (PM). Members of the REMORIN family carry out multiple functions related to biotic and abiotic stimuli (Jarsch and Ott, 2011; Lefebvre et al., 2010; Raffaele et al., 2009; Gui et al., 2014). In contrast to a highly variable N-terminal region, putatively implicated in protein-protein interactions (Marin et al., 2012; Toth et al., 2012), all REMORINS contain a conserved C-terminal region that encompasses a predicted coiled-coil domain (Bariola et al., 2004; Raffaele et al., 2007; Reymond et al., 1996), as well as a short membrane anchor domain (Gronnier et al., 2017; Perraki et al., 2012).

Coiled-coil domains are frequent motifs observed in proteins promoting multimerization towards homo- and also hetero-oligomers (Crick, 1953; Kohn et al., 1997; Lupas and Bassler, 2017). Proteins of the REMORIN family can oligomerize *in vivo* and *in vitro* into a

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https://doi.org/10.1016/j.jsb.2018.02.003 Received 19 November 2017; Received in revised form 25 January 2018; Accepted 12 February 2018 1047-8477/ © 2018 Published by Elsevier Inc.

D. Martinez et al.

filamentous state, possibly mediated by the coiled-coil domain (Bariola et al., 2004; Perraki et al., 2012). The *in vivo* functions of REMORIN oligomerization are not understood but they might include protein clustering to assure the nanodomain-mediated tasks. Nanoclustering of proteins is proposed to play an essential role in the segregation and functionalization of membrane rafts (Simons and Sampaio, 2011). Subtype Group 1 REMORINS form trimeric coiled-coil superstructures *in vitro* (Perraki et al., 2012) that lead to the formation of filamentous complexes (Bariola et al., 2004).

Our studies on the membrane anchoring mechanism and nanodomain targeting have recently revealed unconventional protein-membrane interactions mediated by the last 28 residues of the C-terminal region (REMORIN C-terminal anchor, REMCA) (Gronnier et al., 2017) that anchors to the surface of the plasma membrane without providing any transmembrane segment. The role of REMORIN oligomerization during this process remains obscure.

Magic-angle-spinning (MAS) solid-state nuclear magnetic resonance (SSNMR) and cryo-electron microscopy (cryo-EM) are both powerful emerging techniques useful to investigate assembled biomolecules in their native states. These techniques have the complementary characteristics of providing atomic data (reviewed for example in (Habenstein and Loquet, 2015; Loquet et al., 2013; Meier and Bockmann, 2015; Tycko and Wickner, 2013; Weingarth and Baldus, 2013)) and detailed shape/symmetry information (reviewed for example in (Bai et al., 2015; Earl et al., 2017; Jiang and Tang, 2017; Vonck and Mills, 2017)), respectively.

We have here taken advantage of several biochemical, biophysical and bioinformatic tools to decipher the role and the structure of REMORINs' assembly. We report that multimerization of REMORINs into trimers confers their membrane affinity and could participate in the nanoclustering into membrane domains. Establishing homogeneous filament formation of the protein core involved in oligomerization allowed us to combine SSNMR and cryo-EM to gain insights into the supramolecular architecture of oligomerized REMORIN filaments.

We can thus add another piece to the puzzle of the unconventional mechanism of membrane binding and nanodomain recruitment of the REMORIN family. The coiled-coil superstructures of the REMORINs hold functions resulting from the assembled complexes rather than from their monomeric subunits.

2. Material and methods

2.1. Expression, purification and filament formation of recombinant StREM1.3 $_{\rm 86-198}$

E. coli BL21 (DE3) strains were transformed with a pET24-StREM1.3(86-198)-His₆ vector, containing the DNA encoding for residues 86-198 of REMORIN1.3 of Solanum tuberosum, and plated onto LB agar plates containing 30·g/mL kanamycin. A flask containing 10 mL of LB medium was inoculated with a single colony and incubated overnight at 37 °C under shaking at 200 rpm. The culture and expression of uniformly labelled ¹⁵N,¹³C StREM1.3(86-198)-His₆ (hereafter called REM_{H6(86-198)}) was performed in M9 medium supplemented in ¹⁵NH₄Cl and ¹³C_{6-D}-glucose as nitrogen and carbon sources. The labelled M9 medium was inoculated with 2% (v/v) of the unlabelled LB preculture and incubated at 37 °C under shaking until the OD₆₀₀ reaches 0.7-0.8. At this stage, 1 mM IPTG was added to the culture for 20 h protein expression at 18 °C. Cells are then harvested by centrifugation (6000g, 10 min, 4 °C) and resuspended in the lysis buffer (20 mM HEPES, 150 mM NaCl, 20 mM imidazole, 1 mM PMSF, 1 tablet of Complete (Roche), 0.02% NaN₃, pH 7.4). Cells were lysed by sonication on ice for 3 min, 30% magnitude (30 s pulses, 30 s intervals). The suspension was centrifuged at 15 000g for 15 min at 4 °C to recover the supernatant. The affinity purification was realised with the Äkta Pure 25 HPLC system (GE Healthcare Life Sciences) using a His-trap HP affinity column equilibrated with 5 column volumes of washing buffer

(20 mM HEPES, 150 mM NaCl, 20 mM imidazole, 0.02% NaN₃, pH 7.4). The protein was eluted with a linear gradient from 0 to 100% of elution buffer (20 mM HEPES, 150 mM NaCl, 500 mM imidazole, 0.02% NaN₃, pH 7.4) which led to a final yield of 10 mg of pure ¹⁵N,¹³C labelled protein per liter of culture (Fig. S1). Protein filaments were obtained by dialyzing overnight at room temperature the eluted sample against the assembly buffer (10 mM HEPES, 50 mM NaCl, 0.02% NaN₃, pH 7.4). Protein filaments of REM(86-198), i.e. REM_{H6(86-198)} lacking the N-terminal His6-tag, were prepared as follows. Affinity purification was performed as described above using a different washing buffer (50 mM Tris, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.02% NaN_3 , pH = 8.1) and elution buffer (50 mM Tris, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 500 mM imidazole, 0.02% NaN₃, pH = 8.1). REM_{H6(86-198)} contains a TEV cleavable His₆-tag at its Nterminus. Eluted proteins and the TEV protease were mixed in a 100:1 (w/w) ratio, incubated at 4 °C overnight under shaking and dialyzed against 1L of assembly buffer as described above to obtain REM(86-198) filaments.

For ssNMR studies, filaments of REM(86-198) lacking the N-terminal His6-tag were prepared with a different protocol to enhance purity / yield of the sample. Expression and affinity purification was performed as described for filaments of uniformly ¹⁵N, ¹³C-labelled REM_{H6(86-198)}. Eluted protein fractions were pooled and adjusted to 0.5 mM EDTA and 1 mM DTT. Eluted proteins and TEV protease were mixed in a 100:1 (w/w) ratio, incubated at room temperature for 3 h under shaking and dialyzed overnight at 4 °C against the following buffer: 20 mM HEPES, 150 mM NaCl, 0.02% NaN3, pH = 7.4. After dialysis, urea was added to the proteins up to 7 M urea and $\text{REM}_{(86-198)}$ was retrieved on a His-trap HP column with the following washing buffer: 20 mM HEPES 150 mM NaCl 7 M urea pH = 7.4; and elution buffer: 20 mM HEPES, 150 mM NaCl, 7 M urea, 500 mM imidazole, pH = 7.4. REM₍₈₆₋₁₉₈₎ was enriched in the flowthrough. Uncleaved $\operatorname{REM}_{\operatorname{H6(86-198)}}$ and TEV protease, containing a His₆-tag, bound to the column and were then eluted. Fractions containing REM₍₈₆₋₁₉₈₎ were pooled and concentrated in an Amicon Ultra-15 centrifugal filter unit with MWCO = 3 kDa up to 0.8 mM, then dialyzed at room temperature against the assembly buffer (10 mM HEPES, 50 mM NaCl, 0.02% NaN3, pH = 7.4). The sample was then incubated at 37 °C for 3 h under shaking then at room temperature for 7 days.

2.2. Coiled-coil prediction and sequence alignment

StREM1.3₍₈₆₋₁₉₈₎ coiled-coil domain was identified using the software COILS (Lupas et al., 1991) and helical wheel diagrams for coiledcoils were built with DrawCoil 1.0 (http://www.grigoryanlab.org/ drawcoil/).

Arabidopsis REMORINs were retrieved from (Raffaele et al., 2007). Protein alignment was computed using MUltiple Sequence Comparison by Log-Expectation (MUSCLE; (Edgar, 2004)) using BLOSUM62 matrix, an –sv profile scoring method with following parameters; Anchor spacing:32, diagonal break:1, diagonal length:24, diagonal margin:5, gap extension penalty:-1, gap open penalty:-12, hydro:5 and hydro factor1.2) through the JABAWS server (Troshin et al., 2011). Sequence alignments were rendered via the Jalview plugin (Waterhouse et al., 2009) (Fig. S2).

2.3. Microsomal purification and Western blot analyses

Microsomal and soluble fractions from *Nicotiana benthamiana* leaves expressing GFP-StREM1.3 constructs were prepared as described in Perraki et al., 2012. Each fraction was analyzed by western blot using antibodies against StREM1.3 (Raffaele et al., 2009).

2.4. In vivo confocal imaging

Live cell imaging was performed using a Leica SP5 confocal laser

D. Martinez et al.

scanning microscopy system (Leica, Wetzlar, Germany) equipped with Argon, DPSS and He-Ne lasers and hybrid detectors. Two days after agro-infiltration *N. benthamiana* leaves samples were transferred between a glass slide and a cover slip in a drop of water. GFP fluorescence was observed with excitation wavelengths of 488 nm and emission wavelengths of 490 to 550 nm. Experiments were performed using strictly identical confocal acquisition parameters (e.g. laser power, gain, zoom factor, resolution, and emission wavelengths reception), with detector settings optimized for low background and no pixel saturation. Pseudo-colored images were obtained using the "Red hot" look-up-table (LUT) of Fiji software (http://www.fiji.sc/).

2.5. TEM and cryo-EM

REM_{H6(86-198)} filaments were applied to previously glow-discharged carbon-coated copper 300 mesh grids and stained with a 2% uranyl acetate (w/v) solution. REM_{H6(86-198)} filaments were also prepared for cryo-EM. For the latter, lacey carbon copper grid were submitted to a standard glow discharge procedure and flash-frozen into a liquid ethane bath using EM GP (Leica). Specimens were observed under low-dose conditions using a cryo holder (Gatan, USA). Observations were performed with a FEI Tecnai F20 electron microscope operating at 200 kV and images were acquired using a digital 2 k x 2 k USC1000 camera (GATAN). Measurements of the repetitive patterns were calculated on FFT images of 10 selected areas. REM₍₈₆₋₁₉₈₎ filaments lacking the N-terminal His₆-tag were stained and applied to carbon-coated copper grids as described above. TEM images were recorded under low-dose conditions on a FEI CM120 120 kV FEI electron microscope using a Gatan USC1000 2 k X 2 k camera.

3. 3D modelling

The 3D model of StREM1.3₍₁₁₇₋₁₆₆₎ was constructed by homology using the server I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Zhang, 2008). Coiled-coil trimers were assembled using the SymmDock server (Schneidman-Duhovny et al., 2005) and a supplementary energy minimization step was performed with Yasara (http:// www.yasara.org/minimizationserver.htm) (Krieger et al., 2009).

3.1. Solution NMR spectroscopy

 1 H, 15 N SOFAST-HMQC (band-Selective Optimized-Flip-Angle Short-Transient Heteronuclear Multiple-Quantum Correlation (Schanda et al., 2005)) experiment was carried out at 313 K on a protein sample containing 200 μ M REM₍₈₆₋₁₉₈₎, 20 mM HEPES, 150 mM NaCl, 0.02% NaN3, pH 7.4. Spectra were recorded on a Bruker Avance Neo 700 MHz spectrometer equipped with a 5 mm TXI 1H/13C/15 N/2H probe. The spectrum was acquired with 512 scans, 128 and 2048 complex points in F1 and F2 dimensions, respectively. Frequencies were calibrated according to DSS signal. The NMR data were processed using the TOP-SPIN 4 software (Bruker Biospin).

3.2. Solid-state NMR spectroscopy

MAS solid-state NMR experiments were performed on a 600 MHz (REM_{H6(86-198)}) and 800 MHz (REM₍₈₆₋₁₉₈₎) ¹H Larmor frequency spectrometer (Bruker Biospin) using 3.2 mm MAS probes. Solid-state NMR rotors were filled with 10 mg of REM_{H6(86-198)} or (REM₍₈₆₋₁₉₈₎) filaments and span at 11 kHz for spectra acquisition. Sample temperature was set between 0 and 5 °C according to DSS signal used as an internal reference (Bockmann et al., 2009). A ramped CP with a 1 ms (REM_{H6(86-198)}) and 0.7 ms (REM₍₈₆₋₁₉₈₎) contact time was used for the ¹H-¹³C cross-polarization (CP) transfer. An acquisition time of 20 ms and 2 k scans were used for 1D ¹H-¹³C CP. For the 2D spectrum, the ¹³C-¹³C polarization transfer was performed with proton-driven spin diffusion (PDSD) applied for a mixing time of 50 ms to detect intra-residue

correlations. Proton decoupling during the acquisition was achieved using a SPINAL-64 decoupling sequence (Fung et al., 2000). Acquisition times of 20 ms and 6.5 ms (REM_{H6(86-198)}), 20 ms and 7.5 ms (REM₍₈₆₋₁₉₈₎) were chosen for the direct and indirect dimensions, respectively. The total number of scans was 960 and 640 for a total experiment time of 6 and 7 days approximately, for REM_{H6(86-198)} and REM₍₈₆₋₁₉₈₎ respectively. Secondary chemical shifts were calculated using random coil chemical shifts described by Wang and Jardetzky (Wang and Jardetzky, 2002). All data were processed using Topspin 3.2 (Bruker Biospin) and analyzed with CCPNMR Analysis software (Skinner et al., 2016). Backbone and sidechains ¹³C chemical shifts were predicted from the protein 3D coordinates of the coiled-coil model spanning residues 117–166 using the software ShiftX2 (Han et al., 2011).

4. Results

4.1. REMORIN coiled-coil trimerization promoted by the highly-conserved *C*-terminal is essential for membrane targeting

REMORINs share a highly variable N-terminal domain in terms of length and conservation of the primary sequence and a highly conserved C-terminal region (Bariola et al., 2004; Raffaele et al., 2007; Reymond et al., 1996). Previous work on a Solanum tuberosum RE-MORIN of group 1 isoform 3 (StREM1.3) showed that the C-terminal region encompasses two distinct domains (Fig. 1A): the short C-terminal anchor [171-198] (Perraki et al., 2012) specifically binds plasma membrane phosphatidylinositol 4-phosphate (PI4P) and mediates StREM1.3 PM nanodomain organization (Gronnier et al., 2017), while the region [86-170] contains a segment predicted to be implicated in the oligomerization of the protein (Bariola et al., 2004; Perraki et al., 2012). The latter domain displays a strong propensity to form a coiledcoil super helix (Fig. 1B). In such structures, alpha helical monomers wind around each other to optimize protein-protein contacts and stabilize the multimer assembly (Crick, 1953; Kohn et al., 1997). All the members of the REMORIN family share these predicted structural properties and have strong sequence similarities between their Cterminal regions (Raffaele et al., 2007). We compared and analyzed the sequences of StREM1.3 with 5 Arabidopsis thaliana REMORINs from different groups (Groups 1, 3, 4, 5 and 6) to identify conserved elements that might be essential to the formation of coiled-coil super-helices (Figs. 1B and S2). This comparison revealed a sequence similarity of 59% with the most divergent, i.e. AtREM3.1, and 94% sequence similarity with AtREM1.3. Based on the chemical properties of the amino acids, we identified three candidates that could potentially be involved in the helix-helix interfaces within the coiled-coil domain (L126, L137 and L155). The hydrophobic character of the residues at these three positions is highly conserved between the different members of the REMORIN family. Fig. 1C shows the coiled-coil helical wheel at position L155 for the StREM1.3 trimer. L152 and L155 form a hydrophobic patch, which stabilizes the coiled-coil structure. Polar and charged residues (H156, K157 and E158) are exposed either to the solvent or to an opposite charge residue, again potentially stabilizing the structure.

We have recently reported structural insights into the role of the Cterminal peptide REM-CA during PM anchoring and nanodomain organization (Gronnier et al., 2017) and we here aimed at testing the impact of StREM1.3 trimerization on its membrane-binding properties. Based on the sequence analyses, we introduced Prolines, in order to destabilize the coiled-coil superstructure (Chang et al., 1999), at positions L126, L137 and L155. *In vivo* confocal imaging visualized the subcellular localization of GFP-StREM1.3, GFP-StREM1.3^{L126P}, GFP-StREM1.3^{L137P} and GFP-StREM1.3^{L155P} (Fig. 1D). As expected, the wildtype protein localized exclusively at the plasma membrane. However, for all mutants, fluorescence was detected in the cytosol, which indicates that the protein is no longer strictly associated with the plasma membrane. To further confirm this observation we performed cell fractionation of tissues expressing either GFP-StREM1.3, GFP- ARTICLE IN PRESS



Journal of Structural Biology xxx (xxxx) xxx-xxx



Fig. 1. Mutations of conserved amino acid motifs in the coiled coil impairs PM targeting. (A) Domain organization in StRem1.3. (B) Coiled-coil propensity of StRem1.3C-terminal region (109–183) realised with COILS. Sequence alignment of REMORIN homologs in *S. tuberosum* and *A. thaliana*. Protein sequences are ranked according to the conservation score. Hydrophobic residues potentially involved in helix contacts are highlighted in red. Conserved amino acids between the different REMORINs are shown in blue (dark blue for the most conserved to light blue for the less conserved). (C) Helical wheel corresponding to a coiled-coil trimer centred to the 152–158 region. Residues type are colored as follows: yellow, hydrophobic; blue, basic; red, acid; green, polar. Wheels were drawn using DrawCoil 1.0. (D) Confocal images presenting secant views of *N. benthamiana* epidermal cells expressing GFP-StREM1.3^{L126P}, GFP-StREM1.3^{L135P}, GFP-StREM1.3^{L135P}, transiently expressed in tobacco epidermal cells. Scale bars: 20 μm. (E) Tobacco leaf cells expressing each construct were fractionated into soluble (sol.) and microsomal (m) compartments by centrifugation. Proteins were analyzed by Western blot with antibodies against StREM1.3. The protein loading control is presented in Fig. S8.

StREM1.3^{L126P}, GFP-StREM1.3^{L137P}, or GFP-StREM1.3^{L155P} (Fig. 1E). Western blotting with anti-REM antibody (Raffaele et al., 2009) clearly confirmed that StREM1.3 is only found in the microsomal fraction whereas the three mutants are visible in both microsomal and soluble fractions. Altogether, these observations suggest that oligomerization might be required for REMORINS' PM localization.

4.2. Building blocks of assembled $REM_{H6(86-198)}$ form organized fibers as revealed by TEM and cryo-EM

To investigate the assembly features and the structure of REMORIN oligomers, we focused on the conserved C-terminal region (residues 86 to 198), including the coiled-coil domain that most likely is responsible for the assembly (Bariola et al., 2004), and REMCA. We expressed and purified StREM1.3C-terminal moiety (REM_{H6(86-198)}) in E. coli. After the purification, we recovered different fractions containing REM_{H6(86-198)} protein and analyzed them with gel-electrophoresis under denaturing conditions (Fig. S1). The fractions containing pure REM_{H6(86-198)} were pooled and the self-assembly was triggered by dialysis. In order to provide an in-depth analysis of the mesoscopic shape as well as structural features of the assembly, we chose several biophysical techniques including negative staining transmission EM (TEM), cryo-EM and SSNMR. TEM and cryo-EM gave us first insights into the morphology of REM_{H6(86-198)} assemblies (Fig. 2A and B, respectively), revealing ordered REMORIN fibers with a repetitive pattern. The repeating elements are propagated both horizontally and vertically. The periodicities correspond to 13 nm along and approximately 5 nm perpendicular to the fiber axis. Interestingly, along the fibers, their width can vary (Fig. S3) and their endings are irregular. The variable width engendering irregular endings of the fibers suggests an association and juxtaposition of thin filaments (arrow Fig. 2C). This implies that the thin filaments self-assemble into large fibers in a constructive manner leading to the repetitive patterns.

Importantly, filaments of REM₍₈₆₋₁₉₈₎ without the N-terminal His₆tag do not show specific lateral association (Fig. 2D). However, the SSNMR data on REM₍₈₆₋₁₉₈₎ lacking the His₆-tag clearly confirm that the molecular structure of REM₍₈₆₋₁₉₈₎ in laterally associated (REM_{H6(86-198)}) and single filaments (REM₍₈₆₋₁₉₈₎) does not vary essentially, because the spectral fingerprint is conserved between both filaments (Fig. S4). The lateral association of REM_{H6(86-198)} filaments allows for the analysis of the repetitive patterns promoted by the His₆-tag, indicative of the assembly features.

4.3. The REMORIN coiled-coil domain gains a partially well-organized structure upon trimerization

To decipher the molecular basis underlying the filament formation, we performed a 3D modelling of the StREM1.3 coiled-coil domain based on sequence homology using the I-TASSER server (Zhang, 2008). The modelling was restrained to the region with the higher coiled-coil propensity [117–166] defined by the software COILS (Lupas et al., 1991). The 3D model converged to a regular alpha helix composed of



Fig. 2. Electron micrographs of REM_{H6(86-198)} and REM₍₈₆₋₁₉₈₎ filaments. (A) Negative staining TEM and (B) cryo-EM show filaments of REM_{H6(86-198)} with two repetitive patterns perpendicular to each other. Width and thickness of fibers vary over the length of the filaments. (C) CryoEM of the sharp extremity of a REM_{H6(86-198)} fiber shows similar repetitive motifs. Scale Bar of (A), (B) and (C) is the same: 100 nm (D) Negative-staining TEM micrograph of thin filaments of REM₍₈₆₋₁₉₈₎, lacking the N-terminal His6-tag. Scale Bar: 100 nm.

residues 133 to 166. $\text{REM}_{(117-166)}$ monomers were assembled into a trimer by geometry based docking using the SymmDock server (Schneidman-Duhovny et al., 2005). Fig. 3A shows the trimeric coiled-coil structure of the REMORIN obtained *in silico*. A patch composed of hydrophobic residues, which point towards the trimer core, defines the interface between the three monomers and electrostatic interactions stabilize the architecture.

4.4. SSNMR data on REM_{H6(86-198)} filaments support the 3D model of the coiled-coil domain and identify a short segment in β -strand conformation

To determine whether the 3D model obtained *in silico* reflects the structure adopted by REM_{H6(86-198)} in the filaments, we analyzed ¹⁵N,¹³C-labelled filaments using liquid and solid-state NMR. Solution NMR analysis revealed that REM_{H6(86-198)} is unstructured in solution and adopts a 3D fold only upon assembly into multimeric complexes

(Fig. S5). SSNMR is an emerging technique providing atomic structural information on molecular assemblies in their native states. We conceived a protocol to successfully produce, purify and assemble suitable quantities (~10 mg) of 15 N- and 13 C-labelled REM_{H6(86-198)} in vitro to conduct multidimensional MAS SSNMR on the REMORIN in its filamentous state. The very intense signal observed in a one-dimensional ¹H-¹³C cross-polarization spectrum revealed the presence of a rigid core in the structure of the assembled proteins (Fig. S6). A two-dimensional ¹³C-¹³C PDSD (proton-driven spin diffusion), Fig. 3B, acquired with a short mixing time (50 ms) set up to detect intra-residues ¹³C-¹³C correlations, reveals the SSNMR fingerprint of the REM_{H6(86-198)} structure in filaments. The intense signals again indicate that the filaments contain a rigid protein core and the appearance of well-resolved individual peaks points to a well-defined atomic structure adopted by the monomers composing the assembly. Our in silico 3D model of REM₍₁₁₇₋₁₆₆₎ suggests that the REMORIN monomers oligomerize into a-helical coiled-coil trimers promoted by the coiled-coil domain. Based on the 3D model, we used the ShiftX2 software (Han et al., 2011) to predict the expected protein ¹³C chemical shifts for the coiled-coil domain REM₍₁₁₇₋ 166) and reported them on the 2D ¹³C-¹³C SSNMR spectrum recorded on REM_{H6(86-198)} filaments (Fig. 3B, red crosses, left spectrum). The majority of the predicted chemical shifts match well with the experimentally observed signals. This strongly supports the model of a α -helical tertiary fold in the filament core, reflecting the coiled-coil domain. However, a non-negligible part of the signals in the 2D spectrum remain unassigned and do not appear in spectral regions where signals from α helical structural segments are located (Fig. 3B, blue crosses, right spectrum). Among them, a detailed analysis was possible for Thr, Glu, Lys, Val, Ser, Leu, Ile, and Ala amino acids for which the resonances reflecting β-strand secondary structures are isolated in the spectrum. By comparing these residue-specifically assigned amino acids with the primary sequence of REM_{H6(86-198)} we identified the unique motif in the REM_{H6(86-198)} primary sequence (T₈₆-EKRVSLIKA-A₉₅) adjacent to the coiled-coil region. The theoretical sequential peaks are all visible even if their assignments remain ambiguous. The local conformation of these residues was identified via their secondary chemical shifts that reflect a β-strand conformation where negative values were obtained. The unique amino acid composition of T₈₆-EKRVSLIKA-A₉₅ and the clear βstrand conformation of the identified residues indicate the occurrence of a short β -segment at this location (Fig. 3D). The assignment of the R89 spin system remained ambiguous because of the important peak overlap in this spectral region. Likewise, the region around T₈₆-A₉₅ region might be extended but the chemical shifts of these residues could not be identified due to spectral overlap.

4.5. Cryo-EM data suggest that trimeric coiled-coil $REM_{H6(86-198)}$ arranges itself as aligned building blocks into filaments

Cryo-EM micrographs revealed the repetitive motifs in the REM_{H6(86-198)} filaments and the spectral resolution we obtained on the filaments allowed for the detailed measurement of their dimensions (Fig. 4A). The longitudinal repetitions are characterized by 13 nm in length with a width of approximately 5 nm. The dimensions of the 3D model of the $\operatorname{REM}_{(117\text{-}166)}$ coiled-coil trimer, with 8 nm in length and a width of ~ 1.5 nm without side-chains (Fig. 4A), tie in the distances obtained on the electron micrograph of the REM_{H6(86-198)} fibers. The 3D model of the coiled-coil domain illustrates the tertiary fold of the REM₍₁₁₇₋₁₆₆₎ with the region spanning 132–166 showing well-defined α -helices, slightly twisted to allow protein-protein contacts at positions 155 and 137 (Fig. 4A, B). The segment 117-131 is less ordered than expected for the coiled-coil domain but displays lower structure accuracy for the generated model (Fig. S7). Fig. 4B shows an example of close hydrophobic contacts between Leucine residues in the trimer at the position 155, essential for trimer stability.

Based on the ensemble of our results we propose a cartoon model of the $\text{REM}_{\text{H6(86-198)}}$ building block in filaments, encompassing a coiled-



Fig. 3. 3D model and solid-state NMR of REM_{H6(86-198)} filaments. (A) 3D model of StREM1.3 coiled coil domain predicted with I-TASSER server (Zhang, 2008). Two monomers in magenta are represented in surface mode and the third monomer with cartoon. All the hydrophobic side chains in the monomer are colored in yellow. Residue contacts identified between in StREM1.3 coiled-coil domain are highlighted on the primary sequence (green, hydrophobic contacts; red and blue, electrostatic contacts). (B) 2D PDSD ¹³C-¹³C spectrum of REM_{H6(86-198)} filaments (50 ms mixing time) showing back-calculated ¹³C chemical shifts with ShiftX2 from the 3D model (left spectrum, red crosses) and assigned chemical shift from β-strands (right spectrum, blue crosses). (C) Secondary chemical shift ΔδCα-ΔδC β•••• the T86-E87-K88-R89-V90-S91-L92-I93-K94-A95 amino acid segment in REM_{H6(86-198)}, revealing the presence of a β-strand segment.

coil domain, in which the three membrane-affinity impairing mutations are localized, flanked by a short β -strand and supposedly two segments with less well-defined structures on both sides (Fig. 4A). The N-terminal ending of the coiled-coil domain contains a region of less defined structure and a stretch of 10 amino acids, mainly composed of hydrophobic residues constituting a regular β -strand, whereas the C-terminal REM-CA seems to adopt a condensed, unstructured form. The two endings account for the regions of dense protein concentration in the EM micrographs. The proximity of the β -strands could stabilize the trimer through an inter-strand hydrogen-bonding network.

5. Discussion

Our results have demonstrated an unexpected impact of REMORIN oligomerization via the coiled-coil domain on membrane affinity and we therefore decided to tackle the structure of this best-characterized nanodomain-associated plant protein supramolecular assembly.

By using a coiled-coil prediction server, we identified the region with the highest propensity of forming a coiled-coil and we singled out three hydrophobic residues that could stabilize the trimeric architecture, L126, L137 and L155. *In vivo* monitoring of the localization of REMORIN and of the three substitution mutants by confocal microscopy showed that all three mutations strongly impair membrane affinity suggesting that trimer formation is correlated with REMORIN-membrane interactions and that a cooperative effect between REMORINs plays an important role in membrane binding. Full-length REMORINs form filaments, which were proposed to arise from interactions between coiled-coil trimers of the conserved C-terminal region (Bariola et al., 2004). We show that REM₍₈₆₋₁₉₈₎, lacking the highly variable N-terminal region, also assembles into well-organized filaments. When the N-terminal His₆-tag of REM₍₈₆₋₁₉₈₎ is conserved, i.e. REM_{H6(86-198)}, the filaments arrange into fibers that vary in width over the filament length and end in a thin architecture. This behaviour is consistent with the association or dissociation of trimeric building blocks that build a strong intermolecular network of weak interactions. Importantly, these fibers show very homogeneous repetitive patterns in electron micrographs that enable the analysis of the molecular building blocks in the filaments.

Creating an *in silico* 3D model of the coiled-coil domain allowed us to visualize the underlying 3D structures, to identify stabilizing hydrophobic and electrostatic contacts and to estimate the dimensions of such coiled-coil trimer. In this model, the three Leucines are located in essential positions, explaining the destabilizing effect and membraneaffinity loss of Proline mutations, L126P, L137P and L155P.

To corroborate our working model of trimeric coiled-coil building units, we performed SSNMR on REM_{H6(86-198)} and REM₍₈₆₋₁₉₈₎ filaments, obtaining atomic structural data on the intact filaments. Based on the 3D trimeric model, we compared predicted with observed SSNMR chemical shifts, validating the hypothesis of trimeric coiled-coil units that associate laterally and, furthermore, residue-type and sequential assignments revealed longitudinal interactions between the trimers involving a short β -strand and less well-ordered condensed

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Fig. 4. 3D architecture of $\text{REM}_{(86-198)}$ inside the filaments and associated to PM nanodomains. (A) Cryo-EM micrograph of $\text{REM}_{H6(86-198)}$ fibers depicts 13 nm horizontal repetitive patterns corresponding to a regular assembly of $\text{REM}_{H6(86-198)}$. Scale bar: 20 nm. Schematic representation of $\text{REM}_{H6(86-198)}$ structure inside the filament (β -strands are represented as blue arrows and α -helices as rectangles) and 3D structural model of $\text{REM}_{(86-198)}$ coiled-coil domain. (B) Example of protein contacts inside the coiled coil domain. α -helices are represented in cartoon and Leucine sidechains in sticks. (C) Schematic representation of $\text{REM}_{(86-198)}$ structure when associated to the PM nanodomains. Red stars represent point mutations, which impair the membrane localization of StREM1.3. α -helices are represented as rectangles. The C-terminal membrane anchor REM-CA contains a short α -helice. Point mutations for *in vivo* confocal imaging experiments are highlighted in red.

segments. The dimensions of the building blocks obtained from cryo-EM micrographs are compatible with the trimeric REM_{H6(86-198)} coiledcoil units, derived from the 3D model. Combining the structural data from SSNMR, the *in silico* model, and cryo-EM, we propose a cartoon model that reflects the architecture in REM_{H6(86-198)} and REM₍₈₆₋₁₉₈₎ filaments (Fig. 4A). Furthermore, incorporating the recently proposed model of the REM-CA peptide structure during nanodomain formation and the data obtained on the role and structure of coiled-coil trimers, we propose a cartoon model for the nanodomain-associated REM₍₈₆₋₁₉₈₎ (Fig. 4C). The coiled-coil trimers are stabilized by intermolecular protein-protein interactions including the three Leucine-Leucine contacts of residues 126, 137 and 155 (Fig. 4C, red stars).

6. Conclusions

Protein organization into membrane domains relies on complex and

scarcely in-detail explored structural mechanisms, involving lipid-protein and protein-protein interactions (Simons and Sampaio, 2011). Considered as genuine PM nanodomain-organized proteins in plants, REMORINs attach to the PM and concentrate in nanodomains by an original mechanism via the C-terminal anchor, REM-CA (Gronnier et al., 2017). We here report that REM-CA alone is not sufficient and responsible for membrane targeting. We show that the conserved trimerization region plays a central role and we investigated the structure of this region by conceiving a protocol to obtain filaments of mesoscopic scale that we analyzed by various techniques. The obtained data and proposed cartoon model might also reveal other modes of interaction of REMORINs during oligomerization such as β -strand hydrogen bonding. However, the interactions between trimer coiled-coil domains promoting the filament assembly might reflect interactions that favour the highly specific nanoclustering occurring between REMORINs of the same family (Jarsch et al., 2014). Our findings open an avenue to

D. Martinez et al.

understanding the complex nanodomain-targeting mechanisms of RE-MORIN proteins based on the combination of SSNMR and cryo-EM and aim at contributing to the comprehension of molecular strategies employed by membrane-domain proteins to perform their cellular functions.

Acknowledgments

We acknowledge the financial support by the ANR (ANR-13-PDOC-0017-01 to B.H. and ANR-14-CE09-0020-01 to A.L.), the IdEx Bordeaux "Investments for the future" (ANR-10-IDEX-03-02 to B.H.) and PEPS to B.H. and S.M., the European Research Council (ERC-2015-StG GA no. 639020 to A.L.) and the CNRS (IR-RMN FR3050). A.L. is supported by the Ministère de l'Enseignement Supèrieur et de la Recherche, France (MERS, doctoral grants). TEV protease was a gift from Lionel Beaurepaire and the work has benefited from the facilities and expertise of the Biophysical and Structural Chemistry plateform (BPCS) at IECB, CNRS UMS3033, Inserm US001, Bordeaux University http://www.iecb. u-bordeaux.fr/index.php/fr/plateformestechnologiques».

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsb.2018.02.003.

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Journal of Structural Biology xxx (xxxx) xxx-xxx

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Coiled-coil oligomerization controls nanodomain organization of the plasma membrane REMORINs.

Supplementary Information

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Figure S1. SDS-PAGE (12 % acrylamide gel) of REM_{H6(86-198)} at different stages of His-trap chromatography purification. Molecular weight marker in lane 1; Washing fraction at 25 mM imidazole – lanes 2 and 3; Elution with 400 mM imidazole – lanes 4 to 7; Elution with 500 mM imidazole – lanes 8 and 9; Purified fraction after HPLC as a control – lane 10; the arrow indicates the position of the recombinant REM_{H6(86-198)}.

Figure S2. Residues conservation in REMORINs. MUSCLE multiple alignments of full length StREM1.3 and Arabidopsis REMORINs proteins were visualized using JalView v2.10.2b2. The alignment is colour-coded by percentage identity.



Figure S3. Cryo TEM micrograph of $\text{REM}_{H6(86-198)}$ illustrating the varying filament thickness. Scale bar : 100 nm.



Figure S4. 2D SSNMR on $\text{REM}_{H6(86-198)}$ and $\text{REM}_{(86-198)}$ filaments. Overlay of 2D PDSD ¹³C-¹³C spectra of $\text{REM}_{H6(86-198)}$ (black, recorded on a 600MHz spectrometer) and $\text{REM}_{(86-198)}$ (red, recorded on a 800MHz spectrometer) filaments (50 ms mixing time).



Figure S5. ¹H,¹⁵N SOFAST-HMQC liquid-state NMR spectrum of the unfolded REM_{H6(86-198)} in solution. The spectrum was acquired at 313K on a ¹⁵N,¹³C labelled protein sample at a concentration of 200 μ M in 20 mM HEPES, 150 mM NaCl, 0.02% NaN₃, pH 7.4 in a mixture of 90% H₂O, 10 % D₂O. The spectrum was referenced according to the DSS signal added in the sample.



Figure S6. 1D ¹³C solid-state NMR spectrum of $\text{REM}_{H6(86-198)}$ filaments recorded using a ¹H-¹³C cross-polarization transfer. The spectrum was referenced according to DSS signal added in the sample.



Figure S7. Accuracy graph of the generated 3D model. Residue-level quality of the protein structure prediction has been evaluated by the program ResQ (Yang et al., 2016)



Figure S8. Stain-free of SDS_PAGE showing the loading of proteins for the western blot presented Figure 1.

Yang, J., Wang, Y., Zhang, Y., 2016. ResQ: An Approach to Unified Estimation of B-Factor and Residue-Specific Error in Protein Structure Prediction. Journal of molecular biology 428, 693-701.

Article I: addendum

After working on filaments of REM₈₆₋₁₉₈ to gather structural insights into its coiledcoil oligomerisation domain, we focused on inserting the protein in membranes. Would the structural fingerprint be modified? Would RemCA's structural fingerprint appear as it was first observed (Gronnier et al., 2017)?

I. REM₈₆₋₁₉₈ in liposomes by ssNMR

¹⁵N-, ¹³C- labelled REM₈₆₋₁₉₈ was reconstituted in DMPC/sitosterol/PIPmix (70/15/15 molar ratio) liposomes as described in Article IV. The sample was analysed on a Bruker Avance NEO Solids operating at 600 MHz for proton with a CPMAS 4 mm probe. MAS frequency was 11 kHz. ¹³C-¹³C PDSD spectra were recorded using 20 ms and 7 ms acquisition times in direct and indirect dimensions, respectively. ¹H decoupling during acquisition was performed using a SPINAL-64 decoupling sequence (Fung et al., 2000). All experiments are carried out at 4°C according to water's chemical shift (Wishart et al., 1995).

In the presence of membranes, the N-terminal β -region of REM₈₆₋₁₉₈ disappears while the helical region remains mostly unchanged (Figure 56). Superimposing RemCA's structural fingerprint (Gronnier et al., 2017), we could not match any peaks. In addition, no new peaks were detected. This may imply that: (1) RemCA's conformation in the protein differs from the lone anchor or (2) most of it is too flexible to be detected by ¹³C-¹³C PDSD. Nonetheless, the proteoliposomes preparation was contaminated with filaments of REM₈₆₋₁₉₈ (Figure 57).



Figure 56

¹³C-¹³C PDSD spectra of REM₈₆₋₁₉₈ in liposomes of PC/sitosterol/PIPmix 75/10/15 (molar ratio) with mixing times of 50 ms (black) and 150 ms (red). ¹³C-¹³C PDSD of RemCA (blue) in liposomes containing PIPmix with a 50 ms mixing time from (Gronnier et al., 2017).



Figure 57

Negative staining electron microscopy on ¹⁵N-, ¹³C-labelled REM₈₆₋₁₉₈ in liposomes (Figure 56). Due to the staining protocol, liposomes are deformed and adopt a flat balloon shape. Scale bar: 50nm.

II. Structure of RemCA in micelles

We decided to work on RemCA alone. IsNMR already showed that it was disordered in H₂O and that it would fold differently whether it was in trifluoroethane or in deuterated dodecyl phosphatidylcholine (DPC-d38) micelles (Gronnier et al., 2017). The latter being more relevant in mimicking a biological membrane, we pursued a full structural investigation of RemCA by IsNMR. The following is a revised translation of my master 2 internship report. This work was done under the direct supervision of Dr Denis Martinez, who performed IsNMR experiments and structure calculations.

A. Material and methods

1 mM of synthetic N-acetylated RemCA peptide is mixed with 20 mM of DPC-d38 in 9/1 H₂O/D₂O (V/V). The sample was analysed on a Bruker Avance III spectrometer operating at 800 MHz for proton with a 5 mm TCI cryoprobe controlled by TopSpin 3.1 (Bruker). Pulse sequences used were: ¹H-¹⁵N HMQC, ¹H-¹³C HMQC, ¹H-¹H TOCSY (150 ms mixing time) and ¹H-¹H NOESY (300 ms mixing time) (Levitt, 2008). Spectra were analysed with CCPNMR Analysis (<u>http://www.ccpn.ac.uk</u>) and chemical shift statistics from the BMRB. Structure calculation were handled through the CCPNMR Grid interface (Fogh et al., 2005; Nilges et al., 2008; Rieping et al., 2007; Vranken et al., 2005).



Information provided by the different NMR pulse sequences employed. R is a lateral chain of a residue. In B, C and D, the case of an alanine is illustrated. (A) ¹H- ¹⁵N HMQC informs on the chemical shifts of the highlighted ¹H and ¹⁵N of the amide backbone. (B) ¹H-¹³C HMQC informs us on the chemical shifts of a given ¹³C and the ¹H bound to it. (C) ¹H-¹H TOCSY allows to obtain all the ¹H chemical shifts of a given spin system, i.e. a residue. (D) ¹H-¹H NOESY provides distance constraints between neighbouring ¹H: the intensity of a cross-peaks is correlated to their distance from one another (Nilges et al., 2008).

B. Results

To access RemCA's structure, it was reconstituted in DPC-d38 micelles and analysed by IsNMR. Peak assignment was performed manually with the following methodology: (1) chemical shifts of ¹H bound to backbone nitrogens were determined using the ¹H-¹⁵N HMQC; (2) ¹H-¹H NOESY cross-peaks in the amide region were used to assess connectivities between neighbouring residues; (3) using both the ¹H-¹H NOESY and the ¹H-¹H TOCSY, each residue was identified by its expected chemical shifts (according to the BMRB) and, finally, (4) weak ¹H-¹H NOESY peaks are assigned to more distant connectivities between residues (Figure 58). Figure 59 gives an example of the assignment process for residues 171 to 175. Figure 60 shows the assigned ¹H-¹⁵N HMQC spectrum.



Assignment of residues 171 to 175. (A) Abbreviations used to designate ¹H. (B)
 Connectivities between residues are defined using ¹H-¹H NOESY cross-peaks in the H region. Full arrows: i, i + 1 connectivities. Dashed arrows: i, i + n > i + 1 connectivities (longer range connectivities). (C) Each residue is identified using the ¹H-¹H TOCSY and the expected chemical shifts from the BMRB.





990 peaks were assigned on the ¹H-¹H NOESY, among which 359 ambiguous assignments (Table 4). Preliminary structure calculations converge to a structure bearing, in N-terminal, one α -helix of 4 residues then a second α -helix of 9 residues, kinked by an angle of 96°, followed by a loop and a flexible C-terminal region (Figure 61). The helix break in N-terminal could be an artefact due to the non-physiological micellar environment: it is the major difference with the model from (Gronnier et al., 2017), in which there was only a single N-terminal helix. As a reminder, in the full length protein, a long helix is attached at the N-terminal of RemCA. It is unlikely that both would be connected by a loop partially embedded in the membrane.

Connectivities	i, i	i, i+1	i, i+2	i, i+3	i, i+4	i, i>4
Number of peaks	215	216	88	113	81	277
Ambiguities	40	52	23	30	20	194
Table 4						

Table 4

Number of assigned cross-peaks of the ¹H-¹H NOESY spectrum and amount of ambiguous assignments, sorted by connectivity types, where i is a given residue.





Preliminary structure of RemCA in micelles of DPC-d38. (A) 7 structures were calculated from the same dataset then superimposed. Blue end: N-terminal. Red end: C-terminal. (B) Structure of lowest energy from (A). Dark blue: lysines. Light blue: arginine. Red: aspartate and glutamates. Grey: hydrophobic residues. Aromatic sidechains are represented as sticks. Hydrophobicity and hydrophilicity labelled according to the Kyte-Doolittle scale (Perraki et al., 2012).

III. Structural analysis of RemCA in native-like conditions by ssNMR

We decided to change the membrane mimetic for something more physiologically relevant i.e. multilamellar vesicles (MLVs).

Ordering synthetic RemCA peptides that are fully labelled with ¹³C and ¹⁵N is too expensive, so we decided to produce a fusion protein GFP-(TEV cleavage site)-RemCA in E. coli BL21-DE3. The production of GFP-RemCA in BL21-DE3 cells was induced by the addition of 1 mM IPTG followed by overnight incubation at 18°C under stirring. Cells were lysed and the supernatant was loaded onto a HisTrap column controlled with an Atka Pure 25 HPLC system (GE Healthcare) equilibrated with 20 mM HEPES 150 mM NaCl 20 mM imidazole 0.02% NaN₃ pH=7.4 and eluted with 20 mM HEPES 150 mM NaCl 500 mM imidazole 0.02% NaN₃ pH=7.4. GFP-RemCA was adjusted to 0.5 mM EDTA and 1 mM DTT then TEV protease was added in a ~ 1/200 TEV/GFP-RemCA weight ratio. The mixture was incubated for 2-3h at room temperature then dialysed at 4°C overnight in a MWCO = 1 kDa dialysis tubing made of regenerated cellulose (Spectra Labs). It was adjusted to 7M urea and incubated for 1h at room temperature before being loaded onto a HisTrap column equilibrated with 20 mM HEPES 150 mM NaCl 7 M urea pH=7.4 and with 20 mM HEPES 150 mM NaCl 7 M urea 500 mM imidazole pH=7.4 as elution buffer. Free RemCA flowed through the column without binding to the resin. It was dialysed three times against water.

We were able to produce 3 mg of fully ¹⁵N-, ¹³C-labelled RemCA per litre of labelled culture medium. The peptide was lyophilised, co-solubilised in CHCl₃/MeOH 2/1 (v/v) with DMPC/sitosterol/PIPmix 75/15/10 (molar ratio) (PIPmix is PS/PI/PI4P/PI4,5P₂ 50/20/15/15 (Gronnier et al., 2017)), lyophilised and re-hydrated. The final sample was fitted into a 3.2 mm rotor and analysed by ssNMR on a Bruker Avance NEO operating

at 800 MHz for proton with a CPMAS 3.2 mm probe. Acquisition times were 20 ms and 8 ms in the direct and indirect dimensions, respectively. ¹H decoupling during acquisition was performed using a SPINAL-64 decoupling sequence (Fung et al., 2000).



¹³C-¹³C PDSD (50 ms mixing time) spectrum of RemCA co-solubilised in PC/sitosterol/PIPmix liposomes. Unambiguously assigned spin systems are highlighted. Due to spectral overlap and possible sample aggregation, the assignment of some peaks is deemed too ambiguous and is not shown here.

The residue types of a few spin systems could be identified without ambiguity (Figure 62): two alanines (at least), two isoleucines, two leucines (at least), 191Pro and a threonine. The SNR is quite poor and obtaining unambiguous inter-residue cross-peaks to perform structure calculations will prove difficult. Instead, we will use the simpler but nonetheless robust secondary chemical shift index: by computing the

difference in chemical shift between an assigned C α or C β and the value for such a nucleus in a residue of random coil conformation, we can determine if the residue in a helical conformation or a strand conformation (Figure 63) (Wang, 2002). A positive value in C α is characteristic of helical conformation while a negative one indicates a strand conformation. This reasoning is inverted for C β : strands yield positive values, helices yield negative ones.



Secondary chemical shifts for spin systems identified in Figure 7, based on (Wang, 2002). Top: sequence and fold of RemCA according to (Gronnier et al., 2017). Bottom: secondary chemical shifts of assigned spin systems in Cα and Cβ.
 Positive secondary chemical shift in Cα indicates a helical fold while a negative value indicate a strand conformation. It is the opposite for secondary chemical shifts in Cβ. Notice the change of scale between both plots.

There are five alanines in RemCA. At least of them are assignable as Ala1 and Ala2. Furthermore, there are some less intense signals in the Ala region, indicating sample heterogeneity, multiple conformations or alanines in less rigid conformation. Ala1 is in a blatant helical conformation while Ala2 adopts a strand conformation. According to our model (Gronnier et al., 2017), alanines are either in the N-terminal helix, which could correspond to Ala1's, or in the loop, close to the extended β -strand, which could fit Ala2's secondary chemical shifts. Both isoleucines are in a strand conformation, which is consistent with our model (Gronnier et al., 2017). Similarly, proline 191, which is supposed to be at the junction between the loop and the β segment, displays a strand conformation. There are two identified leucines, with cross-peaks of similar identities but opposite conformations: one is a helical conformation, the other in a strand conformation. Since there are three leucines in the putative N-terminal helix and two in the C-terminal region, these secondary chemical shifts confirm that one part of RemCA is helical and that another part contains an extended β -strand. Overall, this

structural analysis of RemCA by ssNMR does not contradict, and might confirm, the model of (Gronnier et al., 2017).

IV. On a putative role of the N-terminal IDD of *St*REM1.3 on the structure of its filaments

We wished to compare REM₈₆₋₁₉₈ to full length *St*REM1.3 filaments (figure 64). Using the same methodology, with the exception that *St*REM1.3's histag is not cleavable, we compared the fingerprint of both samples (Figure 65).

Most peaks of the β -region in REM₈₆₋₁₉₈ vanish when switching to the full length *St*REM1.3, indicating that the presence of this β -segment was artifactual. Otherwise, the structural fingerprints of both constructs are identical, proving that depletion of the first 85 aminoacids of *St*REM1.3 does not disrupt its propensity to form coiled-coil domains with a wild-type fold. Reciprocally, it implies that this region is not involved in the formation of the coiled-coil structure nor the assembly of the filaments.



Figure 64

Negative staining electron microscopy on ¹⁵N-, ¹³C-labelled *St*REM1.3 filaments (NMR spectrum is given on Figure 65). Scale bar: 50 nm.

V. Conclusion

As a general conclusion, the short N-terminal β -segment of REM₈₆₋₁₉₈ spotted in (Martinez et al., 2018) in filaments is artifactual: it disappears if the protein is in membranes as well as in filaments of the full length *St*REM1.3. Also, the coiled-coil signature is retained between filaments of both constructs, *St*REM1.3 and REM₈₆₋₁₉₈, and between REM₈₆₋₁₉₈ in filaments and in liposomes. Reconstitution of RemCA peptides in lipid bilayers and structural analysis by ssNMR strengthened the validity of our structural model of RemCA in membranes. In addition, preliminary data obtained in micelles provided by IsNMR, although tainted by a micelle-induced artefact, corroborated some of the findings of (Gronnier et al., 2017): an N-terminal helix followed by a loop then a flexible and extended β -strand C-terminal.



¹³C-¹³C PDSD of REM₈₆₋₁₉₈ filaments (black) (Martinez et al., 2018) and *St*REM1.3 filaments (red) at 50 ms mixing time.

Article II

REM1.3's phospho-status defines its plasma membrane nanodomain organization and activity in restricting PVX cell-to-cell movement Artemis Perraki, Julien Gronnier, Paul Gouguet, Marie Boudsocq, Anne-Flore Deroubaix, Vincent Simon, Sylvie Germain-Retana, Anthony Legrand, Birgit Habenstein, Cyril Zipfel, Emmanuelle Bayer, Sébastien Mongrand, Véronique Germain

> PLOS Pathogens 2018 14, e1007378

This second article was much more focused on biology, and much less on biophysics. The thematic was *St*REM1.3's phosphocode and interactome, and how it relates to remorin-mediated PD closure and slowing down of PVX cell-to-cell propagation. *St*REM1.3 was phosphorylated *in vitro* using *N. benthamania* extracts, including purified PMs and [γ -³³P]-ATP further detected by autoradiography. Inhibition by EGTA, a Ca²⁺ chelator, narrowed our field of research to the *Arabidospsis thaliana* calcium-dependent protein kinase 3 *At*CPK3, which is indeed able to phosphorylate *St*REM1.3 *in vitro*. Mass spectrometry-based phospho-mapping failed, so we resorted to systematic mutations of the three most putative phosphorylation sites S74, T86 and S91 (Marin et al., 2012) to Alanine (A), phosphodead, or Aspartic acid (D), phosphomimetic. High resolution microscopy Spt-PALM showed *St*REM1.3^{AAA} mutant was less mobile than *St*REM1.3^{DDD}, with *St*REM1.3^{WT} in between. We assessed these mutants' biological activity by measuring PVX infection foci area in leaves of *N. benthamiana*.

I got involved after the article's first submission, when reviewers asked to perform a finer mutant analysis by testing single-residue mutations (single mutants). Under the supervision and training of Paul Gouguet (PhD student then), who taught me how to use an epifluorescence microscope, we undertook the cloning and analysis of single phosphomutants shown in Figure 3D, from agroinfiltration to observation of viral propagation and data analysis. We were able to show that single phosphodead-mutants (particularly S74) had a WT-like phenotype, hinting at a functional redundancy between these three phospho-residues. I was briefly involved in the re-writing process, mostly for grammar corrections.



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Citation: Perraki A, Gronnier J, Gouguet P, Boudsocq M, Deroubaix A-F, Simon V, et al. (2018) REM1.3's phospho-status defines its plasma membrane nanodomain organization and activity in restricting PVX cell-to-cell movement. PLoS Pathog 14(11): e1007378. https://doi.org/10.1371/ journal.ppat.1007378

Editor: Aiming Wang, Agriculture and Agri-Food Canada, CANADA

Received: July 5, 2018

Accepted: October 3, 2018

Published: November 12, 2018

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files are available from Arabidopsis Genome Initiative (https://www.arabidopsis.org/index.jsp), and GenBank/EMBL (https://www.ncbi.nlm.nih. gov/genbank/) databases under the accession numbers: StREM1.3 (NP_001274989), AtREM1.2 (At3g61260), AtREM1.3 (At2g45820), AtCPK3 (At4g23650). **RESEARCH ARTICLE**

REM1.3's phospho-status defines its plasma membrane nanodomain organization and activity in restricting PVX cell-to-cell movement

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Abstract

Plants respond to pathogens through dynamic regulation of plasma membrane-bound signaling pathways. To date, how the plant plasma membrane is involved in responses to viruses is mostly unknown. Here, we show that plant cells sense the Potato virus X (PVX) COAT PROTEIN and TRIPLE GENE BLOCK 1 proteins and subsequently trigger the activation of a membrane-bound calcium-dependent kinase. We show that the *Arabidopsis thaliana* CALCIUM-DEPENDENT PROTEIN KINASE 3-interacts with group 1 REMORINS *in vivo*, phosphorylates the intrinsically disordered N-terminal domain of the Group 1 REMORIN REM1.3, and restricts PVX cell-to-cell movement. REM1.3's phospho-status defines its plasma membrane nanodomain organization and is crucial for REM1.3-dependent restriction of PVX cell-to-cell movement by regulation of callose deposition at plasmodesmata. This study unveils plasma membrane nanodomain-associated molecular events underlying the plant immune response to viruses.

Author summary

Viruses propagate in plants through membranous channels, called plasmodesmata, linking each cell to its neighboring cell. In this work, we challenge the role of the plasma
Funding: AP was supported by the Greek

fellowship program IKY, https://www.iky.gr/en/. JG, PG and AFD were supported by the Ministère de l'Enseignement Supérieur et de la Recherche, France (doctoral grants). http://www. enseignementsup-recherche.gouv.fr/cid76053/lefinancement-doctoral.html. MB was suported by LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS), https://www6.inra.fr/saclay-plantsciences. VG, SM, EB, VS VG were supported by the French ANR project "Potymove" (ANR-16-CE20-008-01), http://www.agence-nationalerecherche.fr/en/. EB, VG and SM were supported by ANR project (CE19_2014_CONNECT) http:// www.agence-nationale-recherche.fr/en/. CZ was supported by Gatsby Charitable Foundation, http:// www.gatsby.org.uk and the European Research Council (grant "PHOSPHinnATE"), https://erc. europa.eu. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

membrane in the regulation of virus propagation. By studying the dynamics and the activation of a plant-specific protein called REMORIN, we found that the way this protein is organized inside the membrane is crucial to fulfill its function in the immunity of plants against viruses.

Introduction

The cell plasma membrane (PM) constitutes a regulatory hub for information processing [1]. Current knowledge suggests that PM proteins and lipids dynamically associate with each other to create specialized sub-compartments or nanodomains [2], that regulate the cellular responses in space and time [3-5]. For instance, modeling of the localization behavior of a PM-bound receptor and its downstream interactor before and after ligand perception in animal cells suggests that PM-partitioning into nanodomains improves the reliability of cell signaling [6]. In plants a recent example of PM partitioning shows that despite sharing several signaling components, the immune and growth receptors FLS2 and BRI1 are divided into context-specific nanodomains to confer signaling specificity [7]. The REMORIN (REM) family is one of the best-characterized PM nanodomain-associated proteins in plants [7-12]. The association of REMs to the PM is mediated by a short sequence at the extremity of the C-terminus of the protein, called REM-CA (REMORIN C-terminal Anchor) [13, 14]. The REM C-terminal domain contains a coiled-coil (residues 117-152, [15]) which is thought to regulate REM oligomerization [11, 14, 16] and may be involved in regulating REM spatial organization at the PM [15]. Members of the REM family have been associated with plant responses to biotic [9, 17, 18], abiotic stress [19, 20] and developmental clues [12] and current view suggests they could regulate signaling events through nanodomain association [21]. However, the molecular mechanisms leading to REM-associated downstream events remain elusive.

Several REM proteins have been identified as components of the plasmodesmata-plasma membrane subcompartment (PD-PM) [8, 22, 23]. PD are membranous nanopores, crossing the plant cell wall and enabling cytoplasmic, endoplasmic reticulum and PM continuity between adjacent cells. They regulate the intercellular transport of proteins and small molecules during development and defense [24, 25]. The PD-PM is a particular subcompartment of the PM, which displays a unique molecular composition, notably enriched in sterols [26]. The movement of macromolecules through PD can be tightly controlled through modulation of the PD size-exclusion limit (SEL) *via* hypo- or hyper-accumulation of callose at the PD neck region [27–29]. Overexpression of GRAIN SETTING DEFECT 1 (GSD1) encoding a phylogenetic-group 6 REM protein from rice, restricts PD aperture and transport of photo-assimilates [23].

PDs are also the only route available for plant viruses to spread from cell-to-cell. *Potato virus X* (PVX) promotes its cell-to-cell movement *via* modification of PD permeability [30] through the action of TRIPLE GENE BLOCK PROTEIN 1 (TGBp1) [31]. Overexpression of *StREM1.3* (*Solanum tuberosum* REM from group 1b, homolog 3 [32], further referred as REM1.3) hampers TGBp1's ability to increase PD permeability [33]. How REM1.3 obstructs TGBp1 action is still unknown. Here, we used REM1.3 and PVX pathosystem in the solanaceae *Nicotiana benthamiana*, because PVX cannot infect *Arabidopsis* [34] and *N. benthamiana* is a widely used model for research on plant-virus interaction [35]. We previously showed that REM1.3 lateral organization into nanodomains at the PM is directly linked with its ability to restrict PVX movement and regulate PD conductance [36].

REM1.3 was the first REM family member discovered and initially described as a protein phosphorylated upon treatment with oligogalacturonides, which are plant cell wall components and elicitors of plant defense [37, 38] The biological relevance of REM phosphorylation is not known of different REM phospho-statuses suggest that the activity of these proteins could be regulated by phosphorylation during plant-microbe interactions [16, 17, 39, 40].

In the present paper, we show that phosphorylation of REM dictates its membrane dynamics and antiviral defense by the reduction of PD permeability. Our data point towards a model in which viral proteins such as the Coat Protein (CP), TGBp1 from PVX and 30K proteins from *Tobacco mosaic virus* (TMV) elicit the activation of protein kinase(s), which in turn phosphorylate(s) REM1.3 at its N-terminal domain. In turn, REM1.3's phospho-status regulates its spatial-temporal organization at the PM and association with PD. The latter is associated with PD closure by induction of callose deposition at PD pit fields and restriction of viral cell-tocell movement. Last, we further provide evidence that the membrane bound *Arabidopsis* CAL-CIUM-DEPENDENT PROTEIN KINASE 3 (CPK3) interacts with the taxonomic group 1b REMs *in vivo*, phosphorylates REM1.3 *in vitro* and restricts PVX propagation in a REMdependent manner. Collectively, this study brings valuable information about the involvement of PM nanodomains dynamics during the establishment of membrane-bound signaling processes.

Results

PVX triggers changes in REM1.3's membrane dynamic behavior and REM1.3 association with plasmodesmata

Group 1 and group 6 REM have been described as proteins regulating PD size-exclusion limit [8, 23, 33]. REM1.3 plays a role in restricting PVX passage through PD channels [8], [33] counteracting PVX movement proteins which promote PD opening [41]. To study the potential function of REM1.3 at PD in response to PVX infection, we surveyed simultaneously PD callose content and REM1.3 PD localization in healthy or PVX-infected *N. benthamiana* transiently expressing YFP-REM1.3 [42] (S1 Fig). Our analysis showed a significant increase in callose deposition in PVX-infected cells compared to mock conditions (Fig 1A and 1B). This finding suggests the recognition of PVX-encoded elicitors and the mobilization of a plant defense response leading to an increase of callose accumulation at PD pit fields.

Since protein activation is often linked to changes in subcellular localization [3, 44], we next examined whether PVX infection triggers changes in REM1.3 association with PD. Calculation of the PD index (ratio between fluorescence intensity of YFP-REM1.3 at the aniline-labeled PD pit fields and fluorescence at the PM around the pit fields [28], S1 Fig). Fig 1A and 1B showed that despite its role on PD regulation, YFP-REM1.3 is not enriched in the PD region of healthy *N. benthamiana* epidermal cells. We however reproducibly observed a slight increase of YFP-REM1.3 PD index upon PVX infection suggesting that PVX perception modulates REM1.3 localization and association with the PD pitfields (Fig 1A and 1B).

To gain further insights into REM1.3 dynamic localization at the PM upon PVX infection, we applied single-particle tracking Photoactivated Localization Microscopy in Variable Angle Epifluorescence Microscopy mode (spt-PALM VAEM) in living *N. benthamiana* epidermal cells [45] in absence or presence of PVX. We used the photoconvertible fluorescent protein EOS [46, 47] fused to REM1.3 to visualize, track, and characterize mobility behavior of single REM1.3 molecules. In addition, nanoscale localizations of single molecules observed overtime were computed to obtain super-resolution images and analyze REM1.3 organization at a molecular level. By this approach, we recently studied the protein organization and mobility parameters of single EOS-REM1.3 molecules in non-infected conditions and found that



Fig 1. REM1.3 modulates plasmodesmata callose accumulation and displays altered PM organization and dynamic following PVX infection. (A) Representative confocal images of aniline blue stained N. benthamiana leaf epidermal cells transiently expressing YFP-REM1.3 in the absence (mock is infiltration with empty A. tumefaciens) or the presence of PVX at 2 days after infiltration (DAI). Color-coding indicates fluorescence intensity. (B) Left, Pit field aniline blue fluorescence intensity was quantified by ImageJ as described in S1 Fig and expressed as the percentage of the mock control. Right, Quantification of the PD residency of YFP-REM1.3 in the absence (mock) and in the presence of PVX using the PD index [28] as described in S1 Fig. Graphs represent quantifications from 3 independent biological experiments. At least 15 cells per condition were analysed per experiment. Significant differences were determined by Mann-Whitney comparisons test *** p<0.001. (C) Super-resolved trajectories of EOS-REM1.3 molecules (illustrated by different colours) in the PM plane in the absence (Mock) and presence of PVX obtained by high-resolution microscopy spt-PALM. EOS-REM1.3 was transiently expressed in N. benthamiana (D) Diffusion coefficients (D) of EOS-REM1.3 expressed as log(D) in the absence (Mock) and presence of PVX. Statistical significances were assessed by Mann-Whitney test *** p<0.001 using data collected over two independents experiments. (E) Mean Square Displacement (MSD) over time for the global trajectories of EOS-REM1.3 followed during at least 600 ms reflecting two independent experiments. (F) Live PALM analysis of EOS-REM1.3 localization in the absence (mock) and presence of PVX by tessellation-based automatic segmentation of super-resolution images. (G) Computation of EOS-REM1.3 single molecule organization features based on tessellation-based automatic segmentation images. For

REM1.3 nanodomain size distribution for the indicated conditions, the Gaussian fits in absence (mock) and presence of PVX are indicated by lines. Total nanodomain area is expressed as percentage of the total PM surface. Percentage of EOS-REM1.3 molecules localizing into nanodomains, relative to all molecules observed. Localization density refers to the number of molecules observed per μ m² per second. Statistics were performed on at least 10 data sets per condition, from two independent experiments. Significant differences were determined by Mann-Whitney test * p<0.05, *** p<0.001.

https://doi.org/10.1371/journal.ppat.1007378.g001

EOS-REM1.3 displays an immobile and confined PM localization pattern, as commonly observed for plant membrane-associated proteins (Fig 1C-1E) [48], [36]. Reminiscent of these data, previous studies using different techniques described REM-associated PM domains to be predominantly laterally static [36, 48, 49]. Analysis of PVX-infected cells demonstrated an increase of EOS-REM1.3 diffusion coefficient (D) and mean square displacement (MSD), reflecting an increase of REM1.3 mobility (Fig 1C-1E). We next apply mathematical computation (Voronoï tessellation method [36, 50]) to compare the supra-molecular organization of EOS-REM1.3 of live PALM data in mock- and PVX-infected conditions (Fig 1F and 1G). Computation of EOS-REM1.3 single molecule organization features demonstrated a modulation of REM1.3 nanodomain-organization upon PVX infection (Fig 1G). Following PVX infection, the EOS-REM1.3-formed nanodomains are bigger in size, and there is a slight decrease of the proportion of molecules that localized into nanodomains as well as a decrease in the number of nanodomains formed. Overall, in both conditions, EOS-REM1.3 nanodomains represented similar proportions of the total PM surface. Additionally, a decrease in the localization density (number of molecules observed per μ m² per s) showed that upon PVX infection, there was less REM1.3 protein at the PM level. Overall, the changes of REM1.3 distribution under PVX infection *i.e.* enrichment of YFP-REM1.3 in the PD pit field regions, the increase of REM1.3's mobility and the modulation of REM1.3 nanodomain organization, suggest that the plant cell modulates PD-PM and PM nanodomain dynamics to circumvent PVX infection.

Perception of PVX proteins by plant cells leads to the activation of kinase (s) phosphorylating *REM1.3*

REM1.3 overexpression restricts PVX local and systemic spreading in both Solanum lycopersicum [8] and Nicotiana benthamiana [33, 36] (S2A and S2B Fig). Because REM1.3 protein level is not affected by PVX infection (S2C and S2D Fig), we assumed that neither synthesis nor degradation of the protein is modified by PVX, but perhaps post-translational modifications. As REM1.3 was originally discovered as a PM-associated phosphorylated protein [38], we first asked whether REM1.3 could be phosphorylated by leaf protein extracts. Equal protein amounts of microsomal and soluble extracts from N. benthamiana leaves were used as a potential kinase source to phosphorylate affinity-purified full-length 6His-REM1.3 in an in vitro kinase assay in the presence of ATP [γ -³³P]. Autoradiography revealed the presence of a clear band corresponding to a phosphorylated form of 6His-REM1.3 by kinase(s) present in the microsomal fraction (Fig 2A). The intensity of this band was completely abolished by competition with cold ATP, but not cold AMP, indicating a valid experimental set-up to study a genuine transphosphorylation event (S3A Fig). Phosphorylation of 6His-REM1.3 was almost undetectable in soluble fractions, representing cytosolic kinases (Fig 2A). In silico analysis predicted phosphorylation sites throughout REM1.3 sequence (Diphos, DEPP and NETPHOS prediction softwares). In agreement with the location of the sites presenting the highest phosphorylation potential, we experimentally found that REM1.3 was phosphorylated in its N-terminal domain (residues 1–116, hereafter 6His:REM1.3^N) whereas the C-terminal domain



Fig 2. PVX and viral proteins induce REM1.3 phosphorylation in its N-terminal domain. (A, B) *In vitro* protein phosphorylation assays were performed by incubation of recombinant affinity-purified 6His-REM1.3 and *N. benthamiana* extracts with $[\gamma^{-3^3}P]$ -ATP. The samples were run on SDS-PAGE gels and developed by autoradiography. Soluble (Sol) or microsomal (μ) extracts of healthy leaves in (A), or microsomal and PM extracts from healthy and PVX-infected plants in (B) were used. (C) *In vitro* phosphorylation of 6His-REM1.3^N by leaf microsomal extracts of healthy or PVX-infected N. *benthamiana* leaves. Bars show the quantification of phosphorylated 6His-REM1.3N bands from 5 independent repeats. (D) *In vitro* phosphorylation of 6His-REM1.3 by leaf microsomal extracts of healthy or PVX-infected leaves. (E) Experimental flow-chart to study the role virus protein in membrane-bound kinase activation. (F) 6His-REM1.3N phosphorylation by microsomal extracts infected with PVX-GFP or expressing the indicated viral proteins at 4 DAI. Leaves expressing GFP alone, infiltrated with water or with *A. tumefaciens* strain GV3101 alone served as controls. Expression of the viral proteins is presented in S3 Fig. (G) Graph represents the quantification of 6His-REM1.3N bands from three independent repeats (n = 3), as a percentage of the activity induced by *A. tumefaciens* strain GV3101 alone. Error bars show SE, and significance is assessed by Dunnett's multiple comparison test to water control (*, P < 0.1; **, P < 0.05; ***, P < 0.001). Phosphorylated proteins were detected by autoraliography and total proteins by stain free procedure. In all experiments 10µg of total protein extracts and 1µg of affinity purified 6His-REM1.3 or 6His-REM1.3^N were loaded per lane.

https://doi.org/10.1371/journal.ppat.1007378.g002

(residues 117–198, hereafter 6His:REM1.3^C) did not present any detectable phosphorylation (S3B and S3C Fig).

We next tested whether PVX activates the kinase(s) that phosphorylate(s) REM1.3. Our results unveiled that microsomal and PM fractions extracted from symptomatic PVX-infected leaves promoted higher levels of 6His-REM1.3 phosphorylation compared to non-infected plants (Fig 2B and 2C). Studies have shown that functionally different viral components, such as virus-encoded proteins and double-stranded RNA, can trigger plant defense responses [51-56]. We therefore examined whether the PVX genome in its free form was an eliciting signal for kinase activation. We found that the addition of total RNAs extracted from PVX-infected plants in the kinase reaction mix did not alter the levels of 6His-REM1.3 phosphorylation (Fig 2D). We then examined whether the sole expression of individual viral movement proteins was sufficient to trigger REM1.3 phosphorylation (Fig 2E). Importantly, our results demonstrated that the expression of TGBp1 and Coat Protein (CP) fused to GFP triggered the strongest levels of 6His-REM1.3^N phosphorylation to the same extent as the full PVX-GFP construct (Fig 2F and 2G and S3E Fig for controls of viral fluorescent-tagged protein expression as described in [41]). In good agreement, expression of a TGBp1-deleted version of PVX (PVXATGBp1) decreased 6His-REM1.3 phosphorylation levels compared to wild-type PVX extracts (S3D Fig). Expression of TGBp2 and infiltration of the empty Agrobacterium strain alone protein also induced 6His-REM1.3^N phosphorylation, albeit less effective than TGBp1 and CP proteins (Fig 2F and 2G). In accordance with previous reports suggesting REM phosphorylation during plant-microbe interactions [9], Agrobacterium infected N. benthamiana extracts induced much stronger REM1.3 phosphorylation than the water control condition (Fig 2F and 2G). Furthermore, we found that the 30K-RFP protein from Tobacco mosaic virus (TMV) also induces REM phosphorylation (S3D Fig). Similar to PVX-TGBp1, REM1.3 interferes with the ability of TMV-30K to increase PD permeability [33] and overexpression of REM1.3 restricts TMV-GFP cell-to-cell movement in N. benthamiana epidermal cells (S4A Fig).

Altogether our data suggest an additional role of REM-mediated plant response against TMV and possibly to bacteria. Our results also indicate that REM1.3 phosphorylation status is modulated by the perception of viral proteins by plant cells.

Phosphorylation of REM1.3 regulates its function in restricting PVX spreading via PD aperture modulation

Since phosphorylation of REM occurs upon PVX infection, we next aimed to functionally characterize the importance of REM1.3 phosphorylation for the regulation of PVX cell-to-cell movement. Despite our efforts, the identification of *in vivo* phosphorylation sites of REM1.3 appeared technically challenging and remained unsuccessful. *In silico* predictions and *in vitro*

kinase assays however showed that REM1.3^N displays regions of intrinsic disorder and presents the highest potential of phosphorylation (Fig 2C–2F and Fig 3A). For functional characterization, we selected the three putative phosphorylation Serine(S) /Threonine(T) sites present in REM1.3^N, namely S74, T86 and S91, that presented high scores of phosphorylation prediction in intrinsic disorder regions (Fig 3A). S74 and S91 are conserved across the phylogenetic group 1b of REM proteins, suggesting functional redundancy (S5A Fig) [32, 57]. S74 and S91 were the analogous residues identified as phosphorylated *in vivo* in the group 1b REM *AtREM1.3* (At2g45820) of *Arabidopsis thaliana* (hereafter Arabidopsis) in a stimuli-dependent manner [39, 40, 57]. Biochemical analysis showed that **a**-1,4-poly-D-galacturonic acid (PGA)induced phosphorylation of *StREM1.3* occurs on T32, S74 and T86 [58]. T86 is not conserved in Arabidopsis but it is conserved in Solanaceae REM proteins, such as in *N. benthamiana* (S5A Fig). By an *in vitro* kinase assay, we show that phosphorylation occurs within three potential phosphor-residues, since mutation of S74, T86 and S91 to the non-phosphorylatable Aspartic acid (D), generating the 6His-REM1.3^{DDD} mutant abolished REM phosphorylation by the PVX-activated kinase(s) (Fig 3B and 3C).

To discriminate which residues are functionally relevant in the context of PVX-GFP propagation, we generated RFP-tagged REM1.3 phosphomutants, individually mutated at those sites to the non-phosphorylatable Alanine. Transient expression in *N. benthamiana* coupled with PVX-GFP infection assays demonstrated that individual phospho-null mutations at those sites induce a loss of function of REM1.3 in restricting PVX-GFP spreading (Fig 3D). This result suggests that phosphorylation of either S74, T86 and S91 is important for REM1.3 function.

To further characterize the relevance of different REM1.3 phospho-statuses in the context of PVX-GFP propagation and PD-aperture regulation, we analysed RFP-tagged REM1.3^{DDD} to mimic constitutive phosphorylation hereafter termed phosphomimetic mutant, or to Alanine (REM1.3^{AAA}) hereafter termed phosphodead mutant. Infection assays in *N. benthamiana* confirmed that the phosphodead mutant completely lost REM1.3 ability to restrict PVX-GFP cell-to-cell movement, while the phosphomimetic mutant maintained this ability (Fig 3D). TMV-GFP propagation was similarly affected by the phospho-status of REM1.3 (S4A Fig). We then analyzed the capacity of REM1.3 phosphomutants to regulate PD aperture in the absence of viral infection. As previously described [33, 36], RFP-REM1.3 reduces the PD size-exclusion limit as measured by free-GFP cell-to-cell diffusion (Fig 3E). Detailed analysis of REM1.3 phosphorylation mutants demonstrated that the phosphomimetic mutant recapitulated REM1.3 activity towards PD-aperture regulation, while the phosphodead mutant did not (Fig 3E).

Altogether, these results provide strong evidence that REM1.3's phosphorylation state at the evolutionarily conserved positions of S74, T86 and S91 is linked to its function in controlling viral infection and PD conductance.

REM1.3 phospho-status modulates its dynamic lateral segregation in the PM and PD sub-compartments

Both REM1.3 phosphomimetic and phosphodead mutants maintained PM localization, similarly to wild-type REM1.3, when transiently expressed in fusion with YFP in *N. benthamiana* (S4B Fig). Upon PVX infection we observed a modulation of REM1.3 PD-association and PM dynamics (Fig 1), linked to REM1.3 phosphorylation (Fig 2) that is required for REM1.3 function against PVX infection (Fig 3). We then asked whether different REM1.3 phospho-statuses might regulate its lateral organization at the PM and PD compartments in the absence of PVX. We examined the enrichment of REM1.3 YFP-tagged phosphomutants at the PD pit fields, previously calculated by the PD index (S1 Fig) and found that similarly to YFP-REM1.3, none







Fig 3. Mutational analysis reveals three critical phospho-residues required for REM1.3 regulation of PVX-GFP propagation and PD conductance. (A) In silico analysis of REM1.3 protein sequence. Prediction of putative phosphorylation sites was performed by Diphos, DEPP and NETPHOS coupled with published MS data. Predictions highlight three residues S74, T86 and S91 with high probability to be phosphorylated. Disordered prediction was performed by pDONR VL XT. Numbers indicate amino acid position. (B) In vitro kinase assay on recombinant affinity purified 6His-REM1.3 or 6His-REM1.3DDD by incubation with $[\gamma^{-33}P]$ -ATP and microsomal extracts of PVX-infected N. benthamiana leaves, as described in Fig 2. Phosphorylated proteins were detected by autoradiography and total proteins by silver staining. Asterisk * indicates phosphorylation of a N. benthamiana protein of close molecular weight not detected by silver staining. (C) Graph represents the relative quantifications from 4 independent reactions, using WT signal as a reference. (D) Left, Representative epifluorescence microscopy images of PVX-GFP infection foci on N. benthamiana leaf epidermal cells at 5 DAI. Graph represents the mean relative PVX-GFP foci area in cells transiently expressing RFP alone, wild-type RFP-REM1.3 or carrying single serine /threonine mutations to alanine. Co-infiltration of PVX-GFP with an empty A. tumefaciens strain served as mock control. Approximately 160 foci per condition from 3 independent biological repeats were measured. Letters indicate significant differences revealed by Dunn's multiple comparisons test p<0.001. Right, Graph represents the mean relative PVX-GFP foci area in cells transiently expressing wild-type RFP-REM1.3 or triple RFP-REM1.3 phosphodead and phosphomimetic mutants compared to mock control (co-infiltration of PVX-GFP with an empty A. tumefaciens strain). Approximately 250 foci per condition from 5 independent biological repeats were measured Letters indicate significant differences revealed by Dunn's multiple comparisons test p<0.001. Epifluorescence microscopy images show representative PVX-GFP infection foci on N. benthamiana leaf epidermal cells at 5 DAI. (E) GFP diffusion to neighbor cells was estimated by epifluorescence microscopy at 5 DAI in N. benthamiana cells transiently expressing RFP-REM1.3 or phosphomutants. Measurements from 3 independent biological repeats were normalized to mock control (coinfiltration with an empty A. tumefaciens strain). Letters indicate significant differences determined by Dunn's multiple comparisons test p<0.001.

https://doi.org/10.1371/journal.ppat.1007378.g003

of the phosphomutants appeared enriched at the pit field level (Fig 4A and 4C). The phosphodead mutant appeared statistically more excluded than YFP-REM1.3, whereas the phosphomimetic mutant displayed an increase of its PD index (Fig 4C), reminiscent of the REM1.3 localization phenotype under PVX infection (Fig 1A and 1B). Importantly, REM1.3 phosphomutants' association with PD was directly correlated with callose content at PD (Fig 4B). These observations reinforced the hypothesis that REM1.3-mediated increase of callose levels at PD is associated with a dynamic and phosphorylation-dependent redistribution of REM1.3 to the PD surroundings.

We next used spt-PALM VAEM to characterize the localization and mobility behaviour of the EOS-REM1.3 phosphomutants in the PM plane. The analysis of reconstructed trajectories and corresponding super-resolved localization maps indicated slight modifications of lateral mobility behavior between the phosphomutants (Fig 4D and 4E). Quantification of the diffusion coefficient values (D) extracted for each individual molecule revealed that EOS-RE-M1.3^{AAA} displayed a more immobile behavior than EOS-REM1.3^{DDD} and EOS-REM1.3. Consistently, EOS-REM1.3^{DDD} exhibited a higher mobility illustrated by higher diffusion coefficient and mean square displacement values (Fig 4D and 4E). Analysis of the supra-molecular organization of the phosphomutants by Voronoï tessellation (Fig 4F) firstly showed that all mutants displayed similar nanodomain size and localization density compared to EOS--REM1.3WT. Compared to EOS-REM1.3^{AAA}, the EOS-REM1.3^{DDD} nanodomains occupied a smaller area of the total PM and their density in the PM plane appeared slightly reduced (Fig 4F and 4G). A higher number of nanodomains were formed with the EOS-REM1.3^{AAA} mutant. Hence, the phosphomimetic mutations favor a less confined and a more dynamic localization pattern of REM1.3 at the PM, reminiscent to the phenotype of EOS-REM1.3WT in the context of PVX infection (Fig 1C and 1D).

These results suggest that differential REM1.3 phosphorylation is involved in regulating REM1.3 mobility and PM domain organization and support the hypothesis that REM1.3 phosphorylation on S74, T86 and S91 reflects an 'active form' of the protein necessary for REM1.3-mediated defense signaling.



Fig 4. REM1.3's dynamic localization in PD and PM nanodomains is regulated by its phospho-status. (A) Representative confocal mages showing aniline blue staining of callose deposition at the PD pitfields in *N. benthamiana* leaf epidermal cells transiently expressing YFP-REM1.3 or phosphomutants. Color-coding indicates fluorescence intensity. (B) Graphs show aniline

blue fluorescence intensities in cells transiently expressing YFP-REM1.3 and phosphomutants relative to control cells expressing YFP alone. Three independent biological experiments were performed and at least 15 cells per condition and per experiment were analyzed. Letter indicate significant differences revealed by Dunn's multiple comparisons test p<0.001. (C) PD index of YFP-REM1.3 phosphomutants was calculated as described in S1 Fig. Graphs present quantifications from 3 independent biological experiments. Letter indicate significant differences revealed by Dunn's multiple comparisons test p<0.002. (D) Super-resolved trajectories (illustrated by different colours) of transiently expressed EOS-REM1.3, and phosphomutants, transiently expressed in N. benthamiana cells, observed by spt-PALM. Scale bars, 2 µm. (E) Distribution of diffusion coefficients (D) represented as log(D) of the different fusion proteins. Mean Square Displacement (MSD) over time for the global trajectories of each EOS-REM1.3 construct followed during at least 600ms. 27 cells for EOS-REM1.3, 15 cells for EOS-REM1.3AAA and 17 cells for EOS-REM1.3DDD were analyzed in 3 independent experiments. Statistical analysis was performed by Mann-Whitney test * p<0.05 ** p<0.01. (F) Live PALM analysis of EOS-REM1.3 phosphomutants by tessellation-based automatic segmentation of super-resolution images. (G) Computation of EOS-REM1.3 and phosphomutants single molecule organization features based on tessellation-based automatic segmentation images. For REM1.3 and phosphomutants nanodomain size distribution and the Gaussian fits are indicated. Total nanodomain area is expressed as percentage of the total PM surface. Percentage of EOS-REM1.3 molecules localizing into nanodomains, relative to all molecules observed. Localization density refers to the number of molecules observed per μm^2 per second. Statistics were performed on at least 13 data sets per condition extracted from 3 independent experiments. Statistical differences determined by Mann-Whitney test * p<0.05, ** p<0.01.

https://doi.org/10.1371/journal.ppat.1007378.g004

AtCPK3 phosphorylates REM1.3

To gain more insights into the signaling processes leading to REM1.3 phosphorylation, we aimed to biochemically characterize the kinase(s) involved in the phosphorylation of REM1.3. Previous evidence suggested that the kinase(s) phosphorylating REM1.3 are membrane-associated (Fig 2) [38]. We therefore biochemically analyzed the localization of the kinase(s) phosphorylating REM1.3. Plant material from healthy and PVX-GFP-infected leaves was cellfractionated to obtain crude extracts, soluble and microsomal fractions [59] to perform in vitro kinase assays on REM1.3N. Analysis confirmed a maximal kinase activity in purified microsomes (Figs 5A and 2A). Since a kinase in close proximity with its substrate would enhance reaction kinetics [60] and signal fidelity [61], and given that REM1.3 is enriched in detergentresistant membranes (DRM) [8], we investigated whether the kinase activity towards 6His-REM1.3 is enriched in this biochemical fraction. We included "control PM" (C-PM) preparations, submitted to discontinuous sucrose gradients but in the absence of Triton-X100 treatments [62]. In vitro kinase assays on 6His-REM1.3N showed that the kinase activity in C-PM was 5 times inferior than in freshly purified PM not submitted to the sucrose gradient, suggesting that the kinase is not stable during the overnight purification procedure. Only half of the specific activity of the kinase was found in DRMs compared to the C-PM fraction, indicating that the kinase(s) phosphorylating REM1.3 is (are) only partially located in the DRM fraction (Fig 5B).

To gain more information concerning the biochemical characteristics of the kinase phosphorylating REM1.3, we analyzed its activity in the presence of known inhibitors. We firstly tested staurosporine, [63, 64] a general inhibitor that prevents ATP binding to kinases. We found an inhibition of REM1.3 phosphorylation starting at very low concentrations (30 nM) (S6A Fig). We further tested the effect of poly-L-lysine, described to stimulate the activity of the CK2 kinases and inhibit several CDPK kinases [65, 66]. No significant differences on REM1.3 phosphorylation levels were observed under increasing concentrations of poly-Llysine (S6B Fig). The addition of the wide range of Ser/Thr phosphatases inhibitor β -glycerophosphate (BGP) [66] to the reaction mix did not alter the levels of phosphorylated 6His-REM1.3, indicating that the observed data was due to the activation of kinase activity by PVX rather than by inhibition of phosphatases (S6B Fig). Competition assays in the presence of cold AMP and GTP showed that only cold ATP even at 2 mM caused 20-fold depletion in [γ -³³P] incorporation, suggesting that ATP is the major phosphoryl-donor for the kinase (S6B Fig). Addition in the reaction mix of 0,2 mM of EGTA, a chelator of Ca²⁺, strongly inhibited the kinase activity suggesting that the kinase(s) phosphorylating REM1.3 in healthy leaves is



Fig 5. AtCPK3 phosphorylates REM1.3 in a calcium-dependent manner. (A, B) *In vitro* phosphorylation of purified 6His:REM1.3^N by kinase(s) from different cellular fractions of *N. benthamiana* leaves, CEs, leaf crude extracts; Sol, Soluble fraction; μ , microsomal fraction; PM, Plasma Membrane; C-PM: "Control-PM" is PM fraction not treated by TX100, but submitted to sucrose gradient; DRM, Detergent

resistant membranes [62]. The graph represents the relative quantification of 3 independent experiments normalized to the activity in the PM fraction +/- SEM. (C) Quantification of the calcium dose response of kinase activity on 6His-REM1.3^N phosphorylation by *N. benthamiana* microsomal extracts from healthy and PVX infected leaves. (D, E, F) Autoradiography gels show *in vitro* phosphorylation of 6His-REM1.3, 6His-REM1.3N and 6His-REM1.3^C, 6His:REM1.3^{DDD} or 6His:AtREM1.2 by affinity purified GST-AtCPK3 in the presence or the absence of Ca2+. Bands corresponding to autophosphorylation of AtCPK3-GST and transphosphorylation of 6His-tagged group 1 REM variants are indicated. Gels were stained by coomassie blue to visualize protein loading. Asterisk* indicates a non-specific band present in both 6His-REM1.3^C and 6His-REM1.3^N preparation.

https://doi.org/10.1371/journal.ppat.1007378.g005

calcium sensitive (S6C Fig). Calcium is a conserved second messenger in signal transduction during biotic and abiotic stress. In plants, kinases harboring different calcium sensitivities can perceive calcium variations and translate them into downstream signaling activation [67, 68]. To determine whether the PVX-activated kinase phosphorylating REM1.3 is sensitive to calcium regulation, *in vitro* kinase assays from microsomes of healthy and PVX-infected *N*. *benthamiana* leaves were assayed in the presence of free calcium (Ca²⁺) concentrations ranging from 10 nM to 0,1 mM. Fig 5C shows that the kinase(s) displays a high sensitivity to calcium with an optimal activity in the presence of 10 μ M of free Ca²⁺. At this concentration, a 5-fold increase of 6His-REM1.3^N phosphorylation was observed in PVX-infected leaves (Fig 5C). These experiments allowed us to narrow-down the kinase(s) phosphorylating REM1.3 after PVX infection to the group of membrane-bound Ca²⁺-dependent protein kinases [67].

Plants possess three main families of calcium-regulated kinases: calmodulin-binding kinases (CBKs), calcineurin B-like-interacting protein kinases (CIPKs) and calcium-dependent protein kinases (CPKs) [67]. CPKs have the unique feature of calcium sensing and responding activities in one single polypeptide, best characterized in the model plant Arabi*dopsis* [67]. Based on the measured calcium dose response (Fig 5C), we correlated the kinase phosphorylating REM1.3 in N. benthamiana with homologs of Arabidopsis subgroup II AtCPKs [69], and we aimed to capitalize on the knowledge of Arabidopsis CPKs to test REM1.3 phosphorylation. Among the characterized members of subgroup II AtCPKs, we selected the Arabidopsis AtCPK3 as a good candidate to test its putative role in REM1.3 phosphorylation, since previous proteomics studies in Arabidopsis have identified both AtCPK3 and AtREM1.3 as being enriched in PM, PD and DRM fractions [22, 70]. In addition, one study showed that AtREM1.3 from microsomal fractions is phosphorylated in vitro by AtCPK3 [71]. We therefore predicted that REM1.3 might share common functions with the evolutionarily conserved group 1b Arabidopsis REMs [32]. AtREM1.2 and AtREM1.3 are close homologs to REM1.3 and group 1 N. benthamiana REMs (NbREMs) in term of protein sequence [32, 36] and they conserved at least the S74 and S91 phosphorylation sites [39], [40, 57] (S5A Fig). Using super-resolution microscopy, Demir et al. showed that, when co-expressed in Arabidopsis leaves, REM1.3 and AtREM1.3 co-localized in the same PM-nanodomains [72]. Importantly, transient expression of AtREM1.2 and AtREM1.3 in N. benthamiana epidermal cells impaired PVX-GFP cell-to-cell movement, as REM1.3 does (S5B Fig), strengthening the hypothesis that the function of group 1 REMs might be conserved between homologs in different plant species [36].

We assayed the *in vitro* phosphorylation activity of the affinity-purified AtCPK3-GST towards the 6His-REM1.3, the 6His-REM1.3^N and the 6His-REM1.3^C, as well as the homologous substrate 6His-AtREM1.2. Similar to our previous results (S3B and S3C Fig), AtCPK3-GST could phosphorylate strongly both 6His-REM1.3 and 6His-REM1.3N, but not 6His-REM1.3C (Fig 5D). In accordance with the effect of *AtREM1.2* in PVX-GFP propagation (S5B Fig), AtCPK3-GST can also phosphorylate 6His-AtREM1.2 (Fig 5E). Addition of Ca²⁺ is essential for a strong kinase activity as shown by both kinase auto-phosphorylation and transphosphorylation (Fig 5D and 5E). AtCPK3-GST specifically phosphorylated S74, T86 and S91

residues of REM1.3, since the phosphorylation was abolished in the phosphomimetic mutant 6His-REM1.3^{DDD} (Fig 5F).

These results suggest that AtCPK3 is a good candidate for group 1b REM phosphorylation and further support that the S74, T86, and S91 are the phosphorylation sites of REM1.3 (Figs 3A and 5E).

AtCPK3 interacts with group 1b REMs and restricts PVX propagation in a REM-dependent manner

CPKs harbor a variable N-terminal domain, a Ser/Thr kinase domain, an auto-inhibitory junction region and a regulatory calmodulin-like domain. The calmodulin-like domain contains four EF-hand binding motifs that determine the sensitivity of each kinase to calcium [73, 74]. To investigate the role of AtCPK3 in REM-dependent signalling, we generated AtCPK3 mutants presenting altered kinase activities. Deletion of the inhibitory junction region and the regulatory calmodulin-like domain in CPKs creates a constitutive active kinase while mutation of the aspartic acid residue in the catalytic center 'DLK' motif of the kinase domain to an alanine (D202A) creates a catalytically inactive or 'dead' kinase [67] (Fig 6A). We generated AtCPK3 full-length (AtCPK3), constitutive active (AtCPK3CA, residues 1–342) and kinasedead (AtCPK3CAD202A) constructs for transient protein expression (Fig 6A). We evaluated their catalytic activities by expressing them transiently in Arabidopsis mesophyll protoplasts and performing immunoprecipitation coupled to kinase assays using 6His-REM1.3 and histone as a generic substrate [67]. Autoradiography confirmed that *in vivo* purified AtCPK3-CA-HA could trans-phosphorylate both 6His-REM1.3 and histone without the addition of calcium, while the point mutation D202A drastically abolished kinase activity (S7 Fig).

We next examined the sub-cellular localization of both *AtCPK3* and *AtCPK3CA* fused to YFP and found that both proteins disclosed a partial association with the PM, which was further confirmed by their presence, after cell fractionation, in the microsomal fraction at the expected molecular weight (Fig 6B) in good agreement with [71]. We further used AtCPK3CA to test the interaction with group 1b REMs. Bimolecular Fluorescence Complementation (BiFC) experiments showed that AtCPK3CA and REM1.3, REM1.3^{AAA} and REM1.3^{DDD} interact together at the level of the PM *in planta*. Importantly, we also confirmed the interaction of AtCPK3CA with homologous *AtREM1.2* and *AtREM1.3* (Fig 6C). REM1.3/REM1.3 interaction was used as a positive control, and AtCPK3CA /AtCPK3CA as a negative control.

We finally aimed to functionally characterize the *AtCPK3*- and REM1.3-mediated signaling in the context of PVX infection. Transient over-expression of AtCPK3-RFP alone induces a reduction of PVX-GFP infection foci suggesting that *AtCPK3* is indeed important for antiviral responses in plant cells (Fig 6D). Expression of the constitutively-active AtCPK3CA-RFP had a stronger effect on PVX-GFP spreading and to a similar degree with the over-expression of REM1.3 alone (Fig 6D). *AtCPK3*'s function towards PVX movement was observed to be mediated by its kinase activity, as the expression of the catalytically inactive mutant AtCPK3-CA^{D202A} had no effect on PVX-GFP propagation (Fig 6D).

This raised the question whether the effect of AtCPK3CA on PVX propagation was REMdependent. To tackle this question, we stably transformed *N. benthamiana* plants with a hairpin construct, to induce post-transcriptional gene silencing, which resulted in lowering RNA and protein expression of group 1 endogenous NbREMs (S8A and S8B Fig). Consistent with previous studies [8], silencing of group 1 REM correlates with an increase of PVX-GFP cell-tocell movement in inoculated leaves (S8C Fig). No difference was observed by ELISA when measuring PVX accumulation in systemic leaves (S8D Fig). Importantly, PVX assays demonstrated that AtCPK3CA ability to restrict PVX movement was impaired in two independent *N*.





benthamiana epidermal cells. Scale bar shows 10 µm. Western blot against GFP showing AtCPK3-YFP and AtCPK3CA-YFP expression in the microsomal fraction (µ) of *N. benthamiana* leaves. (C) *In planta* Bimolecular Fluorescence Complementation (BiFC) analysis showing interaction of AtCPK3 with Group 1 REMs. REM1.3-YFPN/ REM1.3-YFPC was used as a positive control, and AtCPK3CA-nYFP/AtCPK3CA-cYFP as a negative control. Mean fluorescence intensity at the cell boundary level was recorded using ImageJ. Statistical differences were determined by Mann-Whitney test compared to AtCPK3CA +AtCPK3CA.*** p = 0.0002, **** p < 0.0001. All scale bars indicate 20µm. (D) PVX-GFP spreading in *N. benthamiana* cells expressing RFP-REM1.3 or AtCPK3FL-RFP, AtCPK3CA-RFP, AtCPK3CAD202A-RFP Graph represents the area of PVX-GFP infection foci relative to the mock control (co-infiltration of PVX-GFP with empty *A. tumefaciens*). At least 200 PVX-GFP infection foci from at least 3 independent repeats were imaged at 5DAI. Letters indicate significant differences revealed by Dunn's multiple comparisons test p < 0.001. (E) Effect of AtCPK3CA on PVX-GFP cell-to-cell movement in WT *N. benthamiana* or in transgenic lines constitutively expressing hairpin REM (hpREM) constructs. At least 200 PVX-GFP infection foci from at least 3 independent repeats were imaged at 5DAI. For each *N. benthamiana* line the effect of AtCPK3CA is expressed as a percentage of the corresponding mock control (empty Agrobacteria). Absolute values of the average foci area for each mock control are indicated.

https://doi.org/10.1371/journal.ppat.1007378.g006

benthamiana lines underexpressing group 1 REM levels, (namely lines 1.4 and 10.2 with expression levels decreased respectively by 2 and 20 times) (Fig 6E), indicating that REMs might be the direct substrate of CPK3 *in vivo*.

Altogether, these data suggest that CPK3 and group 1 REMs are major regulators involved in signaling and antiviral defense at the PM level.

Discussion

Protein phosphorylation is a ubiquitous and specific mechanism of cell communication [75]. The addition of a phosphate group on one or more critical residues of a given protein can induce important conformational changes that affect energetically favorable interactions and may lead to changes in its interacting network, localization, abundance and may influence the activity of protein signaling pools [76]. Although, since the initial discovery of REM1.3 in 1989, accumulating evidence suggests that the functions of REM proteins are regulated by protein phosphorylation [38–40]. The biological significance of this phosphorylation remained unclear to this date. REM proteins were among the first plant proteins described which supported the notion of PM sub-compartmentalization to functional protein-lipid nanodomains [8, 11, 77], also named membrane rafts [3, 4, 21]. In the present paper, we used REM1.3 and PVX as an experimental system to study the role of protein phosphorylation and membrane dynamics in the context of stress response.

REM1.3 functions likely involve distinct PM compartments during plant PVX-sensing

Understanding how plants defend themselves against viruses remains a challenging field. The canonical plant immune response against viruses is mainly represented by the mechanism of RNA silencing [78, 79], while additional mechanisms of plant antiviral defense involve hormonal signaling, protein degradation, suppression of protein synthesis and metabolic regulation [51, 78, 80]. Antiviral defense presents similarities to the immune response against microbes [81–83]. Compelling evidence suggests that cell-surface as well as intracellular plant immune receptors recognize viral elicitors [55, 84–89]. An additional number of host cell components have been shown genetically to affect viral replication or cell-to-cell movement [8, 90], indicating that more sophisticated plant defense mechanisms against viruses may exist.

For instance, manipulation of REM levels in transgenic *Solanaceae* suggested that REM is as a positive regulator of defense against the PVX by affecting viral cell-to-cell movement [8, 14, 36]. We recently showed that REM1.3 does not interfere with the suppressor ability of PVX movement protein TGBp1, but specifically affects its gating ability [33]. Group 1 REMs could

be a target for viruses (and other pathogens) to circumvent infection as illustrated by the case of *Rice Stripe Virus* that targets NbREM1 for degradation by 26S proteasome [91]. Nevertheless, in this study we show that REM1.3 protein levels are not altered during PVX infection (S2C and S2D Fig).

In this paper, we provide supporting mechanistic evidence that REM1.3 regulates the levels of callose accumulation at PD pit fields during PVX infection (Fig 1). Whether this function is mediated by a direct interaction with callose synthase/glucanase complexes remains however still unknown. Surprisingly, we found that REM1.3 is not dramatically recruited to PD pit fields, although its PD index is slightly increased after PVX infection (Fig 1). This suggests that association of a sub-fraction of the REM1.3 to the PD-PM region may be sufficient to increase callose accumulation, although we cannot rule out the possibility that REM1.3 may regulate PD permeability *via* a more indirect mechanism. The spt-PALM VAEM microscopy data supports an increase of protein mobility and redistribution to distinct domains during PVX infection (Fig 1). These findings indicate the existence of a mechanism that operates at specific REM1.3-associated PM nanodomains, capable of regulating PD permeability (Fig 1). The dynamic partitioning between PM nanodomains and PD pit fields needs to be further studied.

Plant PVX-sensing induces the activation of a calcium-dependent protein kinase

Since various studies have reported REM phosphorylation during plant-microbe interactions [16, 17, 39, 40], we set out to address which kinase phosphorylates REM and whether REM1.3 phosphorylation plays a role in REM-mediated anti-viral defense. Indeed, our experimental findings show that plant PVX sensing induces the activation of a membrane-bound calcium-dependent protein kinase that in turn phosphorylates REM1.3 (Fig 2, Fig 5). Importantly, we show that the kinase able to phosphorylate REM1.3 is activated specifically by the expression of two PVX proteins, namely CP and TGBp1. Deciphering the exact mechanisms allowing the molecular recognition of those PVX components will be a crucial step toward understanding REM-mediated anti-viral defense. Intriguingly, the finding that the presence of *Agrobacterium* also induces REM1.3 phosphorylation (Fig 2G) is in agreement with previous reports suggesting phosphorylation of REMs under bacterial infection [39, 40] and suggests that phosphorylation should be also a way to regulate -yet unknown functions- of REM1.3 in bacterial defense.

Genetic studies have established that different CPKs comprise critical plant signaling hubs by sensing and translating pathogen-induced changes of calcium concentrations [67, 68]. Biochemical characterization of the kinase phosphorylating 6His-REM1.3 showed that its strong sensitivity to calcium (Fig 5C) corresponds to homologs of phylogenetic subgroup II CPKs from Arabidopsis [67]. CPK3 is a prominent member of subgroup II, shown to function in stomatal ABA signaling [92], in salt stress response [71, 93] and in a defense response against an herbivore [94]. Interestingly, it was suggested that *AtREM1.3* from taxonomical group 1 of REMs could be a substrate for *AtCPK3* in response to salt stress [71]. Here we show that *AtCPK3* can interact *in vivo* with group 1 REM (Fig 6C) and that *AtCPK3* phosphorylates group 1 REM in an *in vitro* kinase assay (Fig 5D). Transient overexpression of *AtCPK3* in *N*. *benthamiana* resulted in a reduction of PVX propagation in a REM-dependent manner, providing compelling evidence that CPK3 together with REM contribute to the plant antiviral immunity. This is the first report demonstrating the participation of CPKs in plant basal immunity against viruses.

Although [95] reports that there is no calcium signal during early recognition of PVX, the activation of CPKs by PVX supports the notion that calcium might be involved in some other late steps of plant-virus interaction like the control of intercellular connectivity. These changes

in calcium concentrations in the cell are sensed by the CPKs and translated via the phosphorylation of REM and/or other unknown downstream components. In *Nicotiana tabacum* calmodulin isoforms are critical for the plant resistance against Tobacco Mosaic Virus and Cucumber Mosaic Virus, further illustrating the existence of virus-specific patterns of calcium signals [96, 97]. More work is needed to identify the CPK family members participating to the response and also the nature and specificity of those PVX-induced calcium changes.

Phosphorylation regulates group 1 REM's function during PVX cell-to-cell movement

AtCPK3 specifically phosphorylated REM1.3 at its N-terminal domain (residues 1-116), a domain displaying a mostly intrinsically disordered secondary structure (Figs 3A and 5). In silico analysis followed by a mutagenesis approach coupled with in vitro kinase assays revealed three major putative phosphorylation sites for REM1.3, namely S74, T86 and S91 on REM1.3. The *in vitro* phosphorylation of REM1.3 (Figs 3A and 5E) is almost totally lost when S74, T86 and S91 are mutated to non-phosphorylable residues, confirming these residues as major REM1.3 phosphorylation sites. Individual phospho-null mutations at those sites impaired REM1.3 ability to restrict PVX cell-to-cell movement to various extent (Fig 3D). The triple phospho-null mutant, YFP-REM1.3^{AAA} totally obliterated REM1.3's capability to restrict PVX cell-to-cell movement (Fig 3D) and to regulate PD permeability (Fig 3E). Reciprocally, REM1.3 triple phosphomimetic mutant, RFP-REM1.3^{DDD} appeared fully functional (Fig 3E and 3F). These results strongly support the functional involvement of single or combined phosphorylation in the N-terminal domain of S74, T86 and S91 to establish REM's function in the context of PVX infection. This is in contrast with LjSYMREM1 from Lotus japonicus which was shown to be phosphorylated at its C-terminal domain *in vitro* by SYMRK [16]. Despite the fact that phosphorylation of REM proteins has been widely reported [16, 17, 39, 40, 57], this work firstly describes an associated role of REM-induced phosphorylation with its function.

Toward the understanding of REMORIN function

Our finding that overexpression of *AtREM1.2* and *AtREM1.3* also restricts PVX-GFP cell-tocell movement (S5B Fig) suggests that REM phosphorylation and its associated functions might be conserved for some REMs of taxonomic group 1b. In good agreement, *AtREM1.2* and *AtREM1.3* localize to the same PM nanodomains [72] and maintain conserved phosphorylation sites with REM1.3 (S5A Fig). By contrast, *AtREM4.1* from subgroup 4, presenting a different N-terminal domain and different expected phosphorylation profile has an opposite effect against geminiviral propagation by promoting susceptibility to *Beet curly top virus* and *Beet severe curly top virus* [17, 57]. This further argues that REMs might be phosphorylated by diverse families of kinases in order to respond to different stimuli [57].

Overexpression of REM1.3 restricts TMV propagation (S4A Fig), and additionally modulates the movement proteins from different virus genera [33, 91]. These findings suggest that the initial hypothesis that REM1.3 causes the sequestration of the PVX virions at the PD [8] might not hold true, but rather that REM1.3 might have a more general role in plant stress and PD regulation (Figs 1 and 3). Interestingly, REM1.3 promotes susceptibility to *Phytophthora infestans* in *N. benthamiana* and localizes exclusively to the PM and the extrahaustorial membrane around non-callosic haustoria [42]. The exact role of REM1.3 as a common regulator of different signaling pathways and its role in PD permeability regulation remains to be determined.

It has been speculated that phosphorylation in intrinsically disorder regions of proteins may act as a molecular switch and confer potential protein-protein interaction plasticity [76, 98]. The intrinsically disordered REM1.3 N-terminal domain exhibits the most sequence variability in REM proteins, presumably conferring signaling specificity [32, 57]. Phosphorylation of AtREM1.3's N-terminal domain could stabilize coil-coiled-associated protein trimerization and protein-protein interactions [57]. Phosphorylated REM1.3 seems to be further targeted to PD-PM to trigger callose deposition. In good agreement, we found that the mobility in the PM of REM1.3 changed depending on its phospho-status (Fig 4). The triple phosphomimetic mutant exhibited a less confined and more mobile behavior at the PM, reminiscent of the WT protein in the context of PVX infection (Fig 4D). Similarly to the role of 14.3.3 proteins in plants [99], REM1.3 could act as a scaffolding protein, interacting with multiple members of a signaling pathway and tethering them into complexes to specific areas of the membrane. Hence, REM1.3 phosphorylation could act as a regulatory switch of protein conformations that would modulate REM1.3 specific interaction patterns and transient signalosomes at the PM. The triple phosphomimetic REM mutant might reflect a 'functionally active' form that constitutes REM-guided signalosomes against PVX-infection at the PM and should be exploited in future studies. The study of the phosphorylation-dependent interactions of REM1.3 (and related phosphocode) in regard to the modulation of REM1.3 PM dynamics and molecular function is the topic for future studies.

Materials and methods

Plant material

Nicotiana benthamiana plants were cultivated in controlled conditions (16 h photoperiod, 25 °C). Proteins were transiently expressed via *Agrobacterium tumefaciens*-mediated transformation for virus and PD functional assays as in [14, 33] or for the localization experiments as described in the appendix. For subcellular localization studies and protein extractions, plants were analyzed 2 or at 4 days after inoculation (DAI) in the phosphorylation assays. Imaging for PVX-GFP spreading assays and plasmodesmata GFP-diffusion experiments were done at 5 DAI. PVX inoculation for test ELISA was performed at 4-week-old *N. benthamiana* plants. Details on molecular cloning and protein work, transgenic lines generation are described in the Appendix.

Cloning and molecular constructs

All vectors constructs were generated using classical Gateway cloning strategies (www. lifetechnologies.com), pDONR211 and pDONR207 as entry vectors, and pK7WGY2, pK7YWG2, pK7WGR2, pK7RWG2, and pGWB14 and pGWB15 as destination vectors [100]. The REM1.31–116, REM1.3117–198 and REM1.3 single S74A, T86A and S91A and triple S74/T86/S91AAA and S74/T86/S91DDD mutants were synthesized in a pUC57 vector (including the AttB sites) by Genscript (http://www.genscript.com/) or GENEWIZ (http:// www.genewiz.com/) and next cloned to Gateway destination vectors. AtCPK3D202A mutant was generated by site-directed mutagenesis as previously described [101] with minor modifications. For BiFC experiments, the genes of interest were cloned into pSITE-BIFC- C1nec, -C1cec, -N1nen, and–N1cen destination vectors [102]. To map the dynamics of single molecules with sptPALM, REM1.3 and phosphomutants were cloned in fusion with EOS in the gateway compatible vector pUBN-Dest::EOS [103]. EOS protein has been widely use for single molecule localization microscopy in mammals, bacteria, and plant cells. It corresponds to the name of a fluorescent protein from the stony coral *Lobophyllia hemprichii* which peculiarity resides in its photoconvertability. The energy of UV light can break the core polypeptidic chain of EOS fluorescent protein inducing changes in EOS spectral fluorescence properties. Due to the stochasticity of EOS photoconversion at low UV radiation (space and number of events/sec can be controlled by modulating UV laser power), single molecules can be converted, localized and tracked.

Generation of transgenic stable hairpin REM and 35S::GFP-REM1.3 *N. benthamiana* lines

Leaf discs were cut from N. benthamiana leaves, transferred on petri plates containing culture medium (complete Murashige and Skoog medium (MS) supplemented with 30g/L saccharose, pH 5,8; phytoagar HP696 (Kalys) 5,5 g/L and the hormones: AIA 0,1 mg/L, BAP 2 mg/L) and incubated for 48 h in the growth room (16 h photoperiod, 30 µmol photons.m2.s-1, 23 °C). For the transformation, the N. benthamiana plants disk leaves were incubated with the Agrobacterium cultures (GV3101 strain) carrying the plasmid of interest for 20 min. The leaf samples were next placed on plates with the complete medium previously described. 48 hours later, the leaf fragments were washed 3 times with sterile water (with 0,1% Tween20). The leaf fragments were next washed with MS complete medium supplemented with Timentin (300 µg/mL). The leaves were next placed on plates with regeneration medium (MS culture medium, as previously described, supplemented with 300 mg/L of timentin and 150 mg/l of kanamycin). The plates were next incubated in the growth room. The explants were transferred to fresh regeneration medium with a maximum periodicity of 7 days until the development of callus. The regenerated seedlings were transferred to a rooting medium (MS, sucrose 30 g/L, phytoagar 5,5 g/L, timentin 200 mg/L, kanamycin 150 mg/L). The regenerated plants (T0) were transferred to the greenhouse for growth and self-fertilization. Homozygous T2 lines carrying a single transgene insertion were selected by segregation analysis on selective Kanamycin media and used for physiological studies and phenotypic characterization. The expression of the GFP-REM1.3 or silencing levels of endogenous NbREMs was controlled by cytological, biochemical and expression analysis. Cytological analysis of the GFP-REM1.3 expression in all leaf cells was performed to avoid chimeric expression, see S2A and S2B Fig.

Transient expression in N. benthamiana

Four-week-old *N. benthamiana* greenhouse plants grown at 22–24 °C were used for *Agrobacter-ium tumefaciens*-mediated transient expression. *A. tumefaciens* were pre-cultured at 28 °C overnight and used as inoculum for culture at initial OD600nm of 0.15 in pre-warmed media. Cultures were grown until OD600nm reached 0.6 to 0.8 values (3–5 h). Cultures were then centrifuged at 3,200 g for 5 min, pellet were washed twice, using water to the desired OD600nm. Bacterial suspensions at OD_{600nm} of 0.2 and 0.1 were used for subcellular localization and Spt-PALM experiments, respectively. The bacterial suspensions were inoculated using a 1-mL syringe without a needle by gentle pressure through a <1mm-hole punched on the lower epidermal surface [104]. Transformed plants were incubated under normal growth conditions for 2 days at 22–24 °C. Transformed *N. benthamiana* leaves were analyzed 2–5 DAI depending on the experiment.

Viral spreading, GFP diffusion assays

PVX-GFP cell-to-cell movement experiments were performed as previously described [14, 36], with minor modifications. Briefly, *A. tumefaciens* strain GV3101 carrying the constructs tested were infiltrated at a final optical density at 600 nm (OD600nm) = 0.2 together with the same strain carrying the plasmid pGr208, which expresses the PVX-GFP complementary DNA harboring GFP placed under the control of a Coat protein promoter, as well as the helper

plasmid pSoup [105] at final OD_{600nm} of 0.001. Viral spreading of PVX-GFP was visualized by epifluorescence microscopy (using GFP long pass filter on a Nikon Eclipse E800 with x4 objective coupled to a Coolsnap HQ2 camera) at 5 DAI and the area of at least 30 of PVX-GFP infection foci was measured using Fiji software (http://www.fiji.sc/) via a homemade macro or ImageJ. The expression levels of transiently expressed constructs were confirmed by Western blot. ELISA tests in systemic *N. benthamiana* leaves were performed similarly to [8] to follow the global virus accumulation. Briefly, GFP-REM1.3 or hpREM plants were mechanically inoculated with PVX, and viral accumulation in systemically invaded leaves (at 3 nodes above the inoculated leaf) was evaluated at 10 or 14 DAI with a specific anti-PVX coat protein antibody (Sediag). Five plants per line for GFP-REM1.3 and 8 for hpREM plants were tested per experiment. GFP diffusion at PD experiments was performed as previously described [33]. All the experiments were repeated at least three times.

Epidermal cells live imaging and quantification. Bimolecular Fluorescence Complementation

Live imaging was performed using a Leica SP5 confocal laser scanning microscopy system (Leica, Wetzlar, Germany) equipped with Argon, DPSS and He-Ne lasers and hybrid detectors. *N. benthamiana* leaf samples were gently transferred between a glass slide and a cover slip in a drop of water. YFP and mCitrine (cYFP) fluorescence were observed with similar settings (*i.e.*, excitation wavelengths of 488 nm and emission wavelengths of 490 to 550 nm). In order to obtain quantitative data, experiments were performed using strictly identical confocal acquisition parameters (*e.g.* laser power, gain, zoom factor, resolution, and emission wavelengths reception), with detector settings optimized for low background and no pixel saturation. Pseudo-colored images were obtained using the "Red hot" look-up-table (LUT) of Fiji software (http://www.fiji.sc/). All quantifications were performed for at least 10 cells, at least two plants by condition with at least 3 independent replicates. BiFC images were taken 2 DAI by confocal microscopy (Zeiss LSM 880). Quantification of fluorescent intensities was performed by ImageJ, as described in [36].

Spt-PALM, single molecule localization and tracking

N. benthamiana epidermal cells were imaged at room temperature (RT). Samples of leaves of 2-week-old plants expressing EOS constructs were mounted between a glass slide and a cover slip in a drop of water to avoid dehydration. Acquisitions were done on an inverted motorized microscope Nikon Ti Eclipse (Nikon France S.A.S., Champigny-sur-Marne, France) equipped with a 100× oil-immersion PL-APO objective (NA = 1.49), a TIRF arm, a Perfect Focus System (PFS), allowing long acquisition in oblique illumination mode, and a sensitive Evolve EMCCD camera (Photometrics, Tucson, USA). Images acquisitions and processing were done as previously described [45].

Single molecule fluorescent spots were localized in each image frame and tracked over time using image processing techniques such as a combination of wavelet segmentation [106] and simulated annealing algorithms [107]. The software package used to extract quantitative data on protein localization and dynamics is custom written as a plug-in running within the Meta-Morph software environment. This plugin is now property of Molecular devices company (https://www.moleculardevices.com/sites/default/files/en/assets/product-brochures/dd/img/metamorph-super-resolution-software.pdf).

Single molecule localization organization analysis, $Log(\delta 1/\delta)$ correspond to the ratio between the local molecule density to overall molecule density at the PM. After correction for artefacts due to multiple single-molecule localization (described in [36] and now presented in materiel and methods section), we computed potential nanodomain by applying a threshold $\delta 1i > 2\delta N$, where δN is the average localization density at PM level and $\delta 1i$ is the density in presumed protein-forming nanodomain, with a minimal area of 32 nm² and with at least 5 localizations per nanodomain.

SR-Tesseler software was used to produce Voronoï diagrams, and subsequently quantify molecule organization parameters as previously recommended [50]. Taking in account fluorophore photophysical parameters, localization accuracy and the first rank of local density of fluorescent molecules, correction for multiple detections occurring in a vicinity of space (w) and blinking tolerance time interval (t) are identified as the same molecule, merged together and replaced by a new detection at a location corresponding to their barycentre. Because first rank of local density of fluorescent molecules was below 0.5 mol/mm² (c.a ranking from 0.1 to 0.3 mol/mm²), we used a fixed search radius w of 48 nm as recommended [50]. To determine the correct time interval t, the photophysics of the fluorophore namely the off-time, number of blinks per molecule and on-time distributions are computed for each cell. For example, for a dataset composed of 618,502 localizations, the average number of blinks per molecule was 1.42, and the number of molecules after cleaning was 315,929. As a control, the number of emission bursts (439,331), counted with t = 0, divided by the average number of blinks per molecule (1.42) was only 2.15% different. After correction for artefacts due to multiple singlemolecule localization, we computed potential cluster using a threshold d1i>2dN, where dN is the average localization density at PM level and dli is the density in presumed protein-forming nanocluster, with a minimal area of 32 nm2 and with at least five localization by cluster.

Over the two independent experiments 54 446 single molecule trajectories have been observed (34 740 Mock / 19 706 PVX). We then computed single molecule mobility behavior (Diffusion coefficient and Mean square displacement) using trajectories of at least 8 time points (tracked for at least 0.16 s; representing 19495 trajectories in total, 12073 for Mock condition and 7422 for PVX condition).

In silico analysis of REM1.3 protein sequence

Prediction of putative phosphorylation sites was performed by Diphos, DEPP and NETPHOS coupled with published data. Disordered domains were performed by pDONR VL XT.

In vitro REM1.3 phosphorylation assays

6His-REM1.3 and mutant recombinant proteins were purified from bacteria using fast flow chelating sepharose resin (Amersham) according to manufacturer's instructions and as in [14]. For the *in vitro* REM1.3 phosphorylation assays about 2 μ g of total plant extracts were incubated with 1 μ g of affinity-purified 6His:REM1.3 protein variants for 10 minutes at room temperature and in a phosphorylation buffer (Tris-HCl 30mM, EDTA 5mM, MgCl₂ 15mM, DTT 1mM, Na₃VO₄ 2,5 mM, NaF 10 mM and 10 μ Ci/reaction ATP [γ -³³P]- (3000Ci/mmol, Perkinelmer). The buffer contained also 10–5 M of free Ca²⁺ which allows the detection of 6His-REM1.3 phosphorylation also in mock conditions. Gradual concentrations of free Ca²⁺ as in [108] were added for Fig 5C. Reactions were performed for 15 minutes in a volume of 25 μ l. The reactions were stopped by the addition of 15 μ l of 6x loading buffer. Proteins were separated by SDS-PAGE and phosphorylation status of REM1.3 was analysed by autoradiography using a phosphor-Imager and quantified by ImageQuant TL program.

In vitro CPK3 kinase assays

CPK3-HA was transiently expressed in mesophyll protoplasts and immunopurified with anti-HA antibodies as performed in [109] while CPK3-GST recombinant protein was purified from bacterial extracts as reported in [69]. For *in vitro* kinase assays, the tagged CPK was incubated with 0.5–1 µg histone or 6His-REM1.3 proteins in the following kinase reaction buffer (20 mM Tris HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT, 50 µM cold ATP, ATP [γ -³³P] 2 µCi per reaction, 1 mM CaCl₂ or 5 mM EGTA) in a volume of 15 µL for 30 min at RT. The reaction was stopped with 5 µL 4X Laemmli buffer, then samples were heated at 95 °C for 3 min. Proteins samples were separated by SDS-PAGE on 12% acrylamide gel. After migration, the gel was dried before exposing against a phosphorScreen to reveal radioactivity on a Storm Imaging system (GE Heathcare). The gel was then rehydrated for Coomassie staining.

Protein work

SDS-PAGE, Western Blot analysis, protein extractions and recombinant protein purification were performed in *E. coli* as in [14]. Cell fractionation and extractions followed the established protocol from [59] and [62]. Anti-REM antibodies were previously described in [8].

Accession numbers

All relevant data are within the paper and its Supporting Information files are available from Arabidopsis Genome Initiative (https://www.arabidopsis.org/index.jsp), and GenBank/EMBL (https://www.ncbi.nlm.nih.gov/genbank/) databases under the accession numbers: *StREM1.3* (NP_001274989), *AtREM1.2* (*At3g61260*), *AtREM1.3* (*At2g45820*), *AtCPK3* (*At4g23650*).

Supporting information

S1 Fig. Callose quantification by aniline blue staining and PD index calculation. (A) Original sample image is an 8-bit, single-channel image.

(B) Masks of total Region Of Interest (ROI) objects before particle analysis were created using the following filters; background subtraction with a rolling ball radius as in [43]; "smooth" twice and an auto-local threshold Max Entropy dark, creating a black and white mask, used for particle detection.

(C) Overlay of outlines of the analyzed ROI (green; after particle analysis with particle size $3-100 \text{ pixel}^2$ circularity (0.3–1) exclude on edge) with the original image. Scale bar indicates $10 \ \mu\text{m}$.

(D) Quantification of PD Index; after aniline blue labeled pit-field detection, YFP-REM1.3 fluorescence intensity was manually measured at pit-field level (ROI2) and surrounding PM (ROI1 and ROI3) using a circle of fixed area ($0.18 \mu m^2$). The PD index was then calculated as the ratio between YFP-REM1.3 pit-field fluorescence (ROI2) and the mean of YFP-REM1.3 fluorescence intensity at surrounding PM (ROI1+ROI3). (TIF)

S2 Fig. Overexpression of GFP-REM1.3 results in reduced PVX accumulation in *N.benthamiana* **and REM1.3 protein levels are not affected by PVX infection.** A. *Top*, Confocal image showing GFP-REM1.3 localisation in the PM in *N. benthamiana* lines *Bottom*, The GFP:REM1.3 expression in three independent transgenic lines #6, 7 and 16 was tested by Western blot against REM and showed that it contains at least three times the amount of endogenous *N. benthamiana* REM.

B. PVX infection assays in independent stably expressing GFP-REM1.3 and wild-type control *N. benthamiana* lines. Viral charge was assayed by test DAS-ELISA using antibodies to PVX coat protein on distal (3 nodes above inoculation) leaves at 14 DAI. Three independent experiments were performed with five plants for each transgenic line and non-transgenic (WT). Error bars show SE, and significance is assessed by Dunnett's multiple comparison test against

WT (*, P < 0.1; **, P < 0.05; ***, P < 0.001).

C, Western blot against REM1.3 was performed on total protein extracts from wild type *N*. *benthamiana* leaves infected by PVX-GFP at 0, 3, 5 and 7 DAI. Stain free loading is indicated below.

D, Confocal images showing PVX-GFP foci at the indicated DAI, tested in C. (TIF)

S3 Fig. Analysis of *in vitro* **6His-REM1.3 phosphorylation and viral proteins expression.** (A) Effect of the addition of ATP or AMP in *in vitro* phosphorylation assays of 6His-REM1.3 by kinase(s) in microsomal (μ) or PM extracts of *N. benthamiana* leaves developed by autora-

(B) 6His-REM1.3^N and 6His-REM1.3 phosphorylation by healthy *N. benthamiana* leaf microsomal (μ) and plasma membrane (PM) extracts.

(C) 6His-REM1.3 $^{\rm N}$ and 6His-REM1.3 $^{\rm C}$ phosphorylation by kinase(s) in microsomal (µ) and soluble extracts.

(D) 6His-REM1.3 was differentially phosphorylated by leaf microsomal extracts expressing the indicated constructs *i.e.* PVX alone, PVX deleted for TGBp1 (PVX Δ TGBp1), 30K protein from *Tobacco Mosaic Virus* (TMV), PVX fused to GFP, and GFP alone at 4 DAI. See the rationale Fig 2E. Control of loading is shown after stain free procedure. In all phosphorylation experiments about 10µg of total protein extracts and 1µg of affinity purified 6His-REM1.3, REM1.3^N or REM1.3^C were loaded per lane.

(E) Controls of expression of fluorescently-tagged viral proteins, namely CP, TGBp1, TGBp2 used in Fig 2.

(TIF)

diography.

S4 Fig. REM1.3 S74 T86 S91 phosphorylation is important to regulate Tobacco mosaic virus movement and REM1.3 phosphorylation mutants maintain PM localization. (A) Representative epifluorescence microscopy images of *Tobacco Mosaic Virus* (TMV-GFP) infection foci in *N. benthamiana* leaf epidermal cells at 5 DAI. Graph represents the relative foci area of REM1.3 or phosphomutants (S74, T86 and S91 into Alanine, AAA or Aspartic Acid, DDD) compared to mock control (co-infiltration of PVX-GFP with an empty *A. tumefaciens* strain). About 78–128 foci per condition were measured in 2 independent biological repeats. Dunn's multiple comparison tests were applied for statistical analysis, p<0.001. (B) Confocal microscopy images of secant views of *N. benthamiana* epidermal cells expressing YFP-REM1.3, YFP-REM1.3AAA and YFP-REM1.3DDD at 2 DAI. Scale bar indicates 10 µm. (TIF)

S5 Fig. Group 1b AtREMs and REM1.3 have similar behavior against PVX cell-to-cell movement in *N. benthamiana* epidermal cells. (A) Clustal alignments of protein sequences of group 1b REMORINs: *AtREM1.2, AtREM1.3, NbREM1.2, NbREM1.3* and REM1.3 (*StREM1.3*). Blue color-coding shows percentage of identity. The REM1.3 S74, T81 and S91 sites are highlighted.

(B) *Left*, Representative epifluorescence microscopy images of PVX-GFP infection foci on *N. benthamiana* leaf epidermal cells transiently expressing RFP-REM1.3, RFP-AtREM1.2 or RFP-AtREM1.3 at 5 DAI. Scale bar indicate 400 μ m. *Right*, Graph represents the relative PVX-GFP infection foci area in the presence of RFP-REM1.3 or Arabidopsis homologs compared to mock control (co-infiltration of PVX-GFP with empty *A. tumefaciens* strain). At least 184 foci per condition in 4 independent biological repeats were measured. Statistical differences are indicated by letters as revealed by Dunn's multiple comparisons test p<0.001. (TIF)

S6 Fig. *In vitro* characterization of REM1.3 phosphorylation conditions. Autoradiography reveals *in vitro* phosphorylated 6His-REM1.3N (A) or 6His-REM1.3 (B) by microsomal extracts of healthy *N. benthamiana* leaves in the presence of increasing concentrations of staurosporine (A) or Polylysine, β -glycerophosphate (BGP), GTP, AMP and ATP (B). (C) Effect of Ca²⁺ and EGTA on 6His-REM1.3^N phosphorylation by kinase(s) in microsomal extracts.

(TIF)

S7 Fig. AtCPK3CAD202A dead mutant does not phosphorylate REM1.3 *in vitro*. AtCPK3-CA-HA and AtCPK3CAD202A-HA were expressed in *Arabidopsis thaliana* mesophyll protoplasts. Immunoprecipitated proteins were incubated with ATP [γ -³³P] and submitted to an *in vitro* kinase assay using 6His-REM1.3 or histone as substrates. *In vitro* kinase assays were revealed by autoradiography. Trans-phosphorylation of the substrates 6His-REM1.3 or histone is indicated. Western blot against HA shows the expression levels of the expressed proteins. (TIF)

S8 Fig. Stable transgenic lines *N. benthamiana* **under-expressing group 1 REMORINs.** (A) Protein expression levels of endogenous NbREMs in the hpREM lines, determined by Western Blot analysis using anti-REM1.3 antibodies. Protein extracts from three independent plants per line were used, namely lines 1.4, 2.1, 10.2.

(B) Expression of endogenous NbREMs in the hpREM lines determined by RT-qPCR analysis. Results are expressed relative to the NbREMs expression levels in the WT background. RT-qPCR signals were normalized to actin levels.

(C) PVX-GFP spreading is accelerated in the hpREM lines. Graph represents the PVX-GFP infection foci area in the different hpREM lines compared to WT. At least three independent experiments were performed. Error bars show +/- SEM. Statistical differences compared to WT were determined by Mann- Whitney test *** p<0.001.

(D) PVX systemic propagation is inversely correlated with REM levels in 4-week-old transgenic N. *benthamiana* leaves. Viral charges were assayed by DAS ELISA using antibodies to PVX coat protein (diluted on 1/100) on distal leaves (at 3 nodes above the inoculated leaves) at 10 DAI. 3 independent experiments were performed with eight plants for each hpREM transgenic line and WT or empty vector control (mock). Error bars show SE, and significance is assessed by Mann-Whitney non-parametric test (*, P < 0.1; **, P < 0.05; ***, P < 0.001). (TIF)

Acknowledgments

Imaging was performed at the Bordeaux Imaging Center, member of the national infrastructure France BioImaging. We acknowledge Dr S. P. Dinesh-Kumar for the gift of TMV-GFP clone, Dr Alicia Zelada for the gift of PVX Δ TGBp1 and viral protein constructs and Ms Coralie Chesseron for greenhouse facilities.

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Each ROI measure 0.18 µm²

Figure S1

D



В

Relative PVX accumulation (%)

100-

75-

50-

25

0-

wт







-

#6

Local

1





Stain free





Figure S3



Figure S4
Α

В

StREM1.3 - MAELEAKKVE NbREM1.3 - MAEVEATKVE NbREM1.2 - MAEVEATKVE AtREM1.3 MAEEQKTSKVD AtREM1.2 - MAEEQKIALE	TEKVVDPAPPAPGP TEKVVDPTPPAPEAPAP TEKVVDPTPPAPEAPAP VESPAVLAPAKEPTPAP SESPAKVTTPAPADTPA	VKEAEPVVETPKEVVAI VKEAEPVVETPKEV - AI VKEAEPVVETPKEV - AI VEVADEKIHNPPPV PAPAEIPAPA <mark>P</mark> APTPAI	DEKAIVAPAL PPF DEKAIVAPAL PPF DEKAIVAPAL PPF DEKAIVAPAL PPF	AEEKEKPDDS EQVKEKSDDS EQVKEKSDDS EQVKEKSDDS EQIFDDS
KAL VVVE TKAPEPADEK KAL VVVEDKAAEPAEEK KAL VVVEDK PAEEK KAL AVVEKPIEEHTPKK KAL TVVEKPVEEPAPAK	K - EG <mark>S</mark> I DRDAVLARVAT K - EGS I DRDAVLARVAT K - EGS I DRDAVLARVAT ASSGSADRDV I LADLEK PASA <mark>S</mark> L DRDVKLADLSK	EKRV <mark>S</mark> LIKAWEESEKSI EKRL <mark>S</mark> LIKAWEESEKSI EKRL <mark>S</mark> LIKAWEESEKSI EKKTSFIKAWEESEKSI EKRL <mark>S</mark> FVRAWEESEKSI	CAENKAQKKVSAIGAV CAENKAQKKVSAIAAV CAENKAQKNVSAIAAV CAENRAQKKISDVHAV CAENKAEKKIADVHAV	VENSKKANLEA VENSKKANLEA VENSKKANLEA VENSKKAAVEA VENSKKAAVEA
EL KKMEEQLEKKKAEYT EL KKMEEQLEKKKAEY I EL KKMEEQVEKKKAEY I QL RK I EEKLEKKKAQYG QL KK I EEQLEKKKAEYA	EKMKNKIALLHKEAEEK EKMRNKIALLHKEAEEK EKMKNKIALLHKEAEEK EKMKNKVAAIHKLAEEK ERMKNKVAAIHKEAEER	RAM I EAKRGEDL L KAEI RAM I EAKRGEDL L KAEI RAM I EAKRGEDL L KAEI RAM V EAKKGEEL L KAEI RAM I EAKRGEDV L KAEI	LAAKYRATGTAPKK LAAKYRATGTAPKKL LAAKYRATGTAPKKL MGAKYRATGVVPKAT TAAKYRATGIVPKA1	LGCF >80 LGCF >60 LGCF 440

PVX-GFP 200 PVX-GFP infection foci area (%) а 150 b b b 100 + RFP-REM1.3 + Mock 50 RFP-REM1.3 -RFP-AtREM1.3 -RFP-AtREM1.2 -0-Mock -RFP-AtREM1.3 + RFP-AtREM1.2

Figure S5



Figure S6







Figure S8

Article II: addendum

We pursued our quest to discover more about *St*REM1.3's interactome and to map, by biophysical means, its phosphorylation sites. Its N-terminal is predicted to be intrinsically disordered (Perraki et al., 2018), however this has never been proven experimentally. If it truly is an IDD, it is tempting to envision it as a domain responsible for many interactions (Uversky, 2013), such as those we have already discussed in Article II, namely kinase *At*CPK3 and, directly or through an uncharacterised signalling pathway, callose synthase. We decided to work solely on this IDD. Thus, the protein of interest here will be a truncated *St*REM1.3: REM1-116.

REM₁₋₁₁₆ was produced in BL21-DE3 cells in minimal medium with 13C-labelled glucose and ¹⁵NH⁴Cl, by addition of 1 mM IPTG at OD₆₀₀ = 0.6-0.8 and incubation at 37°C for 3h. Cells were lysed by sonication and the supernatant was loaded onto a HisTrap column (GE Healtchare) equilibrated in 20 mM HEPES 150 mM NaCl 20 mM imidazole 0.02% NaN₃ pH=7.4 and eluted with 20 mM HEPES 150 mM NaCl 500 mM imidazole 0.02% NaN₃ pH=7.4. Eluted REM₁₋₁₁₆ was adjusted to 1 mM DTT and 0.5 mM EDTA then TEV protease was added in a ~1/200 TEV/REM₁₋₁₁₆ mass ratio. The mixture is incubated for 3h at room temperature then desalted against 10 mM HEPES 50 mM NaCl 0.02% NaN₃ pH=7.5 with a HiPrep column (GE Healthcare). Under native conditions, REM₁₋₁₁₆ was loaded onto a Histrap column equilibrated with 20 mM HEPES 150 mM NaCl 0.02% NaN₃ pH=7.4 and eluted with the same elution buffer as above. Under denaturing conditions, this step was performed in buffers containing 7M urea. Finally, REM₁₋₁₁₆ was desalted again against 10 mM HEPES 50 mM NaCl 0.02% NaN₃ pH=7.5.

In vitro phosphorylation of 0.5 mM of REM₁₋₁₁₆ by *At*CPK3 required adjusting the sample to 10 mM MgCl2, 1 mM CaCl2, 1 mM DTT and 3 mM ATP. The reaction was initiated by the addition of 88 μ M of *At*CPK3 and incubation at 20°C.

IsNMR was performed on a Bruker Avance NEO spectrometer operating at 700 MHz for proton with a TXI 5 mm probe. The sample was adjusted to $9/1 H_2O/D_2O$ to lock the magnetic field. ¹H-¹⁵N HMQC spectra were recorded at 283 K (10°C).

I performed the cloning, production and purification of labelled ¹⁵N-, ¹³C-labelled REM₁₋₁₁₆. Axelle Grélard and Estelle Morvan, engineers of the NMR platform, trained me to perform all the IsNMR experiments and data analysis. The *in vitro* phosphorylation assay by *At*CPK3 was designed and optimised by Dr Marie Boudsocq, who confirmed the phosphorylation of REM₁₋₁₁₆ by biochemical means.

The structural fingerprint of REM₁₋₁₁₆ is characteristic of an IDP (Figure 66). In addition, using either native or denaturing conditions during purification had no impact its fingerprint. Next, we performed the *in vitro* phosphorylation of REM₁₋₁₁₆ by *At*CPK3 with cold ATP in the NMR tube and monitored the evolution of its structural fingerprint. In addition to many chemical shifts being slightly perturbed, three peaks (3 and 4) disappeared and two new peaks appeared (1 and 2) (Figure 67). Even so, the structural fingerprint remains that of an IDP: most peaks are still clogged in the same spectral region. Plotting NMR intensities as a function of time, maximal intensities for

1 and 2 and minimal intensities for 3 and 4 are reached after ~ 4h, meaning that the phosphorylation reaction is complete at that time (Figure 68).



Figure 66

¹H-¹⁵N HMQC spectra of REM₁₋₁₁₆ purified under native (black) or denaturing conditions using 7M of urea (red).



¹H-¹⁵ HMQC spectra of REM₁₋₁₁₆ in *At*CPK3's reaction buffer before addition (black) or 14 h 13 min after addition (red) of *At*CPK3. Notice how the peaks labelled 1,2 and 5 appeared over time while 3 and 4 disappeared. Each spectrum was acquired using 2 scans for an experimental time of 1 min 42 s each.



Kinetics of phosphorylation of REM₁₋₁₁₆ by *At***CPK3.** NMR intensities are plotted as a function of time for peaks 1 to 5 according to (Figure 67). Each data point was obtained from a ¹H-¹⁵N HMQC spectrum acquired using 2 scans for an experimental time of 1 min 42 s. Notice the difference of scale in NMR intensity between (A) and (B).

Recording 3D NMR spectra, namely HNCa and HN(Co)CaCb, will prove capital to establish connectivities between residues and to identify them, thus mapping, for the first time, the phosphorylated residues of *St*REM1.3 through biophysical means. Then, having both phosphorylated and non-phosphorylated REM₁₋₁₁₆, we shall be able to monitor interactions between putative ligands and both forms of the protein. By looking for changes in its structural fingerprint, we could identify their interaction sites on REM₁₋₁₁₆ (to be done).

Article III

Mechanisms governing subcompartmentalization of biological membranes

Julien Gronnier, **Anthony Legrand**, Antoine Loquet, Birgit Habenstein, Véronique Germain, Sébastien Mongrand

> Current Opinion in Plant Biology 2019 52, p114–123

This first review was aimed at showcasing some known cases of plant membrane subcompartmentalisation and how they could be studied. Examples given included remorins, thylakoids, chloroplast envelope and mitochondria cristae. Biophysical tools to study domains are given, with a special focus on ssNMR.

My major contribution was to design Figure 2 and write its caption. I reviewed the main text for grammar corrections and general organisation of ideas. I also offered some technical assistance with Illustrator to make Figure 1.



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Mechanisms governing subcompartmentalization of biological membranes

Julien Gronnier², Anthony Legrand^{1,3}, Antoine Loquet³, Birgit Habenstein³, Véronique Germain¹ and Sébastien Mongrand¹



Membranes show a tremendous variety of lipids and proteins operating biochemistry, transport and signalling. The dynamics and the organization of membrane constituents are regulated in space and time to execute precise functions. Our understanding of the molecular mechanisms that shape and govern membrane subcompartmentalization and interorganelle contact sites still remains limited. Here, we review some reported mechanisms implicated in regulating plant membrane domains including those of plasma membrane, plastids, mitochondria and endoplasmic reticulum. Finally, we discuss several state-of-the-art methods that allow nowadays researchers to decipher the architecture of these structures at the molecular and atomic level.

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Current Opinion in Plant Biology 2019, 52:114–123

This review comes from a themed issue on Cell biology

Edited by Eva Benkova and Yasin Dagdas

https://doi.org/10.1016/j.pbi.2019.08.003

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Introduction

Spatiotemporal organization of the cellular biomolecules is critical to coordinate the numerous activities simultaneously carried out by cells. Biological membranes delimit cells and organelles and constitute specialized subunits that are constantly reshaped to adapt to everchanging environmental conditions and to operate cell functions effectively (Special issue on cell biology edited by Ref. [1]). Cell membranes are composed of a specific set of biomolecules defining their identity. For example, phosphoinositide lipids and small GTPases proteins are major contributors to endosome identity [2,3]. A tremendous body of evidence shows that the motion and the organization of membrane constituents are dynamically regulated on the level of the membrane to form functional domains and this conversely throughout the tree of life [4^{••},5,6]. Thus, it appears that membrane subcompartmentalization into domains is universal and may represent an essential characteristic. Taking into account the knowledge acquired in various model organisms and model systems over the past decades, membrane domains can be defined as membrane regions in which the local composition, lateral organization, and/or dynamics differ in some way from the average membrane properties [7-9]. Such local specificity is dictated by preferential intermolecular interactions, including intra-membrane interactions (i.e lipid-protein, lipid-lipid and protein-protein) and associations with structures peripheral to the membrane for example cortical cytoskeleton and the cell wall in the case of plasma membrane. This also leads to the formation of inter-membrane interaction through Membrane Contact Sites (MCS), important functional platforms for the exchange of lipids and signalling proteins [10,11], see Figure 1. Yet, membranes being constituted of several thousands of molecules surrounded by variable and complex environments, a tremendous mechanistic complexity remains to be uncovered. Here, we review some described mechanisms regulating membrane architecture in plants and discuss recent technological advancements allowing researchers to study membrane organization with molecular and atomic resolution.

Examples of subcompartmentalization of plant membranes Plasma membrane domains

The plasma membrane (PM) is the outermost boundary of the cell, acting as a communication headquarter integrating signal from the environment to the cell interior and *vice-versa*. The PM is asymmetric, as exemplified by the enrichment of sphingolipids in the outer leaflet and phospholipids in the inner leaflet [12,13]. The PM associates with the cortical cytoskeleton network and the cell wall creating a continuum at the cell surface [14]. PM establishes MCS with organelles, notably with ER at the level of PD, see Figures 1a and 2. The lipid and protein composition of the domains formed at these MCS is very





Examples of subcompartmentalization of membranes and membrane contact sites (MCS) in different organelles of plant cells. (a) Scheme of a plant cell showing the membrane contact sites (MCS) between organelles shown by a red dot. Few examples of subcompartmentalization of biological membranes are emphasized in the plasma membrane (b), thylakoids (c), chloroplast envelope (d), mitochondria cristae (e).





Biophysical techniques to study membrane domains.

(a) Examples of major biophysical tools to analyse membrane domains and membrane-associated proteins, subsequently deciphering the molecular mechanisms at play in nanodomain organization. X-ray: shaker family voltage-dependent potassium channel Kv represented in the cartoon, lipids in stick (PDB: 2R9R, [111]). Modelling: C-terminal anchor of StREM1.3 interacting with membranes enriched in phosphoinositol-4-phosphate and sitosterol. ssNMR: model of StREM1.3 nanodomains. FTIR: insertion of C-terminal anchor of StREM1.3 in nanodomain-like membranes [16**]. EM/tomography: observation of ER–PM membrane contact sites at plasmodesmata [89**]. Solution NMR: membrane-embedded domain of the Influenza B BM2 integral protein (PDB: 2KIX, [112]). (b) ssNMR workflow to study membrane domain-associated proteins. Bacterial expression cells (e.g. *E. coli* BL21-DE3) are transformed with a high expression level plasmid coding for the protein of interest. Protein production is achieved in minimal culture media supplemented with isotope labelled metabolites depending on the desired isotopic labelling scheme of the protein (e.g. 13C-glucose, 1,3-¹³C-glycerol, 2-¹³C-glycerol, . . .). Proteins are purified and reconstituted into liposomes of a chosen lipid composition. ssNMR allows obtaining two types of structural data: magic angle spinning (MAS) ssNMR is used to analyze the structure and dynamics of the membrane protein [16**], and ²H (unpublished typical data) and ³¹P (not shown) ssNMR to decipher the dynamics and phase behavior of the membranes of interest comprising deuterated lipids.

specific [11]. Organization of the PM can be rationalized into two types: microdomains and nanodomains. Microdomains are site-specific enrichment of membrane compounds at the cellular level usually referred to as polar domains that control localized cell activities. Nanodomains represent submicrometric heterogeneity of the PM whose visualization often requires the use of high or superresolution microscopy techniques [15,9]. Nanodomains have been proposed to act as dedicated platforms regulating cell signalling notably [4••,16••,9,17•,15]. Mechanisms regulating the organization of PM domains being recently reviewed [9,15,13,18–22], we present here only three case studies to illustrate the molecular mechanisms at play in the organization and dynamics of PM domains.

REMORINs are plant-specific proteins regulating notably immunity [23-26], symbiosis [27,28,29[•]] and development [30] possibly by modulating nanodomain-associated complexes [16^{••},31,32,17[•],29[•]]. REMORINs predominantly associate with the PM [33,23,31,34,35]. In addition, isoforms from group 1 and group 6 REMORINs have been shown to be associated with plasmodesmata (PD) in Rice and in Solanaceae [23,36,30,31]. Electron microscopy immunolocalization, stimulated emission depletion microscopy (STED) and photoactivated localization microscopy (PALM) studies showed group 1 REMORINs are organized into nanodomains of about 70-90 nm in diameter that are sensitive to sterol composition [23,37,16**] and cytoskeleton integrity [38]. Molecular mechanisms at the basis of REMORIN domain organization are being discovered: REMORINs are targeted from the cytosol to the cytosolic leaflet of the PM via a short unconventional sequence at the extremity of the C-terminus of the protein, called REM-CA (REMORIN C-terminal Anchor) [39,34,40], see Figures 1b and 2; REM-CA undergoes conformational changes upon binding of conserved positively charged residues to phosphoinositides and provides to REMORINs biochemical properties indistinguishable from integral proteins [23,39,16^{••}]; REM-CA-sterol-phosphoinositide interactions are required for Group 1 REMORINs supra-molecular organization into functional domains involved in plant response to the Potato Virus X (PVX). Numerous REMOR-INs present cysteine residues that can be S-acylated [41,34,42,43]. While S-acylation of Arabidopsis REMORINS seems to regulate PM affinity but not primarily nanodomain organization [34], the substitution of an S-acylated cysteine of Nicotiana benthamiana REM alters nanodomain organization [43], suggesting functional divergence of REMORIN S-acylation. Oligomerization of group 1 REMORINs into homotrimers is required for PM localization [39,44], suggesting that REMORINs' self-assembly constitute an early step of PM targeting. Furthermore, REMORIN organization seems regulated by intermolecular protein association. Indeed, in Medicago, FLOT4 scaffolds SYMBIOTIC REM1 to recruit the Nod factor co-receptor LYSINE MOTIF KINASE 3 (LYK3) to specific nanodomains controlling root hair infection by *Sinorhizobium meliloti* and the establishment of symbiosis [29[•]]. Interaction of AtREM1.3 with AtHIR1 in *Arabidopsis* suggests that association of SPFH (Stomatin, Prohibitin, Flotillin, HflK/C) proteins with REMORINs may represent a conserved core module shaping PM organization [45]. Finally, phosphorylation of group 1 REMORINs upon infection of *N. benthamiana* by the PVX modulates REM1.3 organization and function [31], probably through the modulation of protein–protein interactions. Thus the genesis and regulation of REMORIN nanodomains appear to rely on several molecular mechanisms such as post-translational modifications, and protein–lipid and protein–protein interactions.

Rho of Plants (ROPs) are the plant-specific subfamily of Rho/Rac small GTP binding proteins, regulating numerous cellular processes such as signalling, trafficking and cytoskeleton dynamics [46,47]. Reversible switch from a GDP-bound state to a GTP-bound state mediated by ROP-GEFs and ROP-GAPs regulates ROPs activity [48]. Polarization of the growth machinery to a predefined root hair initiation domain (RHID) pledges root hair formation in trichoblast cells. ROP2, 4 and 6, are recruited to the RHID before any detectable cell bulging and serve as a landmark for the recruitment of downstream effectors [49,50[•]]. Strikingly, guanine nucleotide exchange factor 3 (GEF3) defines the RHID by guiding ROPs polarization *via* direct protein-protein binding [50[•]]. At the bulging stage, phosphatidylinositol-4-phosphate 5-kinase 3 (PIP5K3), the AGCVIII kinase D6 PROTEIN KINASE (D6PK) and sterol composition modulate ROPs association to the RHID [51,52]. Here, co-regulation of ROP, phosphoinositides and phosphoinositide kinases has been proposed to form a self-organizing system amplifying ROP recruitment and activation [3]. In addition, ROPs associate with the PM via post-translational lipid modifications and direct interaction with membrane lipids mediated by the carboxy-terminal tail [53,47,4**]. For example, ROP6 interacts with phosphatidylserine (PS) via its polybasic tail, a process likely at the basis of nanodomain organization. Recently, using live superresolution microscopy, Platre et al. elegantly showed that variation in PS level during root development stabilized ROP6 into nanodomains to regulate auxin signaling [4^{••}]. In metaxylem vessel cells, ROP-GEF4 locally activate ROP11 to recruit MICROTUBULE DEPLETION DOMAIN 1 scaffold protein which in turn recruits microtubule-depolymerizing kinesin-13A enabling the formation of pits in secondary cell walls [54,55]. IOD13 associates with cortical microtubules (cMTs) and the PM to laterally restrict the localization of ROP GTPase domains, establishing a lateral fence for ROP GTPase [56]. In contrary, CORTICAL MICROTUBULE DIS-ORDERING1-induced disorganization of cortical microtubules impairs the boundaries of PM domains of active ROP11 GTPase [57].

Cellulose microfibers are synthesized by the PM-embedded cellulose synthase (CESA) complexes (CSCs) which are composed of 18–36 cellulose synthase subunits [58]. Cortical microtubules recruit CESA-containing vesicles and guide the trajectory of CSCs at the PM [59–61]. In addition, S-acylation of CESAs influences its immediate membrane environment and conditions their location to the PM [62]. CSCs are tethered to cortical microtubules via two integral components, CELLULOSE SYNTHASE INTERACTING 1 [63–65] which determines the trajectory of CSCs along the cMTs [59] and COMPANION OF CELLULOSE SYNTHASE 1 (CC1), which sustains cellulose synthesis by promoting the formation of a stress-tolerant microtubule array during salt stress [66^{••}].

These examples emphasized that regulation of plasma membrane subcompartmentalization is regulated as part of developmental program, is modulated to respond to environmental clues, relies on the cooperation of multiple factors and is fundamental for function.

Chloroplastic membrane domains

Chloroplasts are organelles composed of a double membrane envelope and thylakoids found in plant cells and algae that conduct photosynthesis. Little is known about how the photosynthetic membrane machinery is arranged in time and space. Microscopy and biophysical shreds of evidence showed the coexistence of domains where lipids are organized in lamellar or hexagonal phases. For example, hexagonal phases have been described in etioplasts of prolamellar bodies or during the transfer of lipids between the envelope and thylakoids, such hexagonal phase domains may be of importance for localizing metabolic activities, for example the violaxanthine-epoxidase in thylakoid domains [67].

Biochemical, 3D reconstruction, in vivo spectroscopy and immunolocalization data, reveal that thylakoids display a heterogeneous subcompartmentalization of photosynthetic complexes in domains which redistribute during state transitions in *Chlamydomonas* [68] and diatoms [69], see Figure 1c. These domains are interconnected, ensuring fast equilibration of electron carriers for efficient and optimal photosynthesis. Underlying molecular events at the basis of thylakoid subcompartmentalization remain unclear. Thylakoids possess a special fatty acid namely trans- Δ 3-hexadecenoic acid (trans-16:1) esterified in phosphatidylglycerol (PG) which may play a role in cementing thylakoids during granum formation and control of light reactions of photosynthesis [70]. Recent 3D cryo-electron tomography showed the thylakoid network of cyanobacteria is organized in domains and forms a synapse-like MCS decorated by ribosomes (but not by phycobilisomes) in tight association with the PM of cyanobacteria. This MCS was named the 'thylapse', for 'thyl(akoid syn)apse', and likely serves for compartmentalization of the different functions of the thylakoids that is photosynthesis or protein

synthesis [71^{••}], see Figure 1d. Because PM of cyanobacteria represents the inner membrane of eukaryotic plastids, thylapses most likely also exist in higher plants.

Chloroplast envelope establishes numerous MCS with other organelles [10]. For example, plastid and mitochondrion envelopes establish membrane connection during phosphate deprivation. The molecular content of this MCS has been recently identified by biochemical and proteomic approaches and showed a big complex of hundred proteins enriched in specific lipids. AtMic60, a conserved protein of the mitochondria inner membrane, plays a crucial role in the lipid transport process by regulating the proximity between mitochondrial membranes via its interaction with the outer membrane protein Tom40 and by destabilizing membranes, likely to promote lipid desorption $[72^{\bullet\bullet}]$. Plastids can also undergo drastic changes in shape under stress, through specialized protrusive membrane domains called stromules (stroma-filled tubules, see Figure 1a) which link plastid envelope with other organelles such as ER, Golgi and nucleus [73,74]. The molecular mechanisms governing stromule formation are not established, but the involvement of cytoskeleton motors has been proposed [75]. Similarly, peroxules, peroxisomal protrusions tethering chloroplasts or mitochondria through specialized membrane microdomains have been evidenced [76,77]. Peroxules also for example link with lipid droplets, see section 'Endoplasmic reticulum domains'. These studies reveal the importance of physical connections through plant membrane domains for establishing complex metabolic pathways.

Mitochondrial membrane domains

Mitochondria are double-membrane-bound organelles. The outer mitochondrial membrane encloses the entire organelle and can be in contact with other organelles for example during phosphate starvation, see above [10]. The inner membrane separates the mitochondrial matrix from the intermembrane space. The structure of the inner mitochondrial membrane is extensively folded. These invaginations are separated from the inner membrane by dynamin proteins to form three domains namely, the inner boundary membrane, the cristae junctions and the cristae membranes [78], see Figure 1e. The latter contains enzymes of the mitochondrial respiratory chain that, instead of being dispersed in the membrane, are organized into a functional supramolecular respiratory domain called respirasome, see Figure 1. ATP synthase dimers sit at the edge of the cristae. Mitochondria inner membrane is rich in cardiolipin (CL), a key phospholipid playing important roles in maintaining the functional integrity and dynamics of mitochondria. Arabidopsis CL localizes to mitochondria and is enriched at specific domains and CARDIOLIPIN SYNTHASE targets to the inner membrane of mitochondria with its C-terminus in the intermembrane space [79]. Mitochondria of ck mutants exhibit altered structural integrity and morphogenesis. In contrast to animal and yeast, plant CL

plays a dominant role in mitochondrial fission and exerts this function through stabilizing the protein complex of DYNAMIN-RELATED PROTEIN3 [79]. In addition, CL induces membrane invaginations which are stabilized by dimers of ATP synthase. In reconstituted systems, bovine ATP synthase is sufficient to deform a lipid bilayer, which is likely the driving force triggering cristae curvature [80]. Recently, dimers of mitochondrial ATP synthase from the green algae *Polytomella* were shown to be required for cristae formation and constitute the main factor in mitochondrial morphogenesis to induce membrane curvature and self-assembly into rows [81[•]]. Finally, mitochondriaassociated ER membrane (MAM) is another structural element that is increasingly recognized for its critical role in cellular physiology and homeostasis of mitochondria [10].

Endoplasmic reticulum domains

The endoplasmic reticulum forms a membrane network virtually in contact with all cell organelles, see Figure 1a. Thus, the ER is actively engaged in organizing membrane domains to perform various functions. For example, ER is known to be organized into smooth and rough domains, the latter being enriched in ribosomes involved in protein production, protein folding, quality control and dispatch. Formation of these domains is regulated by syntaxin proteins [82]. Lipid droplets (LD) are lipid-rich cellular organelles regulating storage and hydrolysis of neutral lipids. LD biogenesis takes place at ER subdomains which are regulated by lipodystrophy proteins called SEIPINs in human, yeast, and plants. SEIPINs reorganize the normal, reticulated ER structure into discrete ER domains that colocalize with LD. In plants, SEIPINs modulate the number and sizes of LD [83,84]. Recent work in plants showed that peroxisome extensions deliver the major TAG lipase Sugar-Dependent 1 (SDP1) to the LD. At early stages of seedling development, SDP1 localizes to a peroxisome membrane domain and then possibly moves to the LD surface through peroxisome tubulations [85]. This constitutes an interesting case of inter-organelle communication and protein transport that is reminiscent of stromule.

In the next chapter, we will briefly describe state-of-the-art biophysical methods that have provided access to the structural basis membrane domain organization.

How to study the molecular mechanisms shaping biological membrane domains in plants?

Membrane subcompartmentalization is intimately linked to the preferential association of membrane constituents. Therefore, establishing the structure-function relationship between the membrane subcompartment components is an essential piece of the puzzle towards understanding the complex interplay of the cells with the extracellular environment. Yet, the intrinsic soft matter state of membrane-related systems in their native environment, such as peripheral or membrane-embedded proteins, hampers the application of numerous techniques in structural biology to visualize molecular association at the atomic level. To provide an overview on a promising route towards understanding the molecular basis underlying membrane subcompartmentalization, we can list tools such as X-ray, crystallography and solution NMR [86-88]. The recent developments of superresolution microscopy (eg. STED, PALM), cryo-electron microscopy (EM) and tomography methods allowed the study of the organization of proteins and lipids and the characterization of membrane subcompartmentalization and MCS [89**,16**,90*] with unpreceded resolution. The complementary biophysical tools to investigate lipid/protein interactions such as Langmuir monolayer, Fourier-Transform InfraRed spectroscopy (FTIR), NMR or modelling are reviewed in [91,11,13]. Figure 2a shows several examples of diverse contributions, including solid-state nuclear magnetic resonance (ssNMR), X-ray crystallography, modelling, FTIR, tomography by EM and solution NMR. The development of lipid and protein imagery by isotope-labeled high-resolution secondary ion mass spectrometry (nano-SIMS) would allow the study of molecular events at play in domain formation and dynamics [92,93]. In plants, nano-SIMS was used to localize elements such as manganese, arsenic, iron, zinc, and cadmium at the nanoscale level [94], but this approach could also be used for lipids and proteins in internal organelles.

Here, we further describe the powerful technique ssNMR that emerges as a tool to understand domain assembly. SsNMR is a versatile technology reporting on membrane and protein structure, sensitive to dynamics and proteinlipid interactions. A major advantage relies in its application on systems in the native bilayer environment, that is reconstituted liposomes that can represent membranes of a chosen lipid composition. The flowchart in Figure 2b illustrates the overall procedure applied to inquire on the previously mentioned aspects of membrane-associated proteins. Reporting membrane biophysical and structural parameters are achieved by well-established membranefocused ssNMR, mainly recorded on ²H and ³¹P nuclei [95,96]. The quadrupolar ²H signal in static ssNMR encodes for the overall lipid mobility and, importantly, the local dynamics along the acyl chain. Upon varying the membrane components (lipid composition, presence or absence of protein) and environment (temperature, pH), ²H ssNMR reveals detailed insights on phase, phase transitions, acyl chain dynamics and membrane thickness and curvature depending on the precise lipid composition and on the presence of a potential interaction partner. The chemical shift of ³¹P nuclei complements and corroborates the data reporting on phase behavior and the impact of potential partner molecules on the lipid head groups. Tackling membrane proteins is based on Magic-Angle spinning (MAS) ssNMR, a method which has seen tremendous advances in elucidating insoluble protein structures, dynamics and interactions in soft matter states such as assemblies, aggregates [97,98,99] or membrane-association $[100^{\bullet\bullet}, 101, 102]$. Since 2002, when the first structure of a microcrystalline protein has been solved by MAS ssNMR [103], the technology has proven very powerful to elucidate protein assemblies such as the first amyloid protein structure [104], bacterial filaments [105,106] and proteinmembrane complexes [107[•]]. A considerable knowledge has already been derived from ssNMR on protein-lipid, lipid-lipid interactions and membrane dynamics and functioning [108-110,96,102]. Most recent technological developments achieving ultra-fast MAS frequencies (>100 kHz) MAS ssNMR allow for observing proton nuclei in protonated protein samples ($\sim 500 \,\mu g$) and should facilitate ssNMR to serve as a common tool for structural biology on membrane/protein related questions. Because of its striking technological evolution, MAS ssNMR has recently been applied in few cases to shed light on protein structures, dynamics and protein-lipid interactions promoted by lipid-dependent membrane features [100**]. Membrane domain formation in plants, relying on the plant protein and lipid interplay (see below the example of REMORIN in PM [16^{••},44]) remains a field to explore by MAS ssNMR.

Conclusions

Virtually all membranes are organized in functional domains that coordinate cell functions. Recent breakthrough in biochemistry, biophysic and microscopy approaches allow nowadays the study of the mechanisms regulating the formation of membrane domains, particularly the interplay between lipids and proteins. The next decade will likely open a vast area of research to understand the roles of membrane organization during plant development and adaptation.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was sustained by CNRS (Centre National de la Recherche Scientifique) and the University of Bordeaux. We thank the European Molecular Biology Organization (EMBO Long-Term Fellowship 438-2018) for financial support to J.G. This work has benefited from the facilities of the Bordeaux Metabolome/lipidome Facility-MetaboHUB to SM, VG (grant no. ANR-11-INBS-0010), a CNRS Momentum project to BH, and an ERC Starting Grantproject "WEAKINTERACT" to AL. We thank Christophe Rocher (LBM, Bordeaux) and Juliette Jouhet (LPCV, Grenoble) for fruitful discussions.

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Article IV

Nanodomain Clustering of the Plant Protein Remorin by Solid-State NMR

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Frontiers in Molecular Biosciences

2019

6

In this work, my first as a first author, we studied the lipid dynamics of artificial lipid membranes in the presence of whole or mutated *St*REM1.3. We already had a hypothetical model of remorin nanodomain formation: (1) homotrimeric *St*REM1.3 binds to and clusters PI4P. These PI4P have mostly saturated acyl chains (Furt et al., 2010). Cholesterol has a higher affinity for saturated than unsaturated phospholipids (Engberg et al., 2016). By analogy, we expect sitosterol to be recruited in this nascent cluster. (2) More PI4P and sitosterol would coalesce into a larger nanodomain. But what are the exact driving forces and molecular determinants in the formation of *St*REM1.3 nanodomains? PIPs can cluster on their own (Bilkova et al., 2017; van den Bogaart et al., 2011), and therefore drive nanodomain formation. Does *St*REM1.3 driven clustering?

²H static ssNMR allows experimental measurement of lipid dynamics using a deuterated reporter lipid, here DMPC-d54 (fully deuterated on both acyl chains). We employed this methodology to monitor the phase behaviour, thermotropism, lipid dynamics and membrane thickness of liposomes in presence of PC, sitosterol, PIPs and PS as well as RemCA WT or mutated. Next, we reconstructed, domain by domain, the full length *St*REM1.3 to assess the impact of each domain on these parameters. Finally, we proposed an updated model for the formation of *St*REM1.3 nanodomains.

Under the help of Mélanie Berbon, I produced and purified both protein constructs REM₈₆₋₁₉₈ and *St*REM1.3. Experiments with peptides and liposome formation were taught to me by Denis Martinez. Under the guidance and training of Axelle Grélard and Estelle Morvan, I was trained to and performed all the NMR experiments in this report, with the exception of ³¹P MAS ssNMR that was performed by Estelle Morvan. For data analysis, interpretation and critical reviewing, I was helped by Dr Denis Martinez and Dr Arpita Tawani. The first draft was made by Dr Birgit Habenstein, who conceptualised the study, and myself, then reviewed by all the authors of this manuscript.





Nanodomain Clustering of the Plant Protein Remorin by Solid-State NMR

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Nanodomains are dynamic membrane subcompartments, enriched in specific lipid, and protein components that act as functional platforms to manage an abundance of cellular processes. The remorin protein of plants is a well-established nanodomain marker and widely serves as a paradigm to study nanodomain clustering. Located at the inner leaflet of the plasma membrane, remorins perform essential functions during signaling. Using deuterium and phosphorus solid-state NMR, we inquire on the molecular determinants of the lipid-protein and protein-protein interactions driving nanodomain clustering. By monitoring thermotropism properties, lipid acyl chain order and membrane thickness, we report the effects of phosphoinositides and sterols on the interaction of various remorin peptides and protein constructs with the membrane. We probed several critical residues involved in this interaction and the involvement of the coiled-coil homo-oligomerisation domain into the formation of remorin nanodomains. We trace the essential role of the pH in nanodomain clustering based on anionic lipids such as phosphoinositides. Our results reveal a complex interplay between specific remorin residues and domains, the environmental pH and their resulting effects on the lipid dynamics for phosphoinositide-enriched membranes.

Keywords: nanodomains, lipid raft, solid-state NMR, membrane protein, plant protein, phosphoinositide, sterol, remorin

INTRODUCTION

The fluid mosaic model of Singer and Nicolson (1972) describes a biological membrane as a bilayer of phospholipids, hydrophobic parts buried and hydrophilic parts exposed, with membrane proteins spanning or anchoring to the bilayer. In this model, lipid heterogeneity, and precise lipid-protein interactions that might lead to cooperative local enrichment of specific components, i.e., nanodomain organization becomes conceivable. Due to their eclectic composition, membranes are key players in cell biology, and have a plethora of functions (Grecco et al., 2011; Ott, 2017), thus constituting prime drug design targets.

The lipid raft hypothesis, that is the lateral segregation of membrane components, was first formulated by Simons and Ikonen (1997). Lipid rafts have different characteristics depending on the organism and bore different names throughout past decades, accounting for the variety of techniques allowing their detection. Here, we will commit to the word nanodomain because remorin, isoform *St*REM1.3, segregates into around 80 nm wide domains (Raffaele et al., 2009). Considering the recent technological advancements in analyzing nanodomains (Sezgin et al., 2017), the description given by Pike (2006), herein named "membrane raft," is still relevant: a

OPEN ACCESS

Edited by:

Anja Böckmann, UMR5086 Microbiologie Moléculaire et Biochimie Structurale (MMSB), France

Reviewed by:

Philip T. F. Williamson, University of Southampton, United Kingdom Manuel Etzkorn, Heinrich Heine University of Düsseldorf, Germany Benjamin James Wylie, Texas Tech University, United States

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Specialty section:

This article was submitted to Structural Biology, a section of the journal Frontiers in Molecular Biosciences

Received: 01 July 2019 Accepted: 30 September 2019 Published: 15 October 2019

Citation:

Legrand A, Martinez D, Grélard A, Berbon M, Morvan E, Tawani A, Loquet A, Mongrand S and Habenstein B (2019) Nanodomain Clustering of the Plant Protein Remorin by Solid-State NMR. Front. Mol. Biosci. 6:107. doi: 10.3389/fmolb.2019.00107

1

small (10–200 nm wide) region of a membrane, not necessarily the plasma membrane (PM), enriched in sterols, and specific proteins and lipids, like phosphoinositides (PIPs) (Furt et al., 2010; Brown, 2017). Nanodomain formation thus relies on the diffusion of the membrane components creating detectable membrane heterogeneity. Importantly, the diffusing, internally ordered lipids can separate into liquid-ordered (Lo) and liquid-disordered (Ld) regions in membranes, including PMmimicking environments (Kaiser et al., 2009). Considering typical nanodomain compositions, these membrane regions should have a tendency of manifesting Lo behavior, usually containing lipids fostering liquid order such as sterols.

Members of the multigenic, aerial plant-specific family of remorins (Raffaele et al., 2007) are well-known protein markers of such nanodomains in plants (Mongrand et al., 2004; Raffaele et al., 2009). This family is made of 6 phylogenetic groups sharing a canonical C-terminal domain, containing a segment with high coiled-coil propensity, and a variable N-terminal domain (Raffaele et al., 2007). Our study will focus on potato (*Solanum tuberosum*) remorin group 1 isoform 3 (*St*REM1.3). It contains an intrinsically disordered N-terminal domain involved in protein-protein interactions (Raffaele et al., 2009) and phosphorylation events (Marín et al., 2012; Perraki et al., 2018), a coiled-coil domain involved in homo-trimerisation (Martinez et al., 2018) and a C-terminal domain called the Remorin Cterminal membrane Anchor (RemCA).

StREM1.3 specifically binds negatively charged liposomes in vitro, as we showed in Perraki et al. (2012). Expression of a plasma membrane (PM)-targeted phosphatidylinositol 4phosphatase MAP-SAC1p in *Nicotiana benthamiana* leaves leads to a strong decrease of a *St*REM1.3's PM targeting and lateral segregation, implying that it has a specific affinity for PI4P of the PM's inner leaflet (Gronnier et al., 2017). Moreover, PM vesicles of *Nicotiana benthamiana* leaves treated with methyl β -cyclodextrin, a chelator of sterols, showed a complete loss of remorin nanoclustering, indicating phytosterols also play a role in the formation of nanodomains (Raffaele et al., 2009). Similarly, treatment with sterol inhibitor fenpropimorph strongly impaired nanodomain clustering, without affecting PM targeting of StREM1.3 (Gronnier et al., 2017).

Membrane anchoring of StREM1.3 proceeds over an unconventional mechanism with a hydrophilic domain tethering to the membrane and organizing into nanodomains (Gronnier et al., 2017). From the latter study, we formulated a hypothetical two-step mechanism for the formation of StREM1.3 nanodomains: (1) the remorin, likely as a homotrimer (Perraki et al., 2012), binds to PI4P of the PM's inner leaflet with a ratio of 1 PI4P moiety per 1 RemCA, therefore clustering the 3 lipids with the remorin; (2) PI4P moieties, bearing mostly saturated acyl chains (Furt et al., 2010), will preferentially attract plant sterols, of which sitosterol is the main representative, while other PI4P moieties gather around the nascent PI4P nanocluster (Picas et al., 2016). Removal of sterols by m-\beta-cyclodextrin on PM vesicles disrupts nanodomain clustering but not PM binding (Raffaele et al., 2009), revealing a dependency of nanodomain formation on sterols. Remorins are also known to form filaments in vitro though no such structure has yet been unambiguously observed in vivo (Bariola et al., 2004; Martinez et al., 2018). In essence, remorins might bind over a complex mechanism involving electrostatic interactions between the positively charged lysines in the RemCA (**Figure 1A**) with negatively charged PI4P head groups (Gronnier et al., 2017), favoring the formation of sitosterol and PIP enriched clusters (**Figure 1B**).

Structural studies of the mechanisms behind protein-lipid nanodomain formation and assembly at the atomic level remain scarce, mostly relying on molecular dynamics (Ackerman and Feigenson, 2015; Gronnier et al., 2017; Javanainen et al., 2017). Indeed, the size, insolubility, non-crystallinity and required native state of the objects of interest disgualify most biophysical techniques, such as X-ray crystallography and liquid-state NMR, to obtain experimental evidence at the atomic level. We use solid-state NMR spectroscopy (ssNMR), a technique tailored to study such objects, both in terms of protein fold and interactions (Habenstein and Loquet, 2016; Loquet et al., 2017) in the native membrane environment (Cady et al., 2010; Baker et al., 2015; Stanek et al., 2016; Ladizhansky, 2017; Lalli et al., 2017; Mandala et al., 2018) and to determine membrane dynamics and interactions at the atomic level using static deuterium NMR on deuterated liposomes (Dufourc et al., 1992; Beck et al., 2007; Huster, 2014; Yao and Hong, 2014; Molugu et al., 2017).

Here, we aim at deciphering the molecular mechanisms by which StREM1.3 anchors to PM to form nanodomains using deuterated liposomes along with deuterium (²H) ssNMR as our main tool. To reflect the lipid-protein interactions and dynamic behavior during nanodomain formation, we chose to work with liposomes of nanodomain-mimicking lipid compositions, including PIPs and sitosterol (Figure 1B). Using different remorin constructs including the membrane anchor RemCA alone, in the wild-type and several mutated versions, Rem₈₆₋₁₉₈, composed of RemCA and the coiled-coil segment, and the intact StREM1.3, we monitored the overall mechanisms and the fine molecular implications of nanodomain assembly guided by remorin. We tested the behavior of RemCA and its critical mutants in various nanodomain-like and unlike lipid environments, assessing the lipid preferences and electrostatic interactions involved in the anchor-membrane association. We then monitored the impact of the coiled-coil trimerisation domain, and its capacity of forming higher-order oligomers, as well as of the intrinsically disordered domain (IDD) of StREM1.3 on its membrane-binding behavior.

MATERIALS AND METHODS

Protein Production and Purification

Synthetic RemCA peptides were ordered from GenScript HK Limited at >90% purity with N-terminal acetylation.

Escherichia coli BL21-DE3 were transformed with a pET24 vector containing the DNA encoding for *St*REM1.3 or REM_{86–198} and plated onto LB-agar plates containing 30 μ g/mL kanamycin. A pre-culture of 100 mL LB medium was inoculated with a single transformed colony and incubated at 37°C overnight (about 20 h). 1L of LB medium is inoculated with the pre-culture at OD₆₀₀ = 0.2 and incubated at 37°C until OD₆₀₀ = 0.7–0.8. Protein production was induced with 1 mM of IPTG at 18°C overnight. Cells were pelleted at 6,000 g for 20 min at 4°C and resuspended in a lysis buffer (20 mM HEPES,



150 mM NaCl, 20 mM imidazole, 1 mM PMSF, 0.02% NaN₃, pH = 7.4) containing protease inhibitors (Complete, Roche). Cells were sonicated on ice at 30% magnitude three times (30s on, 30s off) and centrifuged at 15,000 g for 30 min at 4° C to recover the supernatant.

Purification of *St*REM1.3 was achieved with an Akta Pure 25 HPLC system (GE Healthcare) on a HisTrap affinity column equilibrated in wash buffer (20 mM HEPES, 150 mM NaCl, 20 mM imidazole, 0.02% NaN₃, pH = 7.4). The protein was eluted with a stepwise gradient of elution buffer (20 mM HEPES, 150 mM NaCl, 500 mM imidazole, 0.02% NaN₃, pH = 7.4): 15, 40, 80, and 100% elution buffer. About 20 mg of pure unlabelled *St*REM1.3 were obtained from a 1L culture in LB medium. Purification of REM_{86–198} was performed as described previously in Martinez et al. (2018). After purification, proteins were buffer-exchanged against a reconstitution buffer (10 mM HEPES, 10 mM NaCl, 0.02% NaN₃, pH = 7.4) using a HiPrep gel filtration column (GE Healthcare).

Liposome Reconstitution

Synthetic RemCA peptides were co-solubilized in $CHCl_3/MeOH 2/1$ (V/V) with the appropriate amount of lipids (peptide/lipid molar ratio of 1/20). Organic solvents

were evaporated under an air stream, hydrated, and then lyophilised. Lipid and lipid-peptide powders were rehydrated with deuterium-depleted water or 260 mM Tris buffer. The hydrated samples were submitted to three freeze-thaw-vortex cycles (1 min in liquid N₂, 10 min at ~40°C, 20 s vortexing) for sample homogenization and packed into a ssNMR rotor. This sample preparation has been used for triplicate error assessment on liposomes of the following composition: PC/ β -sitosterol/PIP mix 75/15/10 (molar ratio) and RemCA K192A/K193A (lipid/peptide = 20) (**Figures S1A,C,E**).

For protein reconstitution, pre-formed liposomes were incubated overnight at 30°C with the protein at a protein/lipid molar ratio of 1/20. The milky solution was centrifuged at 100,000 g for 2 h at 4°C to recover the proteoliposomes. To remove filaments, proteins were centrifuged at 12,000 g for 20 min at 4°C before reconstitution and liposomes were then recovered by centrifugation at 12,000 g for 20 min at 4°C. This sample preparation has been used for triplicate error assessment on liposomes of the following composition PC/βsitosterol/PIP mix 75/15/10 (molar ratio) incubated without protein (**Figures S1B,D,F**).

NMR

For ²H static ssNMR, we applied a static quadrupolar spin echo sequence (Davis et al., 1976) at the ²H frequency of 76.8 MHz on a 500 MHz (11.7 T) Bruker Avance III NMR spectrometer, with a 90° pulse of 3.8 μ s, a delay of 40 μ s, a recycle delay of 2 s, a spectral window of 500 kHz and a number of scans of 512 at least. We acquired spectra at different temperatures, ranging from 278 to 308 K. Sample temperature was stabilized

for 20 min prior to data acquisition. All spectra were processed with TopSpin 4.0.6 (Bruker). An exponential window function with a line broadening factor of 300 Hz was applied prior to Fourier transformation. De-Pake-ing procedure (Bloom et al., 1981; McCabe and Wassail, 1997), first order spectral moments M_1 and local order parameters $|2^*S_{CD}|$ along the acyl chains of DMPC-d54 were calculated with NMR Depaker (provided by Dr. Sébastien Buchoux) and refined by spectra simulation with NMR-099 (provided by Arnaud Grélard) as described in Beck et al. (2007). Membrane thickness is calculated as described in Grélard et al. (2013). For DMPC-d54/DMPS 90/10 (molar ratio) liposomes, ²H static ssNMR was performed on a 300 MHz (7.1 T) Bruker Avance III at 121.50 MHz for 31P with a 90° pulse of 3.3 μ s, a delay of 30 μ s, a recycle delay of 2 s, a spectral window of 500 kHz and a number of scans of 512 at least. Sample temperature was stabilized for 10 min prior to data acquisition. Error bars on M_1 and $|2^*S_{CD}|$ are presented in Figure S1 and reflect standard deviations of three independent experiments on the two conditions (see sample preparation 2.2). Error bars for membrane thicknesses are 0.5 Å, based on (Grélard et al., 2013) plus the error contributions from the standard deviations of the $|2^*S_{CD}|$ at each position.

For 31 P ssNMR, we applied a static Hahn spin echo sequence at the 31 P frequency of 162 MHz on a 400 MHz (9.4 T) Bruker Avance III HD spectrometer, with a 90° pulse of 8 µs, a delay of 40 µs, a recycle delay of 5 s, a spectral window of 400 ppm and a number of scans of 1,024 at least. Spectra were processed with TopSpin 4.0.6 (Bruker). An exponential window function with a line broadening factor of 200 Hz was applied prior to Fourier transformation.

 ^{31}P magic-angle spinning (MAS) ssNMR was performed on a Bruker Avance III spectrometer with a 4 mm HX probe at the frequency of 121.52 MHz, a MAS rate of 7 kHz and a 2.5 μs 90° pulse-acquisition sequence. Five hundred twelve scans were acquired for both experiments. Pure H_3PO_4 was used as external reference.

Negative Staining Electron Microscopy

Samples were loaded onto previously glow-discharged carboncoated copper grids and stained with 2% uranyl acetate (w/v) solution. Observations were performed under low-dose conditions on a CM120 120 kV FEI electron microscope using a Gatan USC1000 2k \times 2k camera. Clichés were analyzed with the Fiji distribution of ImageJ2 (Schindelin et al., 2012; Rueden et al., 2017).

RESULTS

Nanodomain Segregation by RemCA Involves Both PIPs and β-Sitosterol

Using ²H ssNMR, we monitored thermotropism, lipid dynamics, and membrane thickness of liposomes containing





consecutively the different membrane components that might impact on remorin-driven nanodomain assembly. The lipid systems of choice included, consecutively, perdeuterated phosphatidylcholine (PC, here DMPCd54), phosphoinositolphosphates (PIPs), β-sitosterol and phosphatidylserine (PS, here DMPS), Figures 1B,C. Deuterium spectra reveal the quadrupolar splittings that can be assigned to the positions of the ²H along the acyl chain of the deuterated lipid. The splitting of the so-called Pake doublet depends on the dynamics of the Carbon-Deuterium bond and can be translated into the local order parameters $(|2^*S_{CD}|)$ along the acyl chains of DMPC-d54 by de-Pake-ing (Davis, 1983). To accurately measure the impact of RemCA WT peptides on lipid dynamics we detected the quadrupolar splittings and then computed the order parameters $|2^*S_{CD}|$ in presence and in absence of RemCA. We chose the physiologically relevant temperature of 298K (25°C). Addition of RemCA to liposomes containing PC/PIPs and PC/β-sitosterol did not visually modify the quadrupolar splittings (Figures 2A,B) whereas RemCA has a clear impact on membranes containing PC/β-sitosterol/PIPs and PC/PS/βsitosterol/PIPs (Figures 2C,D). Membrane-protein interactions generally entail a modification of the lipid dynamic behavior. The selective impact of RemCA points out the importance of β-sitosterol and PIP being simultaneously present to allow for its nanodomain association.

Assessing the order parameters along the acyl chain provides a detailed view on the impact of RemCA on the membrane dynamics (Figure 3). No significant change in $|2^*S_{CD}|$ was

observed in PC/PIPs and PC/β-sitosterol liposomes upon addition of RemCA (Figures 3A,B), despite a slight increase of the order parameter in the presence of β -sitosterol. PC/ β sitosterol/PIPs and PC/PS/β-sitosterol/PIPs liposomes display lower |2*S_{CD}| along their acyl chains in the presence of RemCA, indicating a PIP- and sterol-dependent interaction between RemCA and the membrane (Figures 3C,D). The chosen lipids display a consecutively rigidifying effect on the membrane (Figure S2). RemCA then increases the lipid mobility in the membranes of the complex lipid mixtures PC/B-sitosterol/PIPs and PC/PS/B-sitosterol/PIPs. Addition of PS does not significantly enhance the observed effect of RemCA on liposomes containing β -sitosterol/PIPs (Figure 3D). To monitor whether the impact of the peptide depends exclusively on the simultaneous presence of PIPs and βsitosterol, we tested its effect also on PC/PS-containing liposomes and we observe no detectable change of the $|2^*S_{CD}|$ (Figure S3). The representative error for this type of sample preparation (Figure S1C) is below 0.9% at carbon positions 2-6.

The local order parameter $|2^*S_{CD}|$ can further be translated into the average membrane thickness (Grélard et al., 2013) at 298K (**Figure 4**). PC/PIPs and PC/ β -sitosterol liposomes without and with RemCA display similar membrane thicknesses (46.3 Å vs. 46.5 Å and 49.3 Å vs. 50.4 Å) whereas the membrane thickness of PC/ β -sitosterol/PIPs and PC/PS/ β -sitosterol/PIPs decreases slightly in the presence of RemCA (51.4 Å–48.9 Å and 52.7 Å–50.9 Å, respectively).



The first order spectral moments M_1 , which can be extracted from the deuterium spectra, report on the bilayer phase behavior and thermotropism (Figure S4). The inflection point between higher M₁ values indicating a gel phase to lower ones pointing to a fluid phase reports on the phase transition. For all lipid compositions, the phase transition temperatures (Tm) seem to remain close to the Tm of DMPC-d54 alone, 293K, but no clear phase transitions were detected in the presence of β -sitosterol. We observe a clear thermotropic transition in the absence of sterols, but only a slow descent of M₁ in their presence. Sterols might broaden the primary transition or it might be below the chosen temperature range (Dufourc, 2008). The presence of RemCA WT peptides does not have a significant effect on M₁ values nor on Tm across the range of temperature considered, 278-308 K. ³¹P NMR confirms the presence of lamellar phases in liposomes in all samples, as well as small vesicles in presence of PIPs (Figure S5).

Taken together with the $|2^*S_{CD}|$ analysis, our results show that upon interaction with membranes of relevant lipid compositions (PC/ β -sitosterol/PIP and PC/PS/ β -sitosterol/PIP), RemCA WT binds to the lipid bilayer without disrupting its structural integrity and lipid phase, while exerting a noticeable effect on its internal dynamics, reflected by the variations of the order parameters.

pH Dependency of Protein-Lipid Interactions in Nanodomain Association

At low pH (pH \approx 3–4), polyphosphate PIPs lose parts of their negative charges present at pH = 7–8 (Kooijman et al., 2009). Though, to our knowledge, no values on the pH dependency of the charges for all PIPs of the PIP mix have been documented, in analogy to PI(3,5)P₂, the PIPs of the PIP mix (including

PI4P) should be neutralized at pH \approx 3–4. Moreover (Redfern and Gericke, 2004) showed that di-palmitoyl phosphoinositol monophosphates were able to segregate into phosphatidylinositolenriched microdomains at pH = 7-9.5 but not at pH = 4. To obtain insights into the role of electrostatic interactions during the RemCA-membrane interplay, we decided to test how the pH value influences the impact of RemCA on nanodomainmimicking membranes. To assess the lipid interactions during RemCA-membrane association, we co-solubilised synthetic RemCA WT peptides in various liposome preparations (%/% molar ratio): DMPC/PIP mix 90/10, DMPC/B-sitosterol 85/15, DMPC/\beta-sitosterol/PIP mix 75/15/10 and DMPC/DMPS/βsitosterol/PIP mix 65/10/15/10. Peptides used in our study carried impurities (<10% w/w), such as TFA, which acidified the samples to pH = 3-4 during peptide-containing liposome preparation, as judged by pH paper. We therefore chose to buffer our samples to an approximate pH = 7-8, as judged by pH paper. To reveal the RemCA/membrane interactions at low pH, we also rehydrated our samples in pure 1% acetate pH = 3 during proteoliposome reconstitution (final pH = 3). Monitoring phase behavior (Figure 5A), spectral line shapes (Figure S6), and local order parameters |2*S_{CD}| (Figure 5B) at low pH revealed the significant effect of the head group charges on RemCA-lipid interactions. RemCA, when in contact with low pH nanodomain-mimicking membranes has the inverse effect on the phase behavior, as observed by an increase of the first spectral moment M1 (Figure 5A), and local order parameter (Figure 5B) as compared to neutral pH (Figure 3C and Figure S4C). Thus, the impact of the peptide on membrane dynamics is significantly modified because of the lower pH. ³¹P MAS ssNMR revealed significant chemical shift perturbations as a function of pH (Figure S7). Based on (Kishore and Prestegard, 2003; Müller et al., 2004), we tentatively assigned the four visible signals, from higher to lower chemical shifts: 4phosphate (4-P)-, 4,5-bisphosphate (4,5-P₂)-monoester, PS and PC. The diester groups might be obscured by the PS and PC signal. Assignment of 4,5-P2- remained ambiguous as a second peak was expected. Signals assigned to the monoesters witnessed a heavy shift between spectra at pH = 7-8 and revealing important modifications of their chemical environment which may be attributed to a change in their protonation state (van Paridon et al., 1986).

The acidic pH entails a negligible effect on phase behavior (**Figure 5C**) whereas it significantly modifies the local order parameter at 298K (black curves, **Figure 3C** vs. **Figure 5B**).

K192 and K193 Are Critical Residues in the Targeting of PIPs by RemCA WT

Based on a pre-established list of mutants impaired for both PM and nanodomain targeting (Gronnier et al., 2017), we employed mutated RemCA peptides to evidence the concerted role of lipid-amino acid interactions involving the specific targeting of PIPs. According to previous molecular dynamics experiments, residues K192 and K193 play a key role in the targeting of PI4P by *St*REM1.3. We limited ourselves to five



FIGURE 5 | (A) M₁ as a function of the temperature for liposomes containing PIPs, hydrated with 1% acetate pH = 3 in absence (black) or in presence (red) of RemCA WT at peptide/lipid ratio of 1/25. **(B)** Local order parameters |2*S_{CD}| as a function of the carbon position along the acyl chains of DMPC-d54 in presence of RemCA WT at pH = 3-4. **(C)** M₁ as a function of the temperature for liposomes, in 1% acetate pH = 3 (dotted lines) or with 260 mM Tris pH = 8 (full lines). Liposome composition is: DMPC-d54/β-sitosterol/PIP mix 75/15/10 (molar ratio). Representative error bars are shown in **Figures S1A,C**.

mutants (Gronnier et al., 2017): G188A, a negative control that should show no specific difference to the wild-type peptide; K183S, K193A, K183S/K192A, and K192A/K193A, localizing less efficiently into nanodomains. To trace the influence of each



PIGNE 6 Comparison of ²H quadrupolar spin echo spectra acquired at 298K in presence of RemCA WT and mutated RemCA. Inner line pairs indicate the Pake doublet frequency of the terminal CD₃ while the outer line pairs indicate the plateau region (usually between positions 2 and 8). Liposome compositions are (**A**) DMPC-d54/β-sitosterol 85/15 and (**B**) DMPC-d54/β-sitosterol/PIP mix 75/15/10 (molar ratio), at pH = 7–8. Above RemCA WT, peptides with a similar effect on membranes in presence of PIP mix (K183S, G188A, K192A/K193A) followed by those with no discernible effect (K193A, K183A/K192A).

mutation on the peptide-PIP interaction, each mutant and the WT were co-solubilised in liposomes of two different lipid compositions: PC/ β -sitosterol 85/15 and PC/ β -sitosterol/PIP mix 75/15/10 (molar ratio). We then determined the local order parameter |2*S_{CD}| of DMPC-d54 at 298K for every sample (WT, K183S, G188A, K192A/K193A, K193A, and K183S/K192A) (Figures 6, 7). In absence of PIPs, the effect of the peptides on the quadrupolar splitting and the local order parameter $|2^*S_{CD}|$ of the liposomes does not differ between RemCA WT and the mutants (Figures 6A, 7A,C); the impact of RemCA WT and the mutants is undetectable or entails a slight increase of the local $|2^*S_{CD}|$. In presence of PIPs, we observe two different behaviors (Figures 6B, 7B,D). The wild type peptide (RemCA WT) and a group of mutants (K183S, G188A, and K192A/K193A) have a pronounced effect on the membranes, decreasing $|2^*S_{CD}|$ all along the acyl chains of DMPC-d54. Importantly, this effect is abolished for a second group composed of the mutants K193A and K183S/K192A (Figures 6B, 7B,D), exposing an undetectable or a slightly increasing effect on the [2*S_{CD}]. This observation suggests that mutants from the first group, together with RemCA WT, interact with PIP-containing liposomes whereas peptides from the second group do not (Figures 6, 7). Unexpectedly, the double mutant K192A/K193A still binds to liposomes containing PIPs, possibly through an alternative mechanism involving K183.

The representative error for this type of sample preparation (**Figure S1C**) is below 0.9% at carbon positions 2–6.

Measurements of the membrane thickness reflect a comparable trend, as expected, since they rely on the ²H NMR data. The membrane thicknesses without PIPs are all within the same range of \approx 50 Å. Liposomes containing PIPs are split in the same two groups as described above with RemCA WT, K183S, G188A, or K192A/K193A decreasing membrane thickness more or less significantly (\approx 49 Å) whereas RemCA K193A or K183S/K193A have no effect (\approx 51 Å) (**Figure 8**). Our data corroborate the *in vivo* results that K192 and K193 are critical residues in the targeting of PIPs by RemCA. However, the results reveal that the *in vivo* membrane association of *St*REM1.3 relies on a more complex behavior of the positively charged residues during the trimerised protein-membrane interaction.

Monitoring M_1 as a function of temperature, we detect no important impact of the different mutants and the WT on the phase behavior (**Figure S8**). Above 293K, M_1 values in presence of PIP mix seem to spread out a little more than without PIP mix. All samples contain large liposomes in lamellar phase along with smaller vesicles in presence of PIP mix (**Figure S9**). Our data point to different effects of the different mutations, larger than the error margins for repetition of the identical conditions. However, considering possible uncertainties introduced by other sources (e.g., varying impurities in different peptide stocks) future interpretation of these results should be handled with care.

Oligomerisation Modifies RemCA Behavior on Nanodomain-Mimicking Membranes

We have previously demonstrated (Martinez et al., 2018) that disrupting the coiled-coil region of *St*REM1.3 would partially disrupt membrane targeting. While we hypothesize that the intrinsically disordered domain, responsible for protein-protein interactions, and the coiled-coil homo-oligomerisation domain do not directly interact with the nanodomains, they could indirectly influence nanodomain targeting. To monitor the effect of the two domains, we designed two protein constructs for NMR investigation. We expressed and purified the truncated *St*REM1.3 (REM₈₆₋₁₉₈), bearing only RemCA and the coiled-coil domain (Martinez et al., 2018), and the full length *St*REM1.3 including also the IDD.

We achieved setting up a protocol to produce high quantities of *St*REM1.3 in *E.coli* (about 20 mg/L of culture medium) (**Figures S10, S11A**). Both protein constructs, REM_{86–198} and *St*REM1.3, were purified to high purity (**Figure S11B**). We avoided reconstitution into liposomes by co-solubilisation to mitigate the risk of misfolding and non-native interactions with lipids. Instead, we incubated the proteins with preformed liposomes overnight at 30°C, using two lipid compositions: DMPC-d54/β-sitosterol 85/15 or DMPC-d54/β-sitosterol/PIP mix 75/15/10 (molar ratio). For each proteoliposome sample, about 40–50% of the proteins were pelleted by ultracentrifugation, as could be assessed by

absorbance at 280 nm of the supernatant fractions and SDS-PAGE of the pellets (**Figure S12**). We used deuterium NMR to probe the lipid order in presence of the two remorin domains.

Local order parameters |2^{*}S_{CD}| of PC-d54/β-sitosterol membranes are similar whether in absence or presence of both REM₈₆₋₁₉₈ and StREM1.3, no protein-membrane interactions seem to occur (Figure 9A and Figure S13A). In contrast, DMPC-d54/β-sitosterol/PIP liposomes containing REM₈₆₋₁₉₈ or StREM1.3 witnessed a similar increase of the order parameter |2*S_{CD}| all along their acyl chains (Figure 9B and Figure S13B). In accordance, membrane thicknesses were similar in absence of PIPs but increased comparably in presence of PIPs and one of the proteins (Figure S14). The representative error for this type of sample preparation (Figure S1D) is below 3.7% at carbon positions 2-7. The samples contain large liposomes in lamellar phase with a small isotropic peak in presence of PIPs (Figure S15). All samples displayed a Tm around 293K (Figure 10, representative error in Figure S1B). When compared to the monomeric anchor RemCA, REM₈₆₋₁₉₈ and StREM1.3 show an inverse effect on nanodomain-mimicking membranes, revealing the crucial impact of remorin oligomerisation during nanodomain clustering.

In contrast, both constructs, REM_{86-198} and StREM1.3, display a very similar behavior on the membranes, indicating that the non-phosphorylated intrinsically disordered domain does not influence remorin nanodomain targeting. The sample preparation used for measuring the effects of the two longer constructs should lead to reduced detectable readout range since predominantly the outer leaflet of the liposomes will be accessible. In line with this view, our results indeed show smaller effects for all tested conditions. Consequently, the data and interpretation should be handled with care.

Transient Higher-Order Oligomer Formation Could Stabilize Nanodomain Clustering

When incorporating the constructs REM₈₆₋₁₉₈ and StREM1.3 into liposomes, negative staining electron microscopy revealed the presence of protein filaments (Figure S16). Notably, this observation mostly concerned samples without PIPs (Figures S16A,B,E,F) whereas in the presence of PIPs very few filaments were observed (Figures S16C,D,G,H). Since there is no significant effect of either protein on liposomes in absence of PIPs and filament formation is significantly reduced in the presence of PIPs, we assume that the filaments do not directly interfere with liposomes. Nevertheless, we attempted to remove filaments by pelleting at lower centrifugal force before reconstitution. Removing the filamentous objects before reconstitution into liposomes caused both proteins to remain mostly in solution when exposed to the preformed liposomes, as assessed by absorbance at 280 nm (2.5 and 1.7 in solution before reconstitution, 2.8 and 1.7 in supernatant after reconstitution and liposome pelleting for REM₈₆₋₁₉₈ and StREM1.3, respectively). Absorbance measurement may be biased by remaining small liposomes. However, few proteins were still incorporated into the liposomes, for REM₈₆₋₁₉₈ only in the presence of PIPs,



bars are shown in **Figure S1C**.



as the band seems to be absent, even though it might be hidden under the liposome smear (**Figure S17**). No filaments were found on the liposomes (example given for REM_{86-198} , **Figure S18**). We could not detect an effect of this low protein amount on the membrane dynamics as reflected in the local

order parameter $|2^*S_{CD}|$ of DMPC-d54 (**Figure S19**). Since filaments do not seem to have a direct impact on liposomes and their formation is reduced in the presence of PIPs, transient higher-order oligomer formation might stabilize protein-lipid interactions during membrane association.



FIGURE 9 | Local order parameters $|2*S_{CD}|$ as function of the carbon position along the acyl chains of DMPC-d54 in presence of REM₈₆₋₁₉₈ or StREM1.3. Liposome compositions are **(A)** DMPC-d54/ β -sitosterol 85/15 and **(B)** DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (molar ratio), at pH = 7–8. Representative error bars are shown in **Figure S1D**.



DISCUSSION

Our results shed light on the detailed mechanisms of *St*REM1.3driven nanodomain clustering. We dissect the roles of the three *St*REM1.3 domains, the C-terminal anchor RemCA, the coiledcoil oligomerisation domain and the intrinsically disordered domain (IDD), implicated in protein-protein interactions.

We have monitored a fluidifying effect of RemCA on the membrane, assessed by the decrease of the local carbon-deuterium order parameter all along the acyl chains of DMPC-d54, only in presence of PIPs and β -sitosterol simultaneously. RemCA's membrane interactions therefore depend on the presence of both PIPs and β -sitosterol during nanodomains clustering, as we have suggested earlier (Raffaele et al., 2009; Gronnier et al., 2017). Moreover, addition of DMPS, another negatively charged phospholipid, does not modify this behavior, corroborating RemCA's genuine specificity for PIP and β -sitosterol.

RemCA peptides lacking K192 or K193, namely RemCA K183S/K192A or K193A, do not show any fluidifying effect on nanodomain-mimicking membranes, highlighting the

important role of these two positively charged residues. In the absence of PIPs RemCA and several mutants show a slight increase of the lipid order parameters in the presence of β -sitosterol, which could be attributed to a low overall membrane binding without the formation of lipid clusters. K183S and K192A/K193A mutants still interact with the nanodomain-mimicking membranes such as the wild-type RemCA. These two mutants behave similarly to K183S/K192A or K193A, when considering their segregation into nanodomains in StREM1.3 in vivo, detected by Single-Particle Tracking Photoactivated Localization Microscopy, spt-PALM (Gronnier et al., 2017). Their behavior on nanodomain-mimicking membranes in vitro therefore suggests a more complex interplay between the different positive charges in the intact StREM1.3 in vivo (Gronnier et al., 2017), possibly influenced by the oligomerisation and the subsequent trimeric exhibition of the anchor RemCA. However, our results are coherent with specific electrostatic interactions between K192 and K193 and the polar head of PI4P from the PM's inner leaflet (Gronnier et al., 2017).

In the K192A/K193A mutant, still displaying specificity for PIPs, the replacement of two consecutive positively charged

lysines by two consecutive hydrophobic alanines might allow an alternative folding and binding mechanism. However, we cannot exclude the possibility of errors that might not have been monitored by our error assessment, so we suggest treating the data with care.

Our findings show that negative PIP head group charges should foster RemCA nanodomain-mimicking membrane association. We therefore tested the impact of the pH on RemCA-membrane interactions. The impact of RemCA on phase behavior and acyl chain mobility at acidic pH is inversed compared to neutral pH, i.e., RemCA reduces acyl chain mobility in the nanodomain-mimicking membrane in acidic conditions. This might rely on an alternative binding mode of RemCA to the lipid bilayer, potentially reflecting an unspecific overall membrane binding, as is also the case for low binding of the peptide and its mutants in the presence of β -sitosterol. The electrostatic PIP-RemCA interactions present at physiological conditions should therefore represent a crucial actor during remorin nanodomain clustering.

We then examined the membrane- and nanodomainassociation of the C-terminal anchor in conjunction with the coiled-coil oligomerisation domain REM_{86-198} and the full-length *St*REM1.3, including the intrinsically disordered region. In line with our knowledge on *St*REM1.3, they only associate to nanodomain-mimicking membranes, i.e., containing PIPs and β -sitosterol responsible for *St*REM1.3 nanodomain segregation. Their effect on the membrane dynamics is very similar, indicating that the intrinsically disordered domain does not directly influence the nanodomain association. However, use of phosphodead *St*REM1.3 S74A/T86A/S91A and phosphomimetic *St*REM1.3 S74D/T86D/S91D mutants revealed a phosphorylation-dependent change in nanodomain organization *in vivo* (Perraki et al., 2018), implying the *St*REM1.3's intrinsically disordered region indirectly modifies the membrane-associating behavior, possibly by altering the interactions with other proteins or itself.

Both REM_{86–198} and the full-length *St*REM1.3 have an inverse effect on nanodomain-mimicking membrane dynamics when compared to RemCA WT. The anchoring domain alone is in a monomeric state and therefore represents the lipid specificity of the peptide alone (**Figures 11A,B**), presumably fostering the formation of lipid clusters. In the absence of RemCA, the lipid dynamics could represent the homogeneous rigidifying effect of intercalated PIP/sitosterol (**Figures S1**) along the reporter DMPC



FIGURE 11 Hypothetic model of a comprehensive nanodomain clustering mechanism consolidating the structural data on StREM1.3. (A) PIP mix and sterols increase lipid order over the whole membrane because of their homogeneous distribution (B) RemCA peptides could bind and cluster PIP altogether and recruit sterols in their vicinity. This would reduce the presence of sterols and PIPs in DMPC-d54-rich regions, lowering the $|2^*S_{CD}|$ of the latter. (C) StREM1.3 forms homo-trimers, bringing RemCA domains in vicinity. This might increase the rigidifying effect more efficiently over the whole membrane, considering the size and the stability of the associated objects.

acyl chain (**Figure 11A**). When RemCA is present, it might cluster the PIP/sitosterol moieties and therefore have a fluidifying effect on the reporter DMPC acyl chains (**Figure 11B**). Including the coiled-coil domain drastically increases the complexity of the native trimeric *St*REM1.3 structure, containing three RemCA anchors. REM₈₆₋₁₉₈ and *St*REM1.3 would reflect the rigidifying impact of the clustered protein on the nanodomainmimicking membranes (**Figure 11C**) with the inner bilayers of the liposomes unaffected and the outer exposed to the trimeric remorin. The protein and lipid clustering might reflect driving mechanisms of nanodomain formation in native membranes. Again, we cannot exclude the possibility of errors that might not have been monitored by our error assessment, in consequence we suggest treating the data with care.

We found that REM₈₆₋₁₉₈ and StREM1.3 formed filaments even at low protein concentration, when we proceeded to liposome reconstitution (Figure S16). Filament formation observed by EM was much reduced by the presence of PIP (Figures S16C,D,G,H) and the pelleted liposomes contained a significantly higher amount of protein in the presence of PIP (Figure S12), indicating that the protein is incorporated into membranes. When removing filaments by moderate centrifugation, the relative amount of proteins incorporated into liposomes drastically decreases, indicating that the presence of transient higher-order oligomers might favor the incorporation of trimeric remorins into the nanodomain-mimicking membranes. Although filaments are unlikely to be stable in vivo, the underlying ability of StREM1.3 to cluster with its peers into higher-order complexes could be biologically relevant. In line with this results, disrupting the coiled-coil region resulted in a partial loss of membrane targeting, indicating this domain is required to ensure a tight binding to the PM (Martinez et al., 2018).

CONCLUSION

Gaining mechanistic insights into the interactions and protein/membrane structures governing nanodomain formation remains difficult because of the complexity of the native protein-bilayer system. Here, we employed a ²H ssNMRbased methodology to tackle the lipid-protein interactions and mechanisms behind nanodomain clustering driven by StREM1.3. Using a divide-and-conquer approach, we deciphered the roles of the membrane anchor RemCA in isolation, REM₈₆₋₁₉₈, including the coiled-coil domain responsible for trimerisation and the intact StREM1.3. We seek to uncover the essential electrostatic interactions between RemCA's positive residues and the negatively charged PIP head groups involved in nanodomain formation. Our data moreover suggest that nanodomain clustering depends on the evolutionary evolved trimeric structure, which can only be partially represented by the monomeric anchor. The trimers of remorins appear to confer nanodomain clustering while the pure protein-lipid association relies mostly on the membrane anchor RemCA. Furthermore, we reveal that transient higher-order oligomer formation might stabilize the *in vivo* association of remorins to nanodomains, which is further supported by the structural conservation of the oligomerisation domain in the six phylogenetic groups (Raffaele et al., 2007).

Our results shed new light on the essential role of certain specific electrostatic protein-lipid interactions and protein oligomerisation properties toward understanding the driving forces in the nanoclustering of StREM1.3. We propose a more general picture of the relevance of oligomerisation, a character often present in nanodomain-segregating proteins. To gain more precise notions on the mechanisms behind lipid domain assembly, it will be vital to understand the structural implications of protein oligomerisation and the lipid-protein interactions from a protein structural point of view.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

ALe, DM, AG, MB, AT, and EM performed the experiments. ALe, DM, ALo, SM, and BH interpreted the data. ALo, SM, and BH designed the experiments. BH conceptualized the research. All authors revised and edited the manuscript.

FUNDING

We acknowledge funding from the Centre National de la Recherche Scientifique CNRS Momentum (BH), IdEx Bordeaux-Chaire d'installation (BH), the region Nouvelle Aquitaine (BH, grant convention N° 2017-1R10305-00013031), the CNRS (BH and SM, PEPS project Idex Bordeaux-PEPS 2316), ALe was supported by a French doctoral fellowship MESRI. Research was supported by European Research Council (ERC-2015-StG GA no. 639020 to ALo), and by the French ANR project Potymove (ANR-16- CE20-008-01 to SM), https://anr.fr/en/.

ACKNOWLEDGMENTS

We thank Marion Decossas and Olivier Lambert for technical assistance and advice for acquisition of EM images.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb. 2019.00107/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental information

Nanodomain clustering of the plant protein remorin by solid-state NMR

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Figure S1. Estimation of the experimental errors for the peptide co-reconstitution method (A, C, E) or liposome preparation for protein constructs (B, D, F) for M_1 (A, B), $|2^*S_{CD}|$ (C, D) and membrane thickness assessment (E, F). Error bars are the standard deviations of three independent experiments, as described in section 2.2.



Figure S2. Comparison of the local order parameters $|2^{*}S_{CD}|$ as a function of the carbon position along the acyl chains of DMPC-d54 for the four lipid systems of this study, at 298K. Liposome compositions are: DMPC-d54/ β -sitosterol 85/15, DMPC-d54/PIP mix 90/10, DMPC-d54/ β -sitosterol/PIP mix 75/15/10, DMPC-d54/DMPS/ β -sitosterol/PIP mix 65/10/15/10 (molar ratio) at pH=7-8. Representative error bars are shown in **Figure S1C**.



Figure S3. Local order parameters |2*S_{CD}| as a function of the carbon positions along the acyl chains of DMPC-d54 in absence (black) or presence (red) of RemCA WT. Liposome composition is DMPC-d54/DMPS 90/10 (molar ratio), at pH=7-8. Representative error bars are shown in **Figure S1C**.



Figure S4. Variation of the first order spectral moments M_1 as a function of the temperature T in absence (black) or presence (red) of RemCA WT. Liposome compositions are (A) DMPC-d54/PIP mix 90/10, (B) DMPC-d54/ β -sitosterol 85/15, (C) DMPC-d54/ β -sitosterol/PIP mix 75/15/10, (D) DMPC-d54/DMPS/ β -sitosterol/PIP mix 65/10/15/10 (molar ratio), at pH=7-8. Representative error bars are shown in **Figure S1A**.



Figure S5. ³¹P Hahn echo spectra in absence (black) or presence (red) of RemCA WT for different lipid systems. Liposome compositions are (A) DMPC-d54/PIP mix 90/10, (B) DMPC-d54/β-sitosterol 85/15, (C) DMPC-d54/β-sitosterol/PIP mix 75/15/10, (D) DMPC-d54/DMPS/β-sitosterol/PIP mix 65/10/15/10 (molar ratio), at pH=7-8


Figure S6. Comparison of ²H quadrupolar spin echo spectra acquired at 298K in absence (black) or in presence (red) of RemCA WT in liposomes containing PIPs, hydrated with 1% acetate pH=3. The inner line pair indicates the Pake doublet frequency of the terminal CD₃ while the outer line pair indicates the plateau region (usually between positions 2 and 8). Liposome composition is: DMPC-d54/βb-sitosterol/PIP mix 75/15/10 (molar ratio), at pH=3







Figure S8. Variation of first order spectral moments M_1 as a function of the temperature T in presence of RemCA WT or mutated RemCA. Liposome compositions are (A, C) DMPC-d54/ β -sitosterol 85/15, (B, D) DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7-8. Representative error bars are shown in **Figure S1A**.



Figure S9. ³¹P Hahn echo spectra in presence of various RemCA mutants. Liposome compositions are (A) DMPC-d54/ β -sitosterol 85/15 and (B) DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7-8.



Figure S10. Histrap purification chromatogram of ¹⁵N-, ¹³C-labelled *St*REM1.3. FT: flow-through. W: wash. Blue curve: absorbance at 280 nm in arbitrary units. Brown curve: conductivity (mS/cm). Green curve: elution buffer percentage.



Figure S11. (A) SDS-PAGE of eluted fractions from figure S8. In: sample loaded into the column. 5 and 6 are pooled and purified further. (B) Example of pure protein samples for REM₈₆₋₁₉₈ and *St*REM1.3 analysed by SDS-PAGE. Coomassie-stained gels.



Figure S12. SDS-PAGE of liposomes containing, or not, PIP mix in presence of $REM_{(86-198)}$ or *St*REM1.3, at pH=7.4. Coomassie-stained gel.



Figure S13. Comparison of ²H quadrupolar spin echo spectra acquired at 298K in absence (black) or presence of REM₈₆₋₁₉₈ (red) or *St*REM1.3 (blue). Inner line pairs indicate the Pake doublet frequency of the terminal CD₃ while outer line pairs indicate the plateau region (usually between positions 2 and 8). Liposome compositions are (A) DMPC-d54/β-sitosterol 85/15 and (B) DMPC-d54/β-sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7.4.



Figure S14. Membrane thickness at 298K calculated from the corresponding $|2^*S_{CD}|$ (Figure 8). Liposome compositions are DMPC-d54/ β -sitosterol 85/15 and DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7.4. Error bars are assessed as described in Figure S1A, C, E.



Figure S15. ³¹P Hahn echo spectra in absence (black) or presence of REM₈₆₋₁₉₈ (red) or *St*REM1.3 (blue). Liposome compositions are (A) DMPC-d54/ β -sitosterol 85/15 and (B) DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7.4.



Figure S16. Negative staining electron microscopy of REM₈₆₋₁₉₈ (A-D) and *St*REM1.3 (E-H) in liposomes DMPC-d54/ β -sitosterol 85/15 (A, B, E, F) and DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (C, D, G, H) (molar ratio), at pH=7.4. Scale bars: (A, C, E, G) 500 nm or (B, D, F, H) 50 nm. Fewer filaments are observed for REM₈₆₋₁₉₈ compared to *St*REM1.3 and the presence of PIP mix seems to reduce their number even more.



Figure S17. SDS-PAGE of liposomes containing, or not, PIP mix in presence of REM₈₆₋₁₉₈ or StREM1.3 when attempting to remove filaments, at pH=7.4. For REM₈₆₋₁₉₈ in liposomes in absence of PIP mix, the protein band might be hidden by the smear but the gel indicates a lack of a band. Red arrows indicate the expected molecular weight of each protein.



Figure S18. Negative staining electron microscopy of REM₈₆₋₁₉₈ in liposomes without filaments. Liposome composition is DMPC-d54/ β -sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7.4.



Figure S19. (A) Comparison of ²H quadrupolar spin echo spectra acquired at 298K in absence (black) or presence of REM₈₆₋₁₉₈ (red) or *St*REM1.3 (blue) without filaments in either case. Inner line pairs indicate the Pake doublet frequency of the terminal CD₃ while outer line pairs indicate the plateau region (usually between positions 2 and 8). (B) Local order parameters $|2^*S_{CD}|$ as function of carbon position along the acyl chains of DMPC-d54 from spectra in (A). Liposome composition is DMPC-d54/β-sitosterol/PIP mix 75/15/10 (molar ratio), at pH=7.4. Representative error bars for (B) are shown in **Figure S1D**.

Article IV: addendum

In our study, we committed to sitosterol, which is the most abundant phytosterols in plant PM, with a role similar to cholesterol in ordering membranes above the phase transition temperature, where, by contrast, stigmasterol decreases the order membranes (Grosjean et al., 2015). What would it change, in terms of order and thermotropism, if we replaced sitosterol with stigmasterol? Lipid systems without and with PIP mix displayed a lesser M₁ in the fluid phase yet retained comparable M₁ in the gel phase, compared to their sitosterol counterparts (Figure 69). This loss of M₁ can be interpreted as a loss of global order parameter, which is confirmed by computing local order parameters $|2^*S_{CD}|$. RemCA WT had no significant effect on liposomes made only of PC/stigmasterol but showed significant increase in global order parameters in the gel phase, computing $|2^*S_{CD}|$ is difficult and very much prone to error. We did not compute them.



Comparison of first spectral moments M₁ between four different lipid systems as a function of temperature: 85/15 PC/sitosterol (black, dashed), 75/15/10 PC/sitosterol/PIPmix (red, dashed), 85/15 PC/stigmasterol (black, full), 75/15/10 PC/sitosterol/PIPmix (red, full). Data on systems with sitosterol comes from (Legrand et al., 2019).



Figure 70

On the effect of RemCA WT in a PC/stigmasterol/PIPs lipid system. (A) Superimposed ²H quadrupolar echo spectra in the designed conditions. Black: liposomes alone. Red: liposomes with a 1/20 RemCA WT/lipid ratio. (B) M₁ as a function of temperature for without or with PIPmix (dashed vs full lines) and RemCA WT (black vs red). (C) |2*S_{CD}| as a function of carbon position along the acyl chains of DMPC-d54. The legend is the same as (B).

So replacing sitosterol with stigmasterol modified the effect of a synthetic RemCA WT peptide on lipid order: with stigmasterol, there is no discernible effect on $|2^*S_{CD}|$ in the fluid phase when there is a significant increase in $|2^*S_{CD}|$ with sitosterol; whereas RemCA WT increased M₁, and thus global order, in presence of stigmasterol in the gel phase while no effect was found in presence of sitosterol. This confirms that these two phytosterols have different effects on lipid order depending on temperature (section I.C.4) (Beck et al., 2007; Grosjean et al., 2015). More generally, the diversity and variability of plant sterols should always be accounted for when dealing with their behaviour in membranes.

Article V

This article is in a draft state. It is about reconstituting *St*REM1.3 nanodomains *in vitro*. Based on all the data and – partially – hypothetical models available on membrane attachment and clustering, we were confident enough to try to reconstruct these nanodomains in a minimal system. This would: (1) confirm our model (see Article IV) on *St*REM1.3' nanodomain clustering and (2) provide an *in vitro* platform for further investigations.

We started by assessing which lipids a GFP tagged REM₈₆₋₁₉₈ would bind to. By forming giant lipid vesicles $(1-20 \,\mu\text{m})$ labelled the fluorescent lipid rhodamine-PE (rhod-PE), we could, qualitatively, determine which negatively charged lipids (PS, PA, PIPs) are required for membrane binding. It appeared that StREM1.3 binds to any lipid bearing terminal phosphates, but notably not PS.

A next step was to disrupt REM₈₆₋₁₉₈'s coiled-coil oligomerisation domain to assess the effect of forcing a monomeric state on its membrane interactions, on giant vesicles, and biological activity, by assessing its ability to hinder the cell-to-cell propagation of the Potato Virus X (PVX). Though *St*REM1.3 was shown to interact with PIPs and PA, we observed the effect of PS synthase knockouts on *St*REM1.3 nanodomain organisation. At last, we attempted to reconstitute and observe nanodomains of *St*REM1.3 in a minimal system by cryoEM and AFM.

I performed the experiments involving giant vesicles. Marie-Dominique Jolivet performed all the *in vivo* assays, with coiled-coil mutants of *St*REM1.3 and PS synthase knockouts (*pss1*). I prepared samples for cryoEM and performed the observations with the tremendous help of both engineers of the CBMN EM platform: Dr Marion Decossas and Dr Tan Sisareuth. AFM was handled by collaborators, namely Dr Marisela Vélez for. The following draft was written by myself, figures included.

I. Introduction

Remorins belong to a multigenic family of plant proteins (Raffaele et al., 2007b). They display a broad range of functions and interactors: from protection against biotic and abiotic stress, inception of symbiosis to hormone signalling response (Gouguet et al., 2020). Their most well-known characteristic is to segregate into ~ 80 nm membrane nanodomains (Gronnier et al., 2017; Legrand et al., 2019) in the plasma membrane's (PM) inner leaflet. A model for the nanoclustering of StREM.3 has been proposed (Gouguet et al., 2020), in which homo-oligomeric remorins bind to phosphatidylinositol 4-phosphate (PI4P) negatively charged head groups via their remorin C-terminal anchors (RemCA) and recruit sterols and other remorins and PI4P in their vicinity to form a membrane nanodomain (Gronnier et al., 2017; Legrand et al., 2019; Perraki et al., 2012), while coiled-coil homo-oligomerisation ensures an efficient PM targeting (Martinez et al., 2018). This nanodomain organisation was shown to be cytoskeletondependent (Szymanski et al., 2015). StREM1.3's N-terminal intrinsically disordered domain (IDD) can be phosphorylated (Perraki et al., 2018) and is thought to mediate protein-ligand and protein-protein interactions (Khan et al., 2017; Nishi et al., 2013; Uversky, 2013).

Granted a sufficient understanding of *St*REM1.3's nanodomains, we will attempt to reconstitute these *in vitro* and to observe them by cryo-electron microscopy (cryoEM) and atomic force microscopy (AFM). This would confirm our proposed nanodomain formation model and provide us with a powerful tool for future biophysical studies.

II. Material and methods

A. Protein purification

REM₈₆₋₁₉₈ and *St*REM1.3 are produced and purified as described in (Legrand et al., 2019).

Production of GFP-REM₈₆₋₁₉₈ is identical to REM₈₆₋₁₉₈ and *St*REM1.3. Cells are broken by sonication and the supernatant is loaded onto a HisTrap affinity column controlled by an Atka Pure 25 HPLC system (GE Healthcare) equilibrated with equilibration buffer (20 mM HEPES 150 mM NaCl 20 mM imidazole 0.02% NaN₃ pH = 7.4) and eluted with a stepwise gradient of elution buffer (20 mM HEPES 150 mM NaCl 500 mM imidazole 0.02% NaN₃ pH = 7.4). Proteins eluted at 80% elution buffer was dialysed against 10 mM HEPES 150 mM NaCl 0.02% NaN₃ pH = 7.4 at 4°C overnight. The sample should turn turbid as GFP-REM₈₆₋₁₉₈ will assemble overnight (MARTINEZ). It is centrifuged at 100000 g 4°C 1h, the pellet, containing both GFP-REM₈₆₋₁₉₈ and contaminants, is resuspended then pelleted again. The last supernatant contains pure GFP-REM₈₆₋₁₉₈.

Production of REM₁₋₁₁₆ in BL21-DE3 cells is induced by 1 mM IPTG during 3h at 37°C. Cells are broken by sonication and the supernatant is loaded onto a HisTrap affinity column controlled by an Atka Pure 25 HPLC system (GE Healthcare) equilibrated with equilibration buffer and eluted with elution buffer (buffer compositions as described above). The eluted protein is adjusted to 0.5 mM EDTA and 1 mM DTT then TEV protease is added in a ~ 1/200 mass ratio. The mixture is incubated for 3h at RT then dialysed at 4°C overnight against 10 mM HEPES 50 mM NaCl 0.02% NaN3

pH=7.5. Proteins were adjusted to 7M urea for 1h at room temperature before being loaded on a HisTrap column equilibrated with 20 mM HEPES 50 mM NaCl 7 M urea pH=7.4 and 20 mM HEPES 150 mM NaCl 7 M urea pH=7.4. TEV-cleaved REM₁₋₁₁₆ flowed through without binding to the resin and was desalted against 10 mM HEPES 150 mM NaCl 0.02% NaN3 pH=7.4 using a HiPrep column (GE Healthcare).

B. Preparation and observation of giant vesicles (GVs)

200 µg of lipids, are mixed in solvent, adjusted to 0.1 mg/mL (final concentration) of rhodamine-dioleoyl-PE (RhodPE), spread drop-by-drop on a Teflon disk and dried for at least 1 h in a vacuum chamber. The remaining lipids are stored at -20°C for future use. The disk is pre-hydrated under a water-saturated nitrogen stream, covered with 5 mL of 300 mM sucrose and incubated at 34°C overnight. Observation chambers are coated with 5% BSA (w/v) for 20 min then rinsed three times with 10 mM HEPES 300 mM NaCl pH = 7.4. With large pipette tip, to avoid shearing, a drop of GV solution is added to the chamber, followed by ~2.10⁻¹² moles of GFP-REM₈₆₋₁₉₈, WT or EEE, if necessary. Lipid systems were 0.5 / 0.26/0.08/0.16 dipalmitoyl-PC (DPPC)/di-linoleoyl-PC (DLPC)/sitosterol/anionic lipid (as indicated) plus rhodPE (molar ratio). All lipids were from obtained from Avanti.

C. Nanodomain reconstitution for cryoEM

Lipids are hydrated to 10 g/L with 10 mM HEPES 150 mM NaCl 0.02% NaN₃ pH = 7.4, submitted to five freeze-thaw-vortex cycles (liquid N₂, 42°C water bath), mixed with GFP-REM₈₆₋₁₉₈ to final concentrations of 0.5 g/L lipids and 1 μ M GFP-REM₈₆₋₁₉₈ then incubated at room temperature for 1-2h. Samples are loaded on glow discharged cryoEM grids, blotted then frozen in liquid ethane using an EM-GP plunge freezer (Leica). Observed are carried out on an FEI20 (Tecnai) operating at 200 keV.

D. Agroninfiltration in Nicotiana benthamiana

Proteins were transiently expressed via infection with $OD_{600} = 0.2$ Agrobacterium tumefaciens (strain GV3101) bearing pK7WGY2-StREM1.3-EEE (N-terminal YFP-tag) in four-weeks-old Nicotiana benthamiana plants grown at 22-24°C in a greenhouse. Leaves are observed 3 days post-infection by confocal microscopy.

E. Confocal microscopy

Observations were performed on a Leica SP5 confocal laser scanning microscopy system (Leica, Wetzlar, Germany) equipped with Argon, DPSS and He-Ne lasers and a hybrid detector. GFP was excited at 488 nm, YFP was excited at 514 nm and rhodPE was excited at 565 nm.

III. Results

A. StREM1.3 specifically binds to PA and PIPs, but not PS

To reconstruct a remorin nanodomain, its lipid binding specificity must be precisely assessed. *St*REM1.3 is located at the PM's inner leaflet, where most negatively charged lipids lie. Expression of SAC1 in *Nicotiana benthamian* leaves, which

removes the last phosphate of PI4Ps, abolishes PM targeting (Gronnier et al., 2017). ²H static ssNMR confirmed a specific interaction between *St*REM1.3, with or without its N-terminal IDD, and PIPs along with an overall increase in lipid order (Legrand et al., 2019).

A truncated GFP-tagged *St*REM1.3 (GFP-REM₈₆₋₁₉₈), without its N-terminal IDD, is mixed with GVs of defined lipid compositions, comprising some rhodamine-PE (rhoPE). Phase separation was favoured by mixing di-palmitoyl-phosphatidyl-choline (DPPC), di-linoleyl-phosphatidyl-choline (DLPC) and sitosterol. Using confocal microscopy, we assessed GFP-REM₈₆₋₁₉₈'s binding ability towards negatively charged lipids (Figure 71). GFP-REM₈₆₋₁₉₈ does not bind to neither PC nor PS, but does bind to PA and various phosphoinositides: PI4P, PI5P, PI(4,5)P₂, PI(3,4,5)P₃ and PIPs from bovine brain (PIPmix) (PIPmix is PS/PI/PI4P/PI4,5P₂ 50/20/15/15 (Gronnier et al., 2017).





To test whether PS could play an indirect role in the nanodomain clustering of *St*REM1.3, we assessed its distribution at the PM in epidermal cells of *Nicotiana benthamiana* leaves expressing wild-type (WT) function PS synthase (Platre et al., 2018) or a knocked-out (KO) version. Preliminary results show that *St*REM1.3 was still able to segregate into nanodomains at the PM, although these appear larger and more diffuse (Figure 72).



Figure 72

PM repartition of GFP-*St*REM1.3 in 5-days old seedling roots of *Arabidopsis thaliana* expressing WT or knocked-out (KO) pss. Scale bars: 10 µm.

B. StREM1.3 must be oligomerised to ensure a tight membrane binding

Mutation of critical hydrophobic residues impairs PM targeting (Martinez et al., 2018). Here, leucines were mutated to glutamates, to remove hydrophobic residues at these positions, instead of prolines, to avoid disruption of coiled-coil helices.

Just like REM₈₆₋₁₉₈-PPP, REM₈₆₋₁₉₈-EEE displays a severely impaired PM targeting *in vivo* (Figure 73). Its biological activity, as assessed by its ability to slow down the cell-to-cell propagation of the Potato Virus X (Perraki et al., 2014, 2018; Raffaele et al., 2009b), remains to be tested. Binding of PIP-enriched vesicles by GFP-REM₈₆₋₁₉₈-EEE *in vitro* seems vastly reduced (Figure 74), although these observations should be repeated to make sure this result is reproducible (n = 2).



Figure 73

Cellular localisation of *St*REM1.3 WT (left) (Gronnier et al., 2017) and coiled-coil disrupted EEE (right). Scale bars: 10 µm.

RhodPE GFP-REM₈₆₋₁₉₈ Merged



Figure 74

Liposome binding assay for GFP-REM₈₆₋₁₉₈-EEE on liposomes containing PIPmix. Notice the homogeneous background noise from the GFP and its absence of signal on the liposome. Scale bars: 10 µm.

C. Close visual of synthetic remorin nanodomains

Considering the mass of structural data available on *St*REM1.3 nanodomains, both from previous sections and literature, we shall now attempt to reconstitute and observe them. Were the GVs from section 1 simply decorated with GFP-REM₈₆₋₁₉₈ or where they organised into nanodomains? Confocal microscopy does not allow to unambiguously distinguish nanodomains about 80 nm wide. Also, GVs, unless they are resting at the bottom of the observation chamber, which is rare, swim in solution. Yet, spt-PALM and STED super-resolution microscopies require an immobile sample. Other methodologies must be employed.

We turned ourselves towards cryoEM and AFM. All we should need are bulk lipids, here DPPC and DLPC, some sterols, here sitosterol, and some PIPs. For cryoEM, liposomes, mostly LUVs (100 - 1000 nm in diameter) and SUVs (< 100 nm in diameter) with some multilamellar vesicles as well, are pre-formed by five freeze-thaw-vortex cycles then mixed with GFP-REM₈₆₋₁₉₈. The rationale of choosing the GFP-tagged version was double: (1) the N-terminal IDD is not involved in membrane interactions (Legrand et al., 2019), (2) REM₈₆₋₁₉₈ alone is too small to be unambiguously detected by cryoEM (13 kDa), the GFP makes it 27 kDa bigger (total size: 40 kDa) and thus easier to spot.



Figure 75

CryoEM images of GFP-REM₈₆₋₁₉₈ **nanodomains.** Notice how the protein decorates both liposomes with a heterogeneous repartition. (A) *Inlet:* schematic description of a GFP-REM₈₆₋₁₉₈ nanodomain. Proteins attach to the outer leaflet of the liposome and can be easily detected on the liposome's equatorial plane. A region

of the liposome surrounding this equatorial plane (dashed line) appears rippled: it could be the rest of the nanodomain outside the equatorial plane. (B) *Inlet:* closer view of a GFP-REM₈₆₋₁₉₈ nanodomain (X5 magnification). Scale bars: 50 nm.

We observe dense objects decorating patches of liposomes (Figure 75A, inlet). In these patches, one can distinguish small cylindrical objects directly and exclusively attached to the outer leaflet of liposomes (Figure 75A-B). Since these objects are absent of samples without GFP-REM₈₆₋₁₉₈, we conclude that these objects are GFP-REM₈₆₋₁₉₈ and these patchy structures are, or at least resemble closely to, GFP-REM₈₆₋₁₉₈ nanodomains. Tomography would be required to assess the distribution of angles between these objects and the bilayer they are attached to. In the top image, the membrane alone is 6.8 ± 0.3 nm thick and the nanodomain is 10.8 ± 1.3 nm thick. In the bottom image, thicknesses are 5.3 ± 0.1 nm for the membrane alone and 10.4 ± 0.3 nm for nanodomains. Thus, these nanodomains seem to be 4-5 nm thicker than the bulk membrane due to the presence of the remorin (Figure 75B, inlet).

In our quest to obtain a reliable reconstitution protocol, and to push the resolution barrier lower, we switched to atomic force microscopy (AFM) to study the products of our nanodomain reconstitution attempts, using the same molecular actors as for cryoEM. Experiments are still ongoing.

IV. Discussion

Membrane targeting of *St*REM1.3 relies on PA and PIPs but not PS (Figure 71). We should note that all three are available at PM's inner leaflet. Thus, the anionic phospholipid must bear a free terminal inorganic phosphate at its extremity to be targeted by RemCA through electrostatic interactions (Gronnier et al., 2017). Knocking out the production of PS provoked minor changes in nanoclustering that could be construed as a consequence of modifications to cell metabolism and signalling induced by the deprivation of PS, rather than implying an interaction between *St*REM1.3 and PS (Figure 72).

Disruption of the coiled-coil, as exemplified by *St*REM1.3-EEE *in vivo*, on *Nicotiana benthamiana* leaves (Figure 73), and GFP-REM₈₆₋₁₉₈-EEE *in vitro* on giant vesicles (Figure 74), greatly reduces membrane targeting. So, to reconstitute nanodomains of *St*REM1.3, the minimal set of partners seems to be: REM₈₆₋₁₉₈, i.e. three RemCA plus a coiled-coil domain to permit a homotrimerisation (Martinez et al., 2018), PIPs, sterols (Gronnier et al., 2017; Legrand et al., 2019) and bulk lipids, such as DPPC and DLPC.

With this minimal set of partners, we were able to decorate liposomes with patches of GFP-REM₈₆₋₁₉₈. These patches shared every expected feature of a nanodomain: (1) a finite width around a few hundred nanometres (roughly) so not all the liposome is covered and (2) it is enriched in GFP-REM₈₆₋₁₉₈ (Figure 75). The limitation in size of these nanodomains would come from the exhaustion of nanodomain components, particularly the protein and PIPs, in its vicinity.

V. Conclusion

We are currently attempting to improve the yield of our protocol for the reconstitution of *St*REM1.3 nanodomains. So far, we were able to reconstitute and observe GFP-REM₈₆₋₁₉₈ nanodomains using cryoEM, but analyses on REM₈₆₋₁₉₈ and StREM1.3, by both cryoEM and AFM are on their way. Once more thorough observations can be performed, studying the arrangement of these remorins inside a nanodomain will provide capital insights into their functioning. Another key methodology to achieve this goal would be electron cryotomography to reconstruct a 3D model of a remorin nanodomain.

Conclusion

1. What is the nanoclustering mechanism of StREM1.3?

As detailed in Article V, *St*REM1.3 binds to negatively charged phospholipids with the exception of PS. In other words, it targets phospholipids with a free terminal phosphate: PIPs and PA. *St*REM1.3 has an ordering effect on lipid membranes mimicking nanodomains (Article IV) (Legrand et al., 2019), which is an expected feature of nanodomains enriched in both cholesterol (Raffaele et al., 2009b) and PIPs with saturated acyl chains (Furt et al., 2010). Indeed, both PIPs and sterols seem important for RemCA to influence membrane dynamics (Article IV) (Legrand et al., 2019). And so we come back to wandering whether this nanoclustering is lipid-, protein-or interactome-driven. Even though PIP clusters may pre-exist (Bilkova et al., 2017; van den Bogaart et al., 2011; Ji et al., 2015), there is an undeniable effect of *St*REM1.3 on lipid dynamics that we attributed to protein-driven nanoclustering. Thus, both mechanisms, PIP- and *St*REM1.3-induced clustering seem at work. The influence of the interactome will be discussed in section 3.

Oligomerisation of *St*REM1.3 was proved to depend on the formation of a coiledcoil domain sustained by hydrophobic contacts, and whose integrity is needed to ensure an efficient PM targeting *in vivo* (Article I) (Martinez et al., 2018). In addition, these coiled-coil domains are the building blocks of *St*REM1.3 filaments. Moreover, the nanoclustering mechanism of *St*REM1.3 is linked to its ability to oligomerise: it cannot be summarised by RemCA alone, only by RemCA plus the coiled-coil domain, (the N-terminal IDD is dispensable). Yet, phosphorylation modifies PM mobility, hinting at a crucial influence of *St*REM1.3's interactome on its PM repartition (Article II) (Perraki et al., 2018). These interactions are postulated to happen mainly at the Nterminal IDD, challenging the idea that this domain would be completely irrelevant to the protein's behaviour at the PM. We will elaborate further on this line of thought in section 3.

Aside from *St*REM1.3 specifically, we still wonder what differences in nanoclustering mechanism produce such a variety of different remorin nanodomains in *Arabidopsis thaliana* (Jarsch et al., 2014). Reminiscing on the diversity of RemCA domains involved herein (Konrad et al., 2014), some of which are acylated or display a quite different repartition of basic, acidic and hydrophobic residues, or both, we could attribute some of these differences in PM repartition to differences in lipid specificity. However, *At*REM6.1 and *At*REM1.2 strongly anti-colocalise even though their RemCA domains both bear a rather basic N-terminal and a rather acidic C-terminal with two palmitoylation sites. In such a case, to test whether the RemCA is the sole determinant of their PM repartition, one could swap the RemCA of one remorin with the other and assess whether they would then colocalise. In the same vein, one could swap coiled-coil domains instead of RemCA: where would a hybrid oligomeric remorin be localised?

Alternatively, we can think that the RemCA is not the sole determinant in PM repartition and colocalisation or anti-colocalisation with other remorins. Once more, the answer we seek might lie within the interactome (section 3).

2. What is the minimal set of partners required to make StREM1.3 nanodomains?

The work towards the reconstitution of minimal *St*REM1.3 nanodomains *in vitro* has begun. We know that PIPs and PA but not PS interact *St*REM1.3 (Article V) and that sterols are essential partners of this nanodomain system (Article IV) (Legrand et al., 2019). Moreover, knockouts of PS synthase mutants, effectively removing all PS content from the cells, do not prevent the formation of *St*REM1.3 nanodomains.

For now, we hypothesise that the necessary partners for the formation of these nanodomains are: REM₈₆₋₁₉₈, a bulk phospholipid like PC, PIPs and sterols. The importance of acyl chain lengths and unsaturations, although important in a case like the Ras nanodomain system (Zhou et al., 2017), was not studied here: the combination of electrostatic interactions with PIPs and hydrophobic effects both involving the RemCA and the PM were already shown to be the major mechanisms of membrane targeting (Gronnier et al., 2017). Also, *St*REM1.3, unlike Ras, does not possess a farnesyl anchor, hence a lesser focus on a hypothetical acyl chain specificity.

3. How interactors of *St*REM1.3 may regulate its relationship with membranes and biological functions?

To understand how StREM1.3's interactome may influence its behaviour in membranes, particularly in regard to phosphorylation (Article II) (Perraki et al., 2018), we undertook the mapping of its phosphorylated residues (Article II: addenda). Working on the N-terminal domain REM₁₋₁₁₆, we described it is an IDP and remain an IDP even after phosphorylation by *At*CPK3.

The question remains: why is *St*REM1.3-DDD, a mutant mimicking a constitutively phosphorylated protein, more mobile than *St*REM1.3-AAA, a mutant mimicking a constitutively not phosphorylated protein (Article II) (Perraki et al., 2018)? If we consider phosphorylation as a molecular switch, modifying the protein's interactome, it is tempting to view the changes in the mobility of StREM1.3-DDD as a consequence of interactors driving the protein out of nanodomains, while *St*REM1.3-AAA would remain strictly within. *St*REM1.3 has an intermediate phenotype, indicating either a turnover between non-phosphorylated and phosphorylated forms or, less likely, an inhibition of *St*REM1.3's phosphorylation when it is in nanodomains through an unknown mechanism.

With the goal of being more biologically relevant, it will be important to find what phosphorylates *St*REM1.3 in *Solanum tuberosum* (potato): the only remorin kinase we know of, for now, is *At*CPK3 from *Arabidopsis thaliana* (Article II) (Perraki et al., 2018). Conversely, is there a phosphatase that can dephosphorylate p*St*REM1.3? Alternatively, we should switch to an *At*REM to take advantage of the many genetic resources available on remorins in *Arabidopsis thaliana*.

One hypothesis to explain how the PM repartition of *St*REM1.3 could be influenced by its interactome should be investigated soon. Unpublished work by Dr Paul Gouguet and Dr Julien Gronnier identified the Actin Depolymerising Factor (ADF) as an interactor of *St*REM1.3-DDD, but not the WT, by both split ubiquitin assay and bifluorescence complementation. ADF, as its name suggests, binds to filamentous Factin and provokes its depolymerisation into G-actin monomers: it disrupts the actin cytoskeleton. Could it be that (1) nanodomains of non-phosphorylated *St*REM1.3 are pinned by actin and that (2) upon phosphorylation of *St*REM1.3, *St*ADF is recruited, disrupting the actin cytoskeleton, therefore freeing *St*REM1.3 from pinning by actin and allowing it to escape the nanodomain, hence the higher PM mobility of *St*REM1.3-DDD (Article II) (Perraki et al., 2018)? (Szymanski et al., 2015) showed that disrupting the actin cytoskeleton modifies the PM repartition of *At*REM1.2 and that *At*REM1.2 and *At*REM1.3, found to be enriched in DIMs under physiological conditions, target DSMs upon disruption of either the actin or the microtubule cytoskeleton. This interplay between *St*REM1.3 and actin would well illustrate the concept of fence and pickets as well as provide a plausible explanation to the difference in nanodomain localisation between different remorins (Jarsch et al., 2014), based on differences of interactome.

4. Final words

*St*REM1.3, likely as a homotrimer in the cytosol through a coiled-coil mediated oligomerisation (Article I) (Martinez et al., 2018), anchors to the inner leaflet of the PM using a combination of electrostatic interactions between the polar heads of PIPs, mostly PI4P, and two positively charged lysines of its three RemCA domains, as well as hydrophobic effects (Gronnier et al., 2017). More sitosterol and PIPs are then recruited to the nascent nanodomain where the lipid order parameter increases (Article IV) (Legrand et al., 2019). Phosphorylation of the N-terminal IDD influences the mobility of *St*REM1.3 at the PM (Article II and addenda) (Perraki et al., 2018).

The reconstitution of *St*REM1.3 nanodomains (Article V) and the study of its phosphorylation and interaction mechanism with *St*ADF (Article II and addenda) (Perraki et al., 2018) are promising outlooks for future studies. The former will give access to a powerful platform to experiment upon nanodomain formation and validate our hypothetical nanoclustering model (Article IV) (Legrand et al., 2019). The latter will elaborate on the biological implications of *St*REM1.3, how they are performed and how they may impact its behaviour at the PM. New interactors could be studied using the same methodology.

At last, now that this manuscript is coming to an end and there is nothing left to spoil, I shall leave the reader with the revised draft of our review on remorins (Gouguet et al., 2020).

Annex: an up-to-date review on remorins

This review was the introduction of the PhD manuscript of Dr Paul Gouguet, my predecessor on the two decades long project to study remorins. It has recently been accepted for publication. From the very start, it was written with the intention to become the most comprehensive and up-to-date review on remorins. It covers all remorin groups and summarises all demonstrated biological functions along with putative ones. The history of their discovery and their biophysics are also discussed to their fullest extent. New axes of research are also discussed.

Most of the writing was done by Dr Paul Gouguet and reviewed by Dr Sébastien Mongrand. Among all the authors, Dr Birgit Habenstein and I stepped in to write about the biophysics of the remorins, and that of *St*REM1.3 in particular. In conjunction with Dr Sébastien Mongrand, we also designed the related figure. A table summing up, in the light of this review, all established functions of remorins will be available; it is not part of the accepted manuscript.

Revised version:

Connecting the dots: from nanodomains to physiological functions

of REMORINs

Plant-specific protein REMORINs

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Abstract

REMORINs (REMs) are a plant-specific protein family, proposed regulators of membrane-associated molecular assemblies and well-established markers of plasma membrane nanodomains. REMs have been shown to play a diverse set of functions in plant interactions with pathogens and symbionts, responses to abiotic stresses, hormones signaling and cell-to-cell communication. In this review, we highlight the established and more putative roles of REMs throughout the literature. We discuss the physiological functions of REMORINs, the mechanisms underlying their nanodomain-organization and their putative role as regulators of nanodomain-associated molecular assemblies. Furthermore, we discuss how REMORINs phosphorylation may regulate their functional versatility. Overall, through data-mining and comparative analysis of the literature, we suggest how to further study the molecular mechanisms underpinning the functions of REMs.

Advances Box

• REMORINs are plant-specific membrane-bound proteins family involved in response to biotic (bacteria, viruses, fungi, oomycetes, mycorrhizae) and abiotic stresses (cold, mannitol, salt...), as well as developmental cues.

• REMORINS are strongly embedded in the inner-leaflet of the plasma membrane by an unconventional mechanism involving anionic lipids and sterols

• REMORINs are proposed as nanodomain-organizing proteins.

• REMORINS are highly phosphorylated proteins containing putative intrinsically disordered regions, which likely play a role in scaffolding protein complexes.

• REMORINs regulate cell-to-cell connectivity through plasmodesmata.

Outstanding Questions Box

• How can REMORIN functional versatility be explained?

• What are the consequences of the phosphorylation(s) of REMORIN's putative intrinsically disordered regions in term of 3D structure, protein-protein interaction and nanodomain organization?

• How do REMORINS regulate cell-to-cell connectivity through plasmodesmata?

One-sentence summary

This review collates and discusses all the available information on the plantspecific plasma membrane-bound family of REMORIN proteins involved in a plethora of cellular processes, and discuss their role as potential scaffolding proteins.

REMORINS DISCOVERY

REMORINS LOCALIZE IN DIVERSE AND COEXISTING NANODOMAINS

Mechanisms of REMs' Association to PM Nanodomains

- 1. The unconventional membrane-anchoring domain REMORIN Cterminal Anchor (REM-CA) and S-acylation
- 2. REMORIN Oligomerization
- 3. REMORIN Association with the Cytoskeleton

THE BIOLOGICAL FUNCTIONS OF REMORINS Established functions of REMORIN proteins Data mining toward the putative functions of REMORIN

STRUCTURAL AND BIOLOGICAL IMPLICATIONS OF REMORIN PHOSPHORYLATION

PERSPECTIVES

- 1 Genetics
- 2 Protein-Protein Interactions
- 3 The PM's complexity

REFERENCES

REMORINS DISCOVERY

REMORINs were first identified in tomato (Solanum lycopersicum) and potato (Solanum tuberosum) by Edward Farmer, Gregory Pearce and Clarence Ryan in 1989, in a will of identifying molecular actors involved in the perception of polygalacturonides (PGA) by plant cells (Farmer et al., 1989). PGA are pectic polysaccharides present in the plant cell wall and released during wounding and pathogen attack to sensitize plant defense. (Voxeur and Höfte, 2016). By in vitro phosphorylation assays on isolated tomato and potato plasma membrane (PM), Farmer et al. found that PGA treatment caused the phosphorylation of a protein of 34 kilodaltons, named phosphorylated protein of 34 kilodaltons or pp34 (Farmer et al., 1989). Pp34 was then used as a marker of the plant response to wounding (Farmer et al., 1991). Unintuitively, the purification (Jacinto et al., 1993) and cloning of pp34 (Reymond et al., 1996) revealed that its sequence turned out to be one of a hydrophilic protein even though it is-tightly bound to the PM. Pp34 was then renamed REMORIN (later named StREM1.3 for Solanum tuberosum REMORIN of Group 1 isoform 3) in reference to remora or "suckerfish" depicted as attaching itself to vessels and larger fish described by J.L. Borges in "Book of Imaginary Beings" (El libro de los seres imaginarios, J.L. Borges, 1969), and reflecting REMORIN's ability to bind to the PM whilst displaying an overall hydrophilic residue profile. Concurrently to the experiments of Farmer et al., Alliote et al. isolated a similar protein in Arabidopsis thaliana termed DNA-binding protein (dbp). This gene would be later known as AtREM1.3 (At2g45820) and was first characterized as a DNA-binding protein due to its highly-hydrophilic nature permitting electrostatic interactions with DNA and its amino-acid composition similar to Histone 1 proteins (Alliotte et al., 1989).

Genome-wide analyses have shown that the REM family is specific to the land-plant lineage (Raffaele et al., 2007). REM proteins present a highly-conserved C-terminal domain and a divergent N-terminal domain, which has been the basis for their phylogenetic classification into 6 separate groups (Raffaele et al., 2007). For example, we count 19 OsREMs in rice *Oryza sativa* and 16 AtREMs in *Arabidopsis thaliana*.

Since their discovery in the late 1980's, REMs have been the subject of ever-increasing attention. Be they directly the subject of studies or found in different screens of "omics" approaches, REMs are consistently found in a large variety of academic inquiries, providing grist to the mill to the idea that they could play a central role in plant development and adaptation. The first REMORIN protein identified in potato, which would be later named StREM1.3 for *Solanum tuberosum* REMORIN of Group 1 isoform 3. REMORIN's polyanion

binding capacity, originally linked to binding cell wall compounds (Reymond et al., 1996) would be later mitigated by the discovery of REMORIN's presence in the inner-leaflet of the PM.

In this review, we address the different characteristics that have been described for REMs as well as an overview of the physiological roles that REMs may participate in. We will discuss how REMs are anchored to the PM and cluster into PM-nanodomains, how they are phosphorylated and the subsequent role of these post-translational modifications. Finally, we provide a number of perspectives on how REMs should be further studied in order to better understand the many physiological conditions involving REMs.

REMORINS LOCALIZE IN DIVERSE AND COEXISTING NANODOMAINS

REMORINS predominantly associate with the PM (Raffaele et al., 2009a; Marín et al., 2012; Jarsch et al., 2014; Konrad et al., 2014; Perraki et al., 2014). Moreover, isoforms from Group 1 and Group 6 REMs have been shown to be partially associated with the plasmodesmata (PD)-PM in rice and in *Solanaceae* (Raffaele et al., 2009a; Fernandez-Calvino et al., 2011; Gui et al., 2014; Perraki et al., 2018). Nonetheless, translocation into the nucleus upon interaction with α -importins (Marín et al., 2012) and re-localization to intracellular foci upon perception of an immunogenic epitope of bacterial flagellum (Albers et al., 2019) have been reported for Group 1 and Group 4 REMs, respectively. However, the ER-PM contact sites observed by bimolecular fluorescence complementation (BiFC) for StREM1.3 are likely artefactual and highlight the risk of using the BiFC to study membrane protein interactions in plants (Tao et al., 2019).

Concerning their PM localization, the identification of Group 1 REMs in tobacco detergentresistant membrane fractions (DRM; a biochemical counterpart of membrane subcompartmentalization) first suggested a lateral organization of REMs into clusters at the PM (Mongrand et al., 2004). REMs' co-purification with DRM appeared to be dependent on the presence of phytosterols as it was reported to be absent of DRM in the sterol methyl-transferase mutant *smt1* albeit still present in this fraction in the sterol glycosylation mutant *ugt80A2;B1* (Zauber et al., 2014).

In relation with the above biochemical data, electron microscopy immunolocalization, stimulated-emission depletion (STED) microscopy and photoactivated localization microscopy (PALM) studies have all showed that Group 1 REMs were organized into nanodomains around about 70-90 nm in diameter in *Arabidopsis* and *Solanaceae* species (Raffaele et al., 2009a; Demir and Horntrich, 2013; Gronnier et al., 2017). Members of other groups in *Arabidopsis*

and *Medicago* are organized in PM domains that can be distinguished in density, size and shape (Jarsch et al., 2014). Interestingly, evolutionary distant REMs (belonging to distinct groups *e.g.* groups 1 and 6) localize in separate nanodomains suggesting that distinct REMs cluster into separate domains in order to play independent functions (Jarsch et al., 2014; Bücherl et al., 2017). It must also be noted that not all REMs are found in DRM-fractions of different plant tissues (Stanislas et al., 2009; Keinath et al., 2010; Srivastava et al., 2013; Takahashi et al., 2013) hinting to a diversity of REM-associated PM-domains.

Mechanisms of REMs Association to PM Nanodomains

A number of works have been performed to understand how highly-hydrophilic REMs could be tightly anchored to the PM and the molecular interactions leading to the clustering of REMs into nanodomains. REMs are anchored to the PM inner-leaflet *via* the unconventional lipidbinding motif called REMORIN C-terminal Anchor (REM-CA) (Raffaele et al., 2009; Konrad et al., 2014; Perraki et al., 2012; Gronnier et al., 2017). In the past few years, several molecular determinants regulating REMs' nanodomain organization have emerged mostly for Group 1 and 2 REMs and need to be further studied to fully understand the diversity of REMs' clustering (Jarsch et al., 2014). There are at least four determinants: the lipid-binding properties of REM-CA domain, REM-CA S-acylation, REM-REM oligomerization and REM-cytoskeleton interactions. Here we review the knowledge obtained to explain the molecular mechanisms at play.

1. The unconventional membrane-anchoring REMORIN C-terminal Anchor domain (REM-CA) and S-acylation

REMs do not contain target-peptides or transmembrane domains (Raffaele et al., 2007) and attached to the PM independently of the conventional secretory pathway (Gui et al., 2015; Gronnier et al., 2017). StREM1.3, SYMREM1 (also termed MtREM2.2) as well as AtREM1.2, AtREM1.3, AtREM4.2, AtREM6.1 and AtREM6.4 have been described to be strictly PM localized thanks to their REM-CA moieties (Raffaele et al., 2009a; Lefebvre et al., 2010; Perraki et al., 2012; Raffaele, 2013; Jarsch et al., 2014; Konrad et al., 2014; Gronnier et al., 2017). In the case of StREM1.3, the last 28 amino acids (*i.e.* REM-CA) partially folds into an alpha helix in the presence of PI4P and sitosterol and also inserts itself into the hydrophobic core of the bilayer (Gronnier et al., 2017). This is driven by protein-lipid interactions between the REM-CA and the PM's inner-leaflet lipids: negatively-charged phosphoinositides (PIPs), notably phosphatidylinositol-4-phosphate (PI4P), and sterols, notably sitosterol (Raffaele et al.,

2009a; Legrand et al., 2019), (Figure 1). REM-CA binds to PI4P with a yet unknown stoichiometry through its Lysine/Arginine residues *via* electrostatic interactions. Association of StREM1.3's REM-CA with negatively-charged phospholipids regulates its nanodomain organization (Gronnier et al., 2017).

In mammals, PIPs have been reported to possess the ability to cluster *in vivo* to form nanodomains (van den Bogaart et al., 2011). It was also shown, *in vitro*, that cholesterol are found in the vicinity of saturated phospholipids rather than of unsaturated ones (Engberg et al., 2016). This mechanism creates a so-called Liquid-ordered domain in the lipid bilayer. In plants, the fact that PIPs bear mostly saturated acyl-chains suggest that phytosterols may also actively participate to the formation of nanodomains (Furt et al., 2010). REMs could be either targeted to pre-existing lipid-mediated nanodomains or participate in the organization of their own lipid environments. Both the latter and former possibilities for REMs' association to PM-nanodomains still remain open questions.

The presence of cysteine residues in the REM-CA of many REMs raised the possibility of membrane association due to S-acylation. AtREM1.2 was the first REM to be found to be Sacylated (Hemsley et al., 2013). AtREM1.2 and AtREM6.4, lost strict-PM localization when their REM-CA Cysteine residues were substituted by Alanine residues (Konrad et al., 2014). Altered localization when S-acylation is abolished via 2-bromopalmitate was also observed with the rice REM OsREM6.6 (Gui et al., 2015). This is reminiscent of the relocalization observed for the S-acylation site mutant NbREM1.1^{C206A} to RFP-ATG8e-tagged vesicles (Fu et al., 2018). It is important to note that NbREM1.1^{C206A} is no longer organized in nanodomains, nor co-purifies with the DRM biochemical fraction (Fu et al., 2018). Yet for SYMREM1, the Cysteine substitution did not reduce its PM-localization and did not change its segregation pattern in the PM, hinting to a more complex PM association mechanism. Altogether, all of the above mentioned data highlight the importance of the REM-CA as a determinant of REM's general membrane affinity as well as REM's organization within these membranes. Interestingly, many REMs do not have predicted S-acylation sites in their REM-CA (Konrad et al., 2014; Gronnier et al., 2017), suggesting a degree of diversity in the mechanisms regulating REMs' PM targeting and nanodomain organization.

2. REM Oligomerization

REM oligomerization is highly important for its targeting and function. REMs were first proposed to form oligomeric structures. The first evidence of the oligomerization was reported in Bariola et al. (2004) where the group 1 REMORIN coiled-coil domain was described to participate in the formation of REM oligomers. Electron microscopy and glutaraldehyde

crosslinking assays evidenced REM multimerization *via* the C-terminal region of two Group 1 REMs *i.e.* StREM1.3 and SIREM1.2 (Bariola et al., 2004). Bariola et al. (2004) proposed *via* analytical ultracentrifugation of recombinant proteins and cross-linking experiments on isolated PM that Group 1 REMs associate into dimers, tetramers or higher-order oligomeric structures *via* their coiled-coil domains. Alternatively, Perraki et al. (2012) developed the hypothesis that StREM1.3 would trimerize either before or after the anchoring event at the PM. This hypothesis was upheld by gel filtration assays with the *E. coli* purified 6His-tagged full-length StREM1.3 protein (Perraki et al., 2012). The exact order of oligomerization of REMs *in planta* is still lacking (Jaillais and Ott, 2020).

In our current view, StREM1.3 is homo-trimeric in solution bundling three REM-CA domains together (Bariola et al., 2004; Martinez et al., 2018). This trimeric hypothesis was further developed in Martinez et al. (2018) upon *in silico* modelling confirmed by Solid-State NMR in conjunction with cryo-Electron Microscopy (cryo-EM) and *in vivo* observations of mutations in the coiled-coil domain that impair PM-association (Figure 1). These *in vivo* observations seem to corroborate the hypothesis that in a minimal state, the trimeric form is necessary for PM anchoring. Cryo-EM further revealed a possible lateral association of the trimeric REMs in the presence of an N-terminal 6His-tag (Martinez et al., 2018), yet the presence of a 6His-tag may also alter the oligomeric behavior of proteins (Amor-Mahjoub et al., 2006). This casts a doubt on the *in vivo* relevance of the trimer of StREM1.3 observed by gel filtration (Perraki et al., 2012). In any case, the existence *in vivo* of homo-oligomers and higher-order oligomers has not been invalidated by the latest data. Formation of higher-order oligomers, *i.e.* oligomers of trimers, as a mean to drive nanoclustering cannot be excluded but has yet to be tested. Interactions between neighboring REM trimers are likely to further stabilize nanodomain clustering.

Group 1 AtREMs have been found to form oligomers in a large-scale study of PM protein oligomerization in leaves (McBride et al., 2017). AtREM1.2, AtREM1.3 and AtREM1.4 were predicted to form oligomeric complexes according to their ratio of the calculated mass to the apparent mass (R_{app}) score which calculates a ratio between the predicted molecular mass of the monomer and the measured molecular mass of the complex in which the protein is found. AtREM1.2, AtREM1.3 and AtREM1.4 were found in different complexes to have a ratio of the calculated mass to the apparent mass scores (R_{app}) ranging on average from 35 to 60 for AtREM1.2, 25 for AtREM1.3 and 30 to 95 for AtREM1.4 (McBride et al., 2017). This data may suggest that in addition to being homo-oligomerized, Group 1 REMs are also forming multi-protein complexes. This idea has already been suggested in the literature, notably in

Lefebvre et al. (2010) where REMs are compared to caveolins considering the common properties they share: small, oligomeric, lipid raft-associated scaffold proteins that can form filamentous structures.

3. REM Association with the Cytoskeleton

In the seminal work of Jarsch et al. (2014), filamentous exclusion zones observed at the surface of the PM have led to think that REMs could be associated with the cortical cytoskeleton. A localization dependent on microtubule polymerization has been reported using the microtubule depolymerizing drug oryzalin. Oryzalin treatment substantially mislocalized AtREM1.2 that was originally at the PM in domains (Szymanski et al., 2015). Moreover, AtREM6.6, that localizes at fibrillar structures and to PM domains, has been shown to be affected in its fibrillar localization by oryzalin treatment (Jarsch et al., 2014; Konrad et al., 2014).

This interplay between the cortical cytoskeleton and PM-nanodomains fits with the "fences and pickets" paradigm (Kusumi et al., 2012), where REMs could play a role as a putative scaffold protein. This interplay with actin has been further demonstrated for other REMs such as SYMREM1. SYMREM1's association to PM-nanodomains has been shown to be destabilized by the actin depolymerizing drug, latrunculin B, yet not by the microtubule depolymerizing drug, oryzalin. SYMREM1's presence at the PM was also shown to be essential for the establishment of FLOT4-LYK3 domains that are dependent on the contact between FLOT4 and the actin cytoskeleton (Liang et al., 2018) (see Figure 2B). The rice Group 6 REM, OsREM6.6 (or GSD1 for grain setting defect 1, see below), has also been shown to associate with the actin cytoskeleton. Treatment with cytochalasin D (a potent inhibitor of actin polymerization) completely disrupted the punctate localization pattern of AtREM1.2 and AtREM1.3 to a more uniform distribution with smaller punctate patterns of lower intensity (Szymanski et al., 2015). Additionally, AtREM1.2 was shown to associate with the actin cytoskeleton under viral infection (Cheng et al., 2020). This data reveals the strong relationship between REMs' anchoring to the PM and the actin cytoskeleton, although the functional mechanism remains to be tackled.

THE BIOLOGICAL FUNCTIONS OF REMORINS Established functions of REMORIN proteins *Plant-Microbe Interactions*

Numerous studies have reported the implication of REMs in plant interactions with microorganisms.

Viruses

The first REM to be shown as implicated in the context of viral infection has been StREM1.3. It has been demonstrated that its over-expression leads to the limiting of the cell-to-cell movement of *Potato Virus X* (PVX), and that its under-expression (RNAi lines) lead to an acceleration of the PVX movement (Raffaele et al., 2009) (Perraki et al., 2018). A general effect on the REM-dependent gating of PD was also shown in the presence of viral movement proteins such as 30K from the *Tobacco Mosaic Virus* (TMV) or Hc-Pro from the *Potato Virus Y* (PVY) (Perraki et al., 2014). In addition to limiting PVX cell-to-cell movement, StREM1.3 has also recently been shown to limit TMV cell-to-cell propagation (Perraki et al., 2018). Over-expression of StREM1.3 was shown not to impair the silencing suppressor activity of the PVX-encoded TRIPLE-GENE BLOCK protein 1 (TGBp1) (Perraki et al., 2012), but rather to induce the increase in callose accumulation at PD pit-fields (Perraki et al., 2018). Furthermore, StREM1.3's capacity in hindering PVX cell-to-cell movement has been shown to be abolished when REM-CA is mutated, thereby modifying its targeting to the PM (Gronnier et al., 2017). Additionally, StREM1.3 was shown to physically interact with the PVX movement protein TGBp1 with or without an impaired REM-CA domain (Perraki et al., 2012).

In Perraki et al., (2018), it was shown that PVX-activated kinases are responsible for StREM1.3's phosphorylation potentially on residues S74/T86/S91 and that expression of the phosphomimetic, but not the phosphoablative mutant of StREM1.3 hampered virus cell-to-cell propagation to similar levels with the wild type. Importantly, the calcium-dependent protein kinase AtCPK3, which could phosphorylate StREM1.3 *in vitro*, could also restrict PVX cell-to-cell movement in a REM-dependent manner. *In vitro*, the StREM1.3 phosphomutants were not phosphorylated by AtCPK3 (Perraki et al., 2018). This suggests that an AtCPK3 ortholog in potato could potentially regulate StREM1.3 *in vivo* for anti-viral defense. Moreover, the phosphorylation mutants had an impact on the localization of REM at PD and on callose deposition, associating phosphorylation status, protein mobility, PD permeability and cell-to-cell viral propagation (Perraki et al., 2018, Figure 2A).

Solanaceae Group 1 REMs have also been studied in the context of *Tenuivirus* infection. *N. benthamiana* NbREM1.1 and NbREM1.2 were shown to be degraded during *Rice Stripe Virus*

(RSV) infection in consequence to the interference of the RSV-encoded protein, NSvc4. The degradation of NbREM1.1 and NbREM1.2 via the autophagy pathway led to RSV circumventing NbREM1-associated resistance (Fu et al., 2018). In contrast, a recent study has underlined the effect of the tobacco REM NtREM1.2 on the cell-to-cell movement of a Tobamovirus, the Tomato Mosaic Virus (ToMV) (Sasaki et al., 2018). ToMV infection and the overexpression of ToMV movement proteins separately affected NtREM1.2's localization by inducing its aggregation at the PM. Dissimilarly to StREM1.3, which is known to localize in a patchy pattern at the PM and is present in PM-nanodomains, NtREM1.2 localized in a uniform fashion throughout the PM regardless of N-terminal or C-terminal fusion to fluorescent tags. Considering the high sequence conservation of the C-terminal region, this suggested that the N-terminal region was involved in NtREM1.2's characteristic localization. Interestingly, NtREM1.2 was shown to interact and colocalize with ToMV's 30-kDa movement protein at PD. Agrobacterium-mediated transient expression of NtREM1.2 was also shown to slightly increase ToMV infection foci size (Sasaki et al., 2018). Moreover, in ToMV infected cells, NtREM1.2 aggregates occur close to tubular ER structures and are associated with ToMV's 30kDa movement protein bodies that appeared to be linked to the ER-Actin network (Sasaki et al., 2018). Finally, A. thaliana AtREM1.2 has been proposed to play a role in Turnip Mosaic Virus (TuMV) cell-to-cell movement by competing with the TuMV protein VPg in the actindependent regulation of PD aperture (Cheng et al., 2020).

Group 4 REMs have been reported as positive regulators of viral infection. AtREM4.1 and AtREM4.2 were shown to be susceptibility factors during *Beet Curly Top Virus (BCTV)* and *Beet Severe curly Top Virus (BSCTV)* infection. This was shown using single- and double-knock-out and overexpressing lines for AtREM4.1 and AtREM4.2. This effect of Group 4 REMs on geminiviral infection could be linked to SnRK1, an important positive regulator of plant stress involved in antiviral defense, which could phosphorylate AtREM4.1 *in vitro* (Son et al., 2014).

Bacterial Symbiosis

The Group 2 REMs merely constituted of a C_domain (c.f. Figure 1A) (Raffaele et al., 2007), have been characterized by their role in the establishment of infection threads (IT) during the symbiosis of *M. truncatula* and *Lotus japonicus* with *Rhizobiaceae* family bacteria by participating in the formation of root nodules (Lefebvre et al., 2010; Tóth et al., 2012). SYMREM1 has been shown to be specifically expressed in *M. truncatula* roots and nodules with an expression strongly induced by Nod factor treatment (Lefebvre et al., 2010), which are lipo-chitooligosaccharides produced by symbiotic bacteria in order to initiate symbiosis

(Oldroyd, 2013). Furthermore, SYMREM1 knock-down lines were shown to strongly reduce nodule establishment and growth (Lefebvre et al., 2010). Analogously, overexpression of Group 2 REM LjSYMREM1 significantly increased the number of nodules per root in *L. japonicus*, establishing these orthologs as key players for symbiosis in legumes (Tóth et al., 2012). In this context, SYMREM1 has been further characterized at the molecular level. It has been described as a scaffold protein due to its interaction with three receptor-like kinases (RLKs) essential for nodule establishment and maintenance *i.e.* NFP, DMI2 and LYK3 (Lefebvre et al., 2010). This could be a recruiting process of RLKs to specific nanodomains to initiate IT along with FLOT4 (Liang et al., 2018). SYMREM1 is believed to stabilize the interactions between FLOT4 and both NFP and LYK3 which would prevent the RLKs' endocytosis and ensure nodulation-promoting signaling (Liang et al., 2018; see Figure 2B).

Fungi and Oomycetes

REMs have been reported to be implicated in interactions with filamentous pathogens/symbionts. StREM1.3 has long been established as a marker of Phytophthora infestans extrahaustorial membrane (EHM) (Lu et al., 2012; Bozkurt et al., 2014). StREM1.3 has been observed to label haustoria, approximately 50 % of the time, and to specifically label non-callosic haustoria (Bozkurt et al., 2014). Moreover, the overexpression of StREM1.3 in N. benthamiana and tomato increased susceptibility to P. infestans infection (Bozkurt et al., 2014). The precise role of StREM1.3 during P. infestans has yet to be precisely determined. Interestingly, recent studies showed the specific recruitment of phosphoinositides (PIPs) to the plant-pathogen interfacial membrane during fungal infection (Qin et al., 2020). Different PIPs were found enriched at the EHM. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) was shown to be dynamically up-regulated at powdery mildew infection sites and recruited to the EHM, whereas PI4P is absent in the EHM. Furthermore, the depletion of $PI(4,5)P_2$ in the *pip5k1 pip5k2* mutant lines inhibited fungal pathogen development and caused disease resistance, independent of cell death-associated defenses, involving impaired host susceptibility. The fact that REMs both bind to PIPs and are involved in immune responses to fungi incites to examine in detail the link between these two observations in future work.

The first report of a possible implication of REMs in plant-fungal interactions was a maize ZmREM6.3 that was found in a QTL (Quantitative Trait Loci) analysis for resistance to northern leaf blight caused by the fungal pathogen *Setosphaeria turcica* (Jamann et al., 2016). A recent study further highlighted the importance of REMs in the resistance to fungal pathogens by identifying ZmREM1.3 in a large scale proteomic analysis (Wang et al., 2019). The comparison between southern corn rust (*Puccinia polysora*) sensitive- and resistant-maize
inbred lines revealed that ZmREM1.3 protein amount was increased in the resistant line whereas it was decreased in the sensitive line. Genetic approaches (overexpression and KO) confirmed that ZmREM1.3 mediated maize resistance to *P. polysora*, through SA/JA signaling and defense gene up-regulation (Wang et al., 2019).

A recent study showed that overexpression of tomato (*Solanum lycopersicum*) SIREM1 increased susceptibility to the necrotrophic fungus *Botrytis cinerea* (Cai et al., 2020). Heterologous expression of SIREM1 increased ROS accumulation and triggered other cell-death regulators, suggesting a positive regulatory role of SIREM1 in programmed cell-death (PCD) and providing clues for understanding the PCD molecular regulatory networks in plants.

Hormone Signaling & Abiotic Stress

The rice (*Oryza* sativa) OsREM4.1 was shown to be up regulated by ABA treatment *via* the bZIP transcription factor OsZIP23, which can bind the OsREM4.1 promoter. OsREM4.1 overexpression inhibited BR signaling by inhibiting OsSERK1 and OsBRI1 complex formation. This REM effect is undermined by the phosphorylation of REM. Under high BR concentrations, BR stabilizes the OsSERK1/OsBRI1 complex, which activates the phosphorylation of OsREM4.1 by OsBRI1, thereby reducing OsREM4.1's affinity for OsSERK1 and enabling the BR signaling cascade. This signaling system maintains a dynamic equilibrium between ABA and BR signaling (Gui et al., 2016), see Figure 2C. In *A. thaliana*, AtREM1.2 and AtREM1.3 have been shown to organize lipid raft nanodomains in a salicylic acid (SA)-dependent fashion. Indeed, by interaction with GRF10, both REMs were shown to participate in PD closure after SA treatment (Huang et al., 2019). Moreover, AtREM1.2 was shown to be crucial for SA-induced asymmetric auxin flux during root gravitropic response as well as for the regulation of clathrin-mediated endocytosis (Ke et al., 2020).

The tomato SIREM1.2 has recently been shown to be involved in fruit ripening by interacting with the ethylene biosynthesis proteins. The overexpression of SIREM1.2 up-regulates key genes involved in ethylene, lycopene biosynthesis and ripening regulators resulting in an effective increase in the ethylene and lycopene content of fruit (Cai et al., 2018).

The functional characterization of a mulberry (*Morus indica*) Group 1 isoform MiREM1 evidenced its transcriptional upregulation under a number of abiotic stresses and hormone treatments. Gain of function *via* the heterologous overexpression of MiREM1 in *A. thaliana* conferred resistance to salt stress and drought (Checker and Khurana, 2013).

The Group 6 REM SiREM6 (*Setaria italica*, fox-tail millet) has been found to be transcriptionally up regulated under salt, cold, ABA and osmotic stress. Yet this up regulation

was weakly observed under drought stress and SiREM6 overexpression did not impact drought tolerance. SiREM6 overexpression in *A. thaliana* increased germination rate and seedling biomass as well as seedling survival under salt stress. These characteristics were linked to an increase in proline content and a reduction in electrolyte leakage.-Additionally, overexpression of SiREM6 in *A. thaliana* enhanced sensitivity to ABA treatment (Yue et al., 2014). In rice (*Oryza* sativa), OsREM6.5 was also found to be highly upregulated during drought stress and ABA treatment (Wu et al., 2006)

In *Populus euphratica*, a salt-resistant poplar, PeREM6.5 was induced by NaCl stress. Interestingly, PeREM6.5 recombinant protein significantly increased the H⁺-ATPase hydrolytic activity and proton transport activity in *P. euphratica* PM vesicles. Yeast two-hybrid assays showed that PeREM6.5 interacted with RPM1-interacting protein 4 (PeRIN4). Notably, the increase of H⁺-ATPase activity induced by PeREM6.5 was further enhanced by PeRIN4 recombinant protein. Finally, overexpression of PeREM6.5 in *A. thaliana* improved salt tolerance. PeREM6.5, by regulating H⁺-ATPase activity in the PM, may therefore enhance the plant capacity to maintain ionic homeostasis under salinity (Zhang et al., 2020).

Cell-to-Cell connectivity via PD

The first direct evidence of the implication of REMs in cell-to-cell connectivity *via* PD was revealed by the ability of StREM1.3 to hinder PVX cell-to-cell movement as well as GFP diffusion in non-virally-infected plants in *N. benthamiana* leaf epidermal cells (Perraki et al., 2012; Perraki et al., 2014, Raffaele et al., 2009). REM transcript and protein levels were shown to increase in mature, ageing and senescing tissues, as well as in "source parts" of tobacco leaves (*i.e.* the tip of the leaf), where a majority of mature- and branched-PD are present (Raffaele et al., 2009b). These results suggest that tobacco Group 1 REMs predominantly associate with mature-branched-PD.

The Group 6 REM OsREM6.6 was shown to regulate PD permeability in rice leading to the filling of the seed. The mutant termed GSD-1 (grain setting defect 1) was shown to have an altered and ubiquitous expression of OsREM6.6, which is natively only expressed in phloem companion cells, thereby inducing an accumulation of starch and free-sugars in the leaf blades and consequently a reduction of starch accumulation in grains (Gui et al., 2014).

Data mining toward the putative functions of REMORINs

Throughout the relatively extensive literature that links REMs to biological functions (from transcriptomic, proteomic, phosphoproteomic data), several patterns may be found.

Considering the overlapping nature of these types of events, we summarize that data in Table 1 by grouping REMs by phylum and by putative function. These links remain to be further experimentally confirmed but deserve to be pointed out in order to pave the way in understanding the biological functions of REMs.

STRUCTURAL AND BIOLOGICAL IMPLICATIONS OF REM PHOSPHORYLATION

Since the first identification of REM as an *in vitro* phosphorylated protein, pp34 (Farmer et al., 1989), members of all REM groups have been detected in phosphoproteomes from diverse tissues and biological contexts, suggesting phosphorylation as a major *in vivo* regulatory mechanism of REMs (see Supplemental Table 1 for a compilation of REM phosphoproteomic data). A recent study established the phosphorylation pattern of 14 AtREMs in 30 different plant tissues (Mergner et al., 2020). Apart from this broad-scale analysis, other studies have shown that, for some residues, the phosphorylation of REMs is modulated by stress conditions such as flg22 and OG treatment, nitrogen deprivation, ABA, H₂O₂, cold, osmotic and salt stresses (Benschop et al., 2007; Kohorn et al., 2016; Menz et al., 2016; Nikonorova et al., 2018; Wang et al., 2020, see Supplemental Table 1), suggesting functional roles of phosphorylation that remain to be explored.

REMs are found to be phosphorylated *in vivo* mainly at the putatively intrinsically disordered region (IDR) located in the N-terminal domain (Figure 3) (Marín and Ott, 2012). IDR regions are flexible and extended protein segments that provide dynamic structural remodeling and protein-protein interaction plasticity (Iakoucheva et al., 2004; Dyson and Wright, 2005; Uversky, 2013). They are often regulated *via* phosphorylation that may act as a regulatory switch leading to structural changes such as folding and the sequestration of binding sites (Bah et al., 2015). Because they facilitate protein recognition and binding by acting as adaptable interaction surfaces, IDR regions may often found in hub proteins that link interaction networks and integrate signals (Kim et al., 2008). REMs' basal and signal-induced phosphorylation regions could putatively regulate interactions with its protein partners upon stimulus (Figure 3). This, and the fact that REMs participate in different plant signaling networks, ground the idea that they act as PM-bound-complex signaling "hubs" able to interact with many different proteins, at specific times and locations, and in response to specific stimuli. However, little is known about how REMs integrate diverse signaling cues in plants.

Only a few studies have drawn links between REM phosphorylation and intracellular signaling. Marín and Ott (2012) showed that AtREM1.3 phosphorylation at S66 located in the

IDR region led to a reduced affinity for importins in yeast two-hybrid assays. However, the impact of this phosphorylation on the structure, and subcellular localization of AtREM1.3 remains to be investigated. Furthermore, Gui et al. (2016) demonstrated the role of OsREM4.1 phosphorylation by OsBRI1 in activation of the brassinosteroid signaling pathway (Gui et al., 2016, Figure 2C). While the phosphosites remain to be identified, this model connects REM phosphorylation, hormone-signaling balance and rice development, giving an important insight on one of the functional roles of REM phosphorylation in rice. In Perraki *et al.*, 2018, it was shown that the phosphorylation of StREM1.3 on residues S74, T86, S91 was essential for its anti-viral function and dynamic nanodomain association (Perraki et al., 2018, Figure 2A). Yet, the *in vivo* phosphorylation at those sites in response to PVX infection still remains to be determined.

Although multiple REM phosphosites have been described in phosphoproteomes or with directed approaches, only few REM-associated kinases have been identified (see Supplemental table 1). A recent large-scale phosphoproteomic study identified 8 Arabidopsis kinases (CPK11, MPK6, OST1, SnRK2.4, SOS2, OXI1, CKL2, CRKL2) that could phosphorylate, in vitro, AtREM phosphopeptides generated in vivo (Wang et al., 2020). Additional kinases responsible for REM phosphorylation have been described in rice, N. benthamiana, M. truncatula, L. japonicus and A. thaliana (SnRK1.2, AtCPK3 and various RLKs) (Mehlmer et al., 2010; Tóth et al., 2012; Son et al., 2014; Gui et al., 2016; Perraki et al., 2018; Albers et al., 2019), see a summary in Figure 4. However, all these data have been obtained in vitro and will require further validation. Notably, in vivo interactions between REMs and protein kinases had already been reported for some of the kinases identified in Wang et al., (2020) (Tóth et al., 2012; Perraki et al., 2018; Albers et al., 2019). These kinases have been reported to phosphorylate a unique REM or several of them (Mehlmer et al., 2010; Tóth et al., 2012; Son et al., 2014; Gui et al., 2016; Perraki et al., 2018; Albers et al., 2019; Wang et al., 2020) (Supplemental Table 1). Interestingly, most of those kinases are associated with DRMs, (Majeran et al., 2018) but some of them are putatively soluble suggesting a PM recruitment.

This protein-protein interaction conferred by regions with high-scores of intrinsic disorder (Raffaele et al., 2007, Marín and Ott, 2012) and phosphorylation (Reymond et al., 1996; Marín and Ott, 2012; Gui et al., 2016; Kohorn et al., 2016; Perraki et al., 2018) (see Figure 3) are characteristics reminiscent of scaffold proteins, such as the 14-3-3 family of proteins. This analogy holds on the cases where REMs' interaction capacity was reported to differ upon phosphorylation of either the interaction partner(s) or the REM isoform itself (Gui et al., 2016; Liang et al., 2018). GRF10 (14-3-3 epsilon, At1g22300) was recently shown to interact with

and mediate AtREM1.2 and AtREM1.3 interaction (Huang et al., 2019). 14-3-3 family proteins are involved in phospho-relay events, preeminently in facilitating and hindering protein-protein interactions by binding to the phosphorylated residues of interaction partners (Lozano-Durán and Robatzek, 2015).

PERSPECTIVES

The amount of data that has been accumulated since the cloning of the first REM in 1989 on the characteristics and the cellular and physiological role of REMs is very heterogeneous. Although many articles have shown REMs expression as being correlated with a number of physiological processes, a clear role for the REM family has not yet arose. Nevertheless, many trails have emerged from the 30 years of research on REMs that show great promise in the search for REMs' functionalities. One of these functionalities stands out, considering the multiple instances where REMs are linked to intercellular connectivity. These instances reinforce the idea that the establishment of REMs as a genetic family could have been important for the adaptation of photosynthetic algae during the land plant invasion that occurred approximately 475 million years ago (Steemans et al., 2009). Indeed, the control of water and solute exchanges between cells and tissues was a major necessary adaptation in order to handle water fluxes in dry land. The development of complex PD along with a complex vasculature necessary for the establishment of root systems and hormone signaling were the fundamental developments that enabled adaptation and establishment of Embryophytes (Rensing et al., 2008). Moreover, the fact REMs are found to be involved in biotic interactions, notably with symbiotic fungi and bacteria is also a clue to their potential involvement in the conquest of land by plants considering the hypothetical importance of symbiotic microorganisms in helping the first rootless land plants to secure nutrients (for review Rensing 2018). (Raffaele et al., 2009a). The recent discovery of Group 1 REMs in a proteomic analysis of A. thaliana extracellular vesicles may potentially change our view of REMORIN's strictly-PM function (Rutter and Innes, 2017; Rutter and Innes, 2018).

1. Genetics

A major weakness in the functional study of REMs is the lack of genetic tools. Overall, REM single mutants have not yet been shown to display any striking or strong phenotypes although AtREM1.2 alone has recently been shown to be involved in SA-dependent gravitropism response (Ke et al., 2020). To study REMs, the most notable problematic is currently the lack

of multiple KO-lines for each isoform although the generation of these lines is currently ongoing with the recently published CRISPR-Cas9 KO-lines for two Group 1 REMs in *N. benthamiana* (Fu et al., 2018, Huang et al., 2019) and the quadruple CRISPR-Cas9 KO-line for all four Group 1 REMs in *A. thaliana* soon available (T. Ott, personal communication). KO lines for each REM Group will be essential for our understanding of the role played by each REM Group that could entail not only different possible cellular functions but also different tissue specificities (*e.g.* Gui et al., 2014). Nevertheless, we cannot exclude that REMs from different sub-groups are at least partially redundant or can functionally substitute for other REMs, even if in the wild-type situation they are doing a different function.

Several REM Groups such as Group 5 and Group 3 could consequently become less illusive as to their function. The generation of KO-lines should be combined with gain-of-function assays as those undertaken in the characterization of OsREM6.6 (or GSD1; Gui et al., 2014) and OsREM4.1 (Gui et al., 2016) in order to develop true functional approaches in deciphering the cellular and physiological purposes of REMs.

2. Protein-Protein Interactions

Phosphorylation of REMs are suspected to be involved in protein-protein interactions (Raffaele et al., 2007; Marín et al., 2012) (Figure 2, Supplemental Table 1). These characteristics are reminiscent of scaffold proteins, notably of 14-3-3 family proteins. Recently, GRF10 (14-3-3 epsilon) was shown to interact with AtREM1.2 and to be crucial for its partitioning at the PM (Huang et al., 2019). REMs and 14-3-3 family proteins could indeed co-depend on each other considering REMs' interaction capacity was reported to vary upon phosphorylation of either the interaction partner(s) or the REM isoform itself (Gui et al., 2016; Liang et al., 2018;). In both cases, the REM isoforms *i.e.* OsREM4.1 or SYMREM1 interact with receptor-like kinases (RLKs), OsSERK1 (Gui et al., 2016) and LYK3 and NFP (Liang et al., 2018), respectively (Figure 2B). The interplay between REMs and RLKs deserves to be further investigated particularly in view of the recent emerging role of RLK signaling in PD physiology (Grison et al., 2019; Cheval et al., 2020)

Considering that phosphorylation is at the center of the interaction events recorded for REMs, it seems crucial to develop phospho-dependent interactomic screens for REMs in order to understand their function. This type of screen should importantly be performed under different stress conditions and in different tissues as REMs' interactions with its cognate partners appear to be highly determined by plant development and stress.

3. The PM's complexity

Important advancements have recently been established in the deciphering of the PManchoring properties of REMs (Perraki et al., 2012; Gronnier et al., 2017; Martinez et al., 2018; Legrand et al., 2019). In as much as REMs are dependent of their C-terminal region or REM-CA for their anchoring to the PM, more must be developed in the understanding of the exact biophysical properties that govern this anchoring. Critically, decrypting the determinants of the PM-nanodomain association of REMs is a top priority, as it has already been shown to be a determining factor of REM function (Gronnier et al., 2017; Fu et al., 2018; Ke et al., 2020). In particular, affiliating the presence of Group 1 REMs in PM-nanodomains with their observed effect on the accumulation of callose at PD pit-fields (Gronnier et al., 2017; Perraki et al., 2018) raises a critical question: how does a protein, present in the PM inner-leaflet, influence enzymatic activities (*i.e.* callose synthesis/degradation) occurring in the cell wall or, at best in the PM outer-leaflet?

Several hypotheses may be explored to answer this question: 1) Can Group 1 REMs or their interaction partners regulate callose synthases or other partners of the callose synthesis complex present at PD pit-fields in order to stimulate callose deposition? 2) Can Group 1 REMs or their interaction partners regulate the activity or localization of B-1,3-glucanases? This latter question can be a particularly interesting hypothesis to investigate considering the different subcellular and extracellular localizations of B-1,3-glucanases (for review: (Zavaliev et al., 2011). Group 1 REMs could potentially regulate the extracellular secretion of B-1,3-glucanases as it was reported in Zavaliev et al. 2013 in response to salicylic acid treatments. Yet some ß-1,3glucanases have been reported to be glycosylphosphatidylinositol (GPI)-anchored proteins within the PM outer-leaflet associated with PD (Levy et al., 2007). The enrichment of GPIanchored proteins in the DRM biochemical fraction (for review on plant GPI-anchored proteins: Yeats et al., 2018) licenses the hypothesis that there could be trans-bilayer coupling between the inner-leaflet PM-domains containing REMs and the outer-leaflet PM-domains containing GPI-anchored B-1,3-glucanases. In this context, the concept of trans-bilayer coupling refers to the presence of each component in a common PM-domain that spans from one PM-leaflet to the other. There could therefore be interaction *in trans* between inner- and outer-leaflet proteins via the interdigitation of saturated and/or very-long-chain acyl chains of the lipids that are present in these PM-domains. This functional mechanism has been demonstrated in animal cells by showing the necessity of long-chain PS species with at least one long saturated chain in order to register a GPI-anchored protein to a lipid-protein complex that virtually anchored the GPI-anchored protein to the actin cytoskeleton (Raghupathy et al., 2015; Skotland and Sandvig, 2019). The registration hypothesis is supported by the altered StREM1.3 PM-dynamics under a phosphorylated state (Perraki et al., 2018), which could be due to the regulation of StREM1.3's PM-lateral-segregation *via* the cortical cytoskeleton. This change in REM PM-patterning could affect the PM-dynamics of PM-outer-leaflet-associated proteins *e.g.* GPI-anchored β -1,3-glucanases. Ultimately, elucidating not only the protein-protein- but also the protein-lipid-interactions necessary for REMs' PM-nanodomain-association constitutes a great challenge that will participate in the unfurling of the complex and dynamic molecular mechanisms that govern REMs particular role(s) at the PM.

New methodologies must be developed in order to study the PM-based events and interactions: these methodologies should include: 1) cutting-edge proteomic and lipidomic techniques (e.g. identifying lipids in close interaction with proteins) including phosphoproteomics and the study of lipidated proteins (myristoylation, palmitoylation, isoprenylation, and GPI-anchoring; reviewed in Ray et al., 2017); 2) biophysical tools to study lipid-protein interactions via surface plasmon resonance (e.g. Lenarčič et al., 2017), liposome binding assays and lipid blotting assays (e.g. Perraki et al., 2012), solid-state NMR, cryoelectron microscopy, atomic force microscopy, computational modeling, Langmuir monolayer tensiometry, and Fourier transform infrared spectrometry (e.g. Gronnier et al., 2017; methods reviewed in Zhou & Hancock 2018); 3) to obtain the structure of these lipid-protein complexes for example by solid-stade NMR; 4) generation of mutants impaired in PM-domain-association and the use of super-resolution imaging to visualize the in vivo segregation and dynamics of PM-components, which should be a focus in order to better understand the molecular interplay occurring at the protein and lipid clustering sites that are PM-domains. These important tools can considerably be assisted by in silico modeling such as molecular dynamics simulations (for review: Ulmschneider & Ulmschneider 2018). Overall, these techniques and methods have been used and described in numerous research articles and reviews that focus on understanding the function of PM-based proteins (Zhou et al., 2015; Zhou et al., 2017; Gronnier et al., 2018; Maxwell et al., 2018).

ACKNOWLEDGEMENTS

This work was sustained by CNRS (Centre National de la Recherche Scientifique) and the University of Bordeaux. This work has benefited from the facilities of the Bordeaux Metabolome/lipidome Facility-MetaboHUB (ANR-11-INBS-0010). AFD, AL, JG and PG were supported by grants from the French Ministère de l'Enseignement Supérieur et de la Recherche (MESR). JG was supported by EMBO LTF 438-2018 and ERC-2017-COG IMMUNO-PEPTALK ID: 773153. SM, BH, MB and VG are supported by ANR project Phospho-REM domain (ANR 2019). SG, SM, VG are supported by ANR project Potymove The IPS2 (MB) benefits from the support of the LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-SPS). BH was supported by the CNRS Momentum.

AUTHOR CONTRIBUTIONS

PG and JG developed the rationale of the review and wrote the text with the help of all the co-authors. AL and BH did the Figure 1 and wrote the corresponding text. PG did the Figure 2. AP did the Figures 3 and 4. MDJ and MB gathered the data for the Supplementary Table 1 and wrote the corresponding text. SM and VG supervised the writing and figures. All authors read and corrected the manuscript.

Plant species	Group of REMs	Methods / Stimuli	Biological effect(s)	Putative associated function(s)	References
Arabidopsis thaliana	AtREM1.2	Protein-protein interaction (<i>in vivo</i> co- purification)	Interaction with RPM1- INTERACTING PROTEIN 4 (RIN4)	Bacterial immunity	(Mackey et al., 2002; Liu et al., 2009a; Lee et al., 2015)
	AtREM1.2	Proteomics and Phosphoproteomics / AvrRPM1	Increased protein amount and phosphorylation	Bacterial immunity	(Widjaja et al., 2009)
	AtREM1.2 AtREM1.3	Protein-protein interaction (<i>in vivo</i> Proximity-dependent biotin identification BioID)	Interaction with HopF2b ^{PtODC3000}	Bacterial immunity	(Khan et al., 2018)
	AtREM1.2 AtREM1.3	Proteomics	Presence in extracellular vesicles	Immunity	(Rutter and Innes, 2017)
	AtREM1.3	Phosphoproteomics / Flagellin (flg22)	Increased phosphorylation	Bacterial immunity	(Benschop et al., 2007)
	AtREM1.3	Transcriptomics / Water deficit, cold, mannitol, salt stress	Transcript up- regulation	Abiotic stress	(Reymond et al., 2000; Bray, 2002; Kreps et al., 2002)
	AtREM1.3	Transcriptomics / auxin	Transcript up- regulation	Development	(Alliotte et al., 1989)
	AtREM1.3	Protein-protein interaction (yeast two- hybrid)	Interaction with Arabidopsis response- regulator 4 (ARR4)	Cytokinin response	(Yamada et al., 1998)
	AtREM1.3	Affinity-based chromatography enrichment with immobilized ergosterol/ ergosterol	Interaction with the fungal MAMP ergosterol	Fungal immunity	(Khoza et al., 2019)
	AtREM4.1 AtREM4.2	Transcriptomics / Drought, osmotic, salt stress and ABA treatment	Transcript up-regulation	Abiotic stress	(Reymond et al., 2000; Bray, 2002; Son et al., 2014).
	AtREM6.7	Transcriptomics / Overexpression of DAYSLEEPER	Transcript up-regulation	Flowering, PD connectivity in the meristematic zones	(Bundock and Hooykaas, 2005)
Curcumis sativus	Group 1 and 4 CsREMs	Quantitative trait loci and transcriptomics / Podosphaera fusca	Transcript up-regulation associated with increased fungal resistance	Fungal immunity	(Xu et al., 2017)
Glycine max	GmREM4	Transcriptomics / Drought treatment	Transcript up-regulation	Abiotic stress, circadian rhythm	(Marcolino- Gomes et al., 2014)

Table 1: Data mining toward the putative functions of REMORINs

Hordeum	Group 1	Transcriptomics / GA	Transcript	Hormone	(Chen and
vulgare	REM	and ABA treatments	down-regulation	response	An, 2006)
Lotus	Group 1	Transcriptomics /	Transcript	Fungal	(Kistner et
japonicus	REMs	Glomus intraradices	up-regulation	symbiosis	al., 2005)
Oryza sativa	OsREM1.5	Transcriptomics / ABA,	Transcript	Hormone	(Lin et al.,
		BR treatments	up-regulation	response	2003)
	OsREM5.3	Quantitative trait loci	Strongly	Panicle size	(Liu et al.,
	(Long		expressed in		2016)
	Panicle 1		young panicle,		
	LP1)		associated with		
			longer panicle		
	OsREM4.1	Transcriptomics /	Transcript	Stress	(Byun et
		overexpression of	up-regulation	adaptation, cold	al., 2015)
		Deschampsia antartica		recovery	
		C-repeat binding factor			
		/dehydration-responsive			
		element binding protein			
		(CBF/DREB), DaCBF7			
	OsREM6.5	drought stress and ABA	Transcript	Stress	(Wu et al.,
		treatment	up-regulation	adaptation,	2006)
	0.000				
Quercus	QrREM4.1	Transcriptomics /	Transcript	Ectomycorrhizal	(Tarkka et
robur		Piloderma croceum	up-regulation	symbiosis	al., 2013)
<u>C</u> - 1	StDEM: 4	Transsistersies /	Turnersint	Destarial	(Vana at
Solanum	STREMa4	Prescriptomics /	I ranscript	Bacterial	(Kong et
tuberosum	StDEM.	Raistonia solanacearum	up-regulation	Immunity	al., 2016)
	SIKEMa4	Transcriptomics/ ABA,	I ranscript	Hormone	(Kong et $(1, 2016)$
Colour	SIDEM1 2	SA, Meja	up-regulation	Enhanced	al., 2010)
booparsioum	SIKEWI1.2	overexpressing CaPIE1	i ranscript	resistance to	(Seong et
iycopersicum		overexpressing Car II'I	up-regulation	cold stress	al., 2007).
				014 54 655	
	SIREM1.2	Proteomics / Verticillium	Increased	Fungal	(Hu et al.,
		dahlia	protein amount	immunity	2019)
			during	5	,
			incompatible		
			interaction		
Triticum	12	Transcriptomics / Cold	Transcript up-	Cold adaptation	(Badawi et
aestivum	members of	acclimation	regulation	1	al., 2019)
	TaREM		during early (7		
			TaREMs) and		
			late (3 TaREMs)		
			cold response,		
			Transcript		
			down-regulation		
			(2 TaREM)		
Zea mays	ZmREM4.1	Transcriptomics / cold	Transcript	Cold sensitivity	(Bilska-Kos
			down-regulation		et al.,
					2016).
	ZmREM4.1	Transcripts localized	Transcript	PD connectivity	(Bilska-Kos
		near PD linking cells	localization		et al., 2016)
		from the Kranz			
		mesophyll and the			
		bundle sheath			



Figure 1



B Group 2 REM in Medicago truncatula : Wild Type MtREM2.2 mutant



C Group 4 REM in Oryza sativa :



Figure 2



Figure 3



--> The PK interacts with REM

→ The PK and REM function together in the biological context --→ The PK and REM function in the same biological context but the link of PK/REM in the function is not yet demonstrated

Figure 4

FIGURE LEGENDS

Figure 1: StREM1.3-enriched PM nanodomains based on structural analysis. (A) Sequence features of all REMs with Intrinsically Disordered Region (IDR) domain at the Nterminal highly variable in length, and a REM-C domain composed of a coiled-coil region (CC) and the REM-C-terminal anchor (REM-CA); (B) StREM1.3 (blue) clusters at the PM's inner leaflet into nanodomains (red rectangle) enriched in phytosterols and phosphoinositides, the most common being sitosterol and PI4P (Palta et al., 1993; Furt et al., 2010; Furt et al., 2011; Gronnier et al., 2017; Legrand et al., 2019). The overall REM orientation is likely to be perpendicular to the membrane plane to minimize steric hindrance, though the precise angle distribution is unknown. Lipids herein are more ordered than the bulk of the PM and the membrane therefore is slightly thicker even though the PM's complex composition may greatly attenuate this tendency (Gronnier et al., 2018, Legrand et al., 2019); (C) StREM1.3 forms homo-trimers through a coiled-coil domain (Martinez et al., 2018) bundling together three REM C-terminal membrane anchors (REM-CA) (Gronnier et al., 2017) and three intrinsicallydisordered regions (IDR) bearing phosphorylation sites (Perraki et al., 2018). REM-CA binds to PI4P through electrostatic interactions involving, notably, positively charged amino-acids K192 and K193, as proposed in the right panel by molecular dynamics (Gronnier et al., 2017). The most C-terminal part (region 2) of REM-CA is embedded inside the inner leaflet, as supported by solid-state NMR and molecular dynamics (Gronnier et al., 2017). Nanodomainassociated PIPs being enriched in saturated acyl chains (Furt et al., 2010), sitosterol is also recruited. The left panel shows hydrophobic contacts between the three L155 residues (red), which are critical to the coiled-coil's structure, supporting membrane-association (Martinez et al. 2019). Membrane binding is accompanied by an enrichment in PI4P and sitosterol in the vicinity of the binding site (Legrand et al., 2019). A relative impoverishment in such lipids in the surrounding bulk membrane would therefore be a logical consequence. Note here that only REM molecules are presented, and the other PM proteins are omitted.

Figure 2. Proposed model for REM-mediated signaling. (A). Group 1 REM in *Nicotiana benthamiana*. Proposed model for the molecular mechanisms involved in the StREM1.3-dependent limiting of PVX cell-to-cell movement according to the data from (Raffaele et al. 2009; Perraki et al. 2012; Gronnier et al. 2017; Perraki et al. 2018). Perception of PVX leads to the production of a calcium burst that activates a group 2 calcium-dependent protein kinase (here CPK3), which in turns phosphorylates group 1 REMs. Group 1 REMs' phosphorylated

state increases its PM-mobility and interacts with phospho-REM Interacting Proteins (RIP). These interactions cause an increase in callose deposition at the plasmodesmata; (B) Group 2 REM in Medicago truncatula. Constitutively-expressed FLOT4 forms a primary PM-scaffold that is unable to recruit LYK3 in the absence of SYMREM1 (MtREM2.2). Nod factor (NF) perception by NFP and LYK3 PM-receptors triggers the activation of a symbiosis-specific signaling cascade that leads to the expression of SYMREM1. Due to its ability to directly bind LYK3, SYMREM1 actively recruits the receptor into the FLOT4 domain. In symrem1 mutants, LYK3 is destabilized and endocytosed upon rhizobial inoculation. Illustrations adapted from Liang et al. 2018; (C) Group 4 REM in Oryza sativa. OsREM4.1 protein is PM-localized in association with OsBRI1-OsSERK1 complex. Upon elevated ABA levels, the ABA-responsive transcription factor OsbZIP23 is activated and upregulates OsREM4.1 expression. OsREM4.1 protein interacts with OsSERK1 and interferes with OsBRI1-OsSERK1 active complex formation, repressing therefore BR signaling initiation. Increased BR levels, causes the binding of BR to the extracellular domain of OsBRI1 and the activation of OsBRI1 kinase (BKI1) to phosphorylate OsREM4.1. The phosphorylated OsREM4.1, has lower binding affinity to OsSERK1, and therefore the OsREM4.1- OsSERK1 complex is dissociated. Therefore, OsSERK1 is able to interact with OsBRI1 to form the OsBRI1- OsSERK1 receptor kinase complex, and activate BR signaling. OsREM4.1 function is similar to BKI1, which upon BR binding to BRI1, is phosphorylated and released from the PM, allowing the BRI1/SERK1 complex to form. Illustrations adapted from Gui et al. 2016.

Figure 3. Phosphorylation of Arabidopsis REMs occurs mostly at putative intrinsically disordered regions at its N-terminus. Graphs show the intrinsic disorder probability on the Arabidopsis REM proteins that were found to be phosphorylated up to date by in vivo studies. In vivo phosphorylation site positions on REM sequences are indicated by asterisk. Intrinsic disorder predictions were calculated using the PrDOS-Protein disorder prediction server (http://prdos.hgc.jp/cgi-bin/top.cgi). Coiled-coil predictions were calculated using the COILS program (https://embnet.vital-it.ch/software/COILS_form.html). REM-CA prediction was estimated based on homology with the REM-CA protein from StREM1.3.

Figure 4. REM proteins are involved in independent signaling pathways and are phosphorylated by different kinases.

Schematic representation of all the known interactions between REM proteins from groups 1, 2, 4 and protein kinases, that were described in plant immunity, symbiosis and hormonal

signaling. Dotted lined arrow (i.e. Functional link to be determined) signifies that the given REM and kinases were identified as being involved in the given biological context yet direct experimental data has yet to be established. See text for details.

SUPPLEMENTAL INFORMATION

Table 1: Compilation of phosphoproteomic datasets on REMs of different plant species. (Nühse et al., 2004; Benschop et al., 2007; Niittylä et al., 2007; Nühse et al., 2007; Sugiyama et al., 2008; Whiteman et al., 2008; Li et al., 2009; Reiland et al., 2009; Chen et al., 2010; Grimsrud et al., 2010; Mehlmer et al., 2010; Nakagami et al., 2010; Reiland et al., 2011; Engelsberger and Schulze, 2012; Lan et al., 2012; Mayank et al., 2012; Tóth et al., 2012; Yang et al., 2013; Son et al., 2014; Wang et al., 2014; Choudhary et al., 2015; Hou et al., 2015; Lu et al., 2015; Marcon et al., 2015; Roitinger et al., 2015; Ye et al., 2015; Gui et al., 2016; Kohorn et al., 2016; Marondedze et al., 2016; Mattei et al., 2016; Nukarinen et al., 2016; Qiu et al., 2016; Vu et al., 2016; Xiang et al., 2016; Qiu et al., 2017; Nikonorova et al., 2018; Perraki et al., 2018; Song et al., 2018; Wang et al., 2018; Albers et al., 2019; Gupta et al., 2019; Cai et al., 2020; Mergner et al., 2020; Wang et al., 2020)

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Remorin Organism Group Isoform		lsoform	Functions	References	
At	1	2	Slows TuMV by closing PD	Cheng 2019	
•	•	•	Higher expression vs bacterial elicitor AvrRPM1	Widjadja 2009	
			Interacts with RIN4 (PAMP-immunity trigger vs P. syringae) and ERD4	Mackley 2002, Liu 2009, Lee 2015	
•	•	•	In EVs during infection, with AtREM1.3 and RIN4	Meyer 2009, Micali 2011	
•	•	•	SA-induced PD closure depends on PM targeting and organisation	Huang 2019	
•	•	•	Binds OGA, OGA-induced phsphorylation	Reymond 1996, Kohorn 2016, Mattei 2016	
•	•	•	Cytoskeleton-dependent PM organisation	Szymanski 2015	
· ·	•	3	More phosphorylated vs bacterial elicitor flg22	Benschop 2007	
•	•	•	In EVs during infection, with AtREM1.2 and RIN4	Meyer 2009, Micali 2011	
•	•	•	Binds ergosterol (toxic for plants)	Khoza 2019	
	•		Expressed in response to water, cold and osmotic stresses	Reymond 2000, Bray 2002 Kreps 2022	
•	•	•	Auxin-induced overexpression in dividing cells	Alliotte 1989	
•	•	•	Interacts with ARR4	Verma 2015	
•	•	•	Binds OGA, OGA-induced phsphorylation	Reymond 1996, Kohorn 2016, Mattei 2016	
•	•	•	Cytoskeleton-dependent PM organisation	Szymanski 2015	
•	4	1 2	Higher susceptibility vs BCT and BSCT	Son 2014	
•	•	1	Higher expression under drought- and osmotic-		
•		2	stresses	Deurs and 2000 Bray 2002 Car 2014	
•		1	Higher expression under ARA treatment	Reymond 2000, Bray 2002, Son 2014	
•	•	2	Higher expression under ABA treatment		
Cs	1		Higher expression vs Powdeny mildew	Vu and Vu 2017	
•	4				
Gm	4		Circadian-dependent transcription under drought stress	Marcolino-Gomes 2014	
Hv	1		Lower expression under ABA or GA treatment	Chen and an 2006	
Lj	2 SY	MREM1	More nodules/root	Toth 2012	
Mi	1		Higher transcription in response to abiotic stress Higher transcription ABA, SA or BR treatment	Checker and Khurana 2013	
Mt	2	2	Expressed in root nodules, response to Nod factors	Lefebvre 2010	
•	•	•	Scaffold for nodulation-related receptor-like kinases NFP, DMI2, LYK3 and flotillin 4	Liang 2018	
Nb	1	1 2	Slows RSV by closing PD	Fu 2018	
Nt	1	2	Speeds TumMV	Sasaki 2018	
Os	1	5	Higher expression under ABA or BA treatment	Lin 2003	
•	4	1	Upkeep of ABA/BR homeostat	Gui 2016	
	•		Higher expression induced by cold-resistance factor DaCBF7	Byun 2015	
•	5	3	Size of panicles	Liu 2016	
•	6	5	Higher expression under drought	Wu 2006	
•	•	•	Higher expression under ABA treatment		
	•	6	Grain Setting defect 1 (GSD1) mutant has plant-wide impaired starch and sugar metabolism	Gui 2014, 2015	
Pe	6	5	Higher expression and resistance to salt stress		
•	•	•	Higher H+-atpase activity	Zhang 2020	
•	•	•	Interacts with RIN4		
Qr	4	1	More expressed with Piloderma croceum	Tarkka 2013	
Si	1		Higher susceptibility vs Botrytis cinerea	Cai 2020	

Remorin Organism Group Isoform		ו Isoform	Functions	References	
•			Higher ROS accumulation Cell death trigger	Cai 2020	
•	•		Lower susceptibility vs Verticillium dahlia	Hu 2019	
•	6		Higher expression under salt, osmotic and cold stress		
•			Higher expression under ABA treatment		
			Overexpression in A. thaliana increased germination rate, seedling biomass and drought resistance	Yue 2016	
•	•		Overexpression in A. thaliana increased ABA sensitivity		
SI	1	2	Interacts with SAM1, ACS2 and ACO1, promotes fruit ripening	Cai 2018	
•	•	•	Favours ethylene and lycopene biosynthesis		
•		•	Higher transcription when CaPIF1 (resistance to bacterial infections and cold) is overexpressed	Seong 2007, Oh 2005	
St	1	3	Slows PVX by closing PD	Perraki 2014, Raffaele, 2009	
•	•	•	Interacts with TGBp1 of PVX	Raffaele 2009	
•		•	Promotes callose deposit at PD, even in absence of virus	Perraki 2014, 2018	
•	•	•	IDP is phosphorylated by AtCPK3	Perraki 2018	
•		•	Enriched in Pms near haustoria (fungal protrusions into plant cells)	Lu and Schomack 2012, Bozkurt 2014	
•	•	•	May localise at PD	Gronnier 2017, Raffaele 2009	
			Binds OGA, OGA-induced phsphorylation	Reymond 1996, Kohorn 2016, Mattei 2016	
•	1	4	Higher expression vs Ralstonia solanacerum	Kong 2016	
		•	Higher transcription under ABA, SA or MeJa treatment	Kong 2016	
Та			Higher expression under cold stress	Badawi 2019	
Zm	1	3	Lower susceptibility vs Puccinia polyspora	Wang 2019	
•	4	1	Higher expression under cold stress	Bilska-Kos 2016	
•	6	3	Genetic correlation with resistance vs Steosphaeria turcica	Jamann 2015	

List of known biological involvement of remorins from (Gouguet et al., 2020), with colour code from Figure 29. Abbreviations: *At, Arabidopsis thaliana* (thale cress); *Cs, Cucumis sativus* (cucumber); *Gm, Glycine max* (soybean); *Hv, Hordeum vulgare* (barley); *Lj, Lotus japonicas*; *Mi, Morus indica* (mulberry); *Mt, Medicago truncatula* (barrel clover); *Nb, Nicotiana benthamiana* (australian tobacco); *Nt, Nicotiana tabacum* (common tobacco); *Os, Oryza sativa* (rice); *Pe, Populus euphratica* (poplar); *Qr, Quercus robur* (oak); *SI, Solanum lycopersicum* (tomato); *St, Solanum tuberosum* (potato); Ta, Triticum aestivum (wheat); *Zm, Zea mays* (corn).

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