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Mechanisms governing subcompartmentalization of biological membranes

Julien Gronnier[2], Anthony Legrand[1,3], Antoine Loquet[3], Birgit Habenstein[3], Véronique Germain[1] and Sébastien Mongrand[1]

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 inter-organelle 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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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 ever-changing 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

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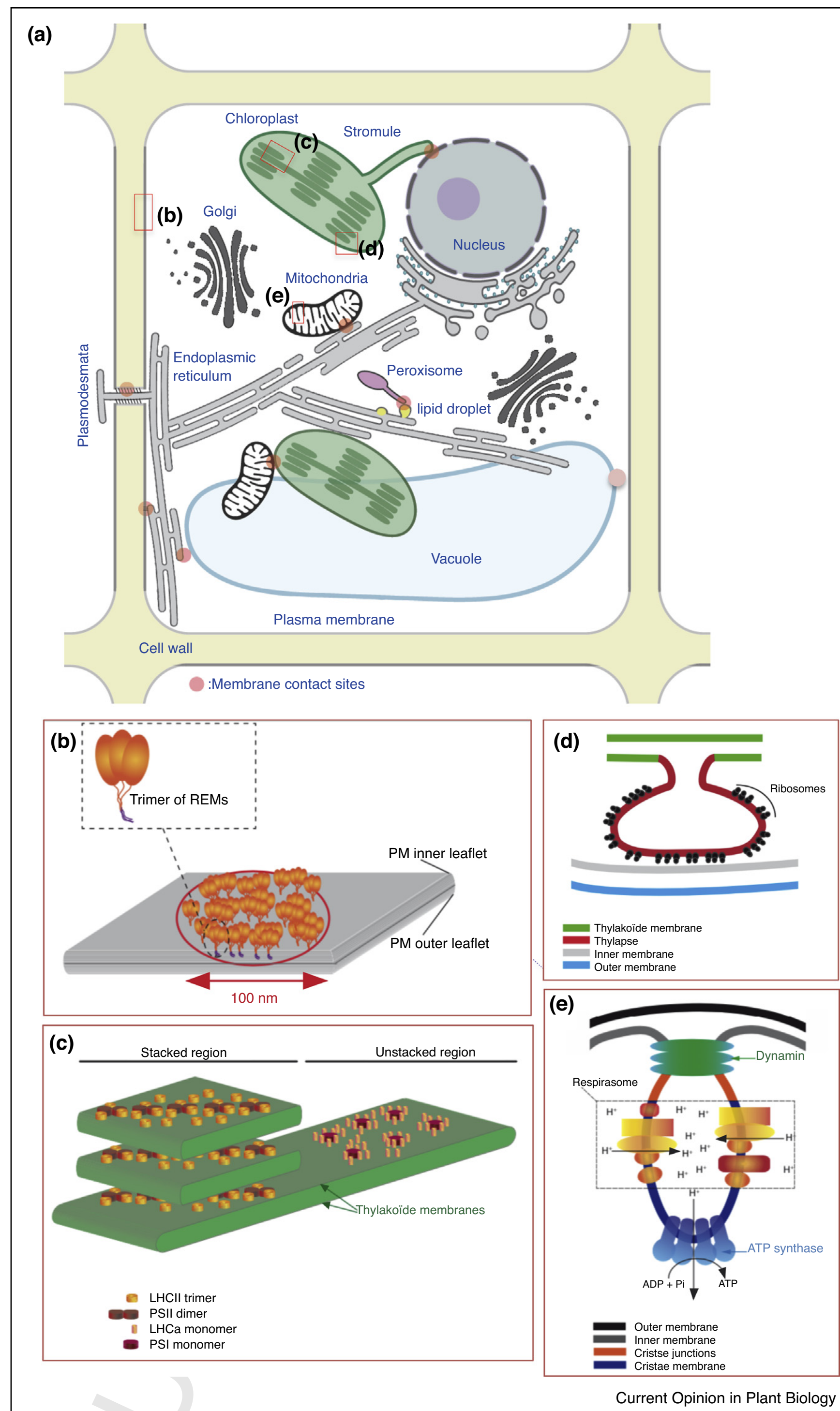
Figure 1



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)**.

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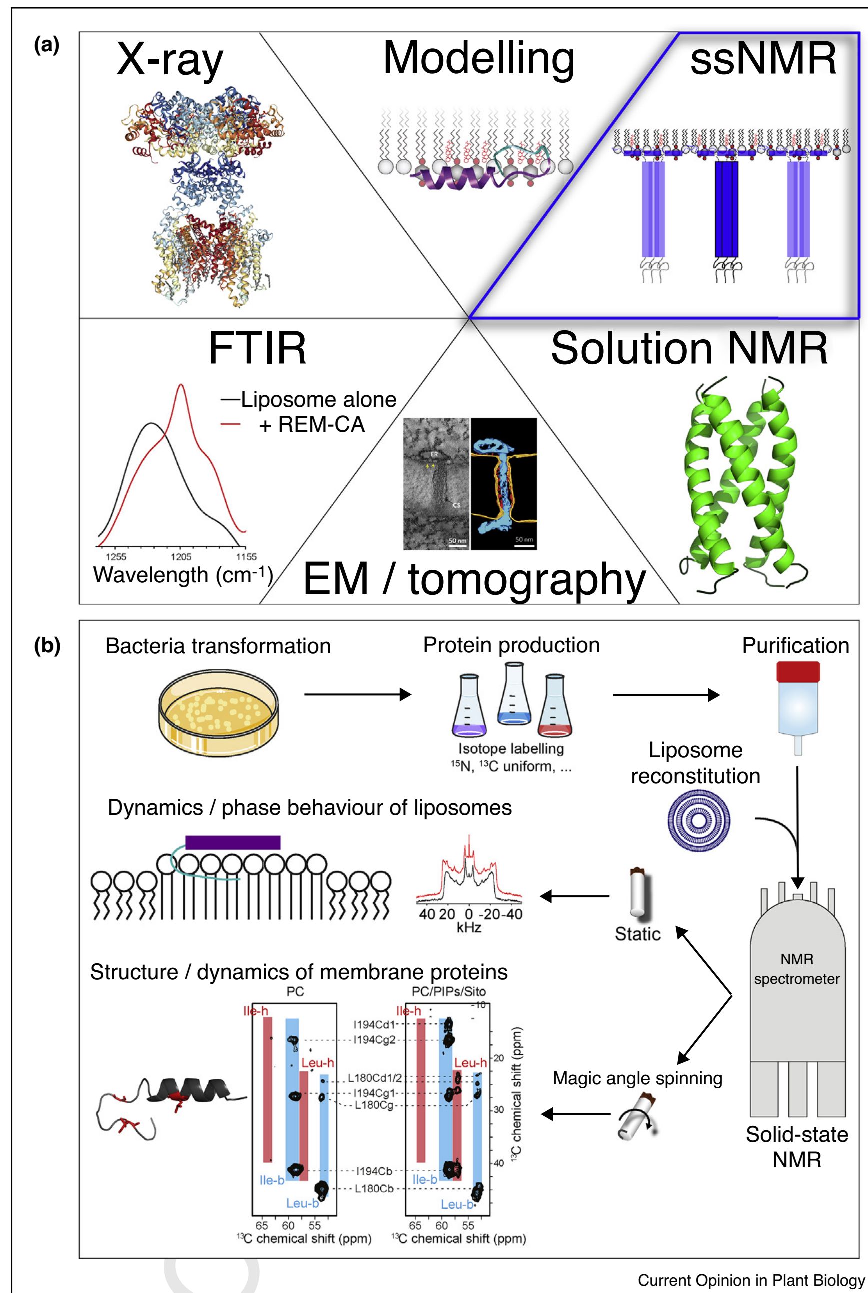
Figure 2



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-^{13}C-glycerol, 2-^{13}C-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 ^{2}H (unpublished typical data) and ^{31}P (not shown) ssNMR to decipher the dynamics and phase behavior of the membranes of interest comprising deuterated lipids.

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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 S*olanaceae* [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 REMORINs 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]. IQD13 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 DISORDERING1-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)

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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-Δ3-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••]. 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 *cls* 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

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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, mitochondria-associated 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 protein–lipid 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 membrane-focused ssNMR, mainly recorded on ^{2}H and ^{31}P nuclei [95,96]. The quadrupolar ^{2}H 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), ^{2}H 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 ^{31}P 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

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415 membrane-association [100••,101,102]. Since 2002, when the
416 first structure of a microcrystalline protein has been solved by
417 MAS ssNMR [103], the technology has proven very powerful
418 to elucidate protein assemblies such as the first amyloid
419 protein structure [104], bacterial filaments [105,106] and
420 protein–membrane complexes [107•]. A considerable knowl-
421 edge has already been derived from ssNMR on protein–lipid,
422 lipid–lipid interactions and membrane dynamics and func-
423 tioning [108–110,96,102]. Most recent technological devel-
424 opments achieving ultra-fast MAS frequencies (≥100 kHz)
425 MAS ssNMR allow for observing proton nuclei in protonated
426 protein samples (~500 μg) and should facilitate ssNMR to
427 serve as a common tool for structural biology on membrane/
428 protein related questions. Because of its striking technologi-
429 cal evolution, MAS ssNMR has recently been applied in few
430 cases to shed light on protein structures, dynamics and
431 protein–lipid interactions promoted by lipid-dependent
432 membrane features [100••]. Membrane domain formation
433 in plants, relying on the plant protein and lipid interplay (see
434 below the example of REMORIN in PM [16••,44]) remains a
435 field to explore by MAS ssNMR.

436 ## Conclusions

437 Virtually all membranes are organized in functional
438 domains that coordinate cell functions. Recent break-
439 through in biochemistry, biophysic and microscopy
440 approaches allow nowadays the study of the mechanisms
441 regulating the formation of membrane domains, particu-
442 larly the interplay between lipids and proteins. The next
443 decade will likely open a vast area of research to under-
444 stand the roles of membrane organization during plant
445 Q5 development and adaptation.

446 ## Conflict of interest statement

447 Nothing declared.

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- • of special interest
- •• of outstanding interest

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Combining super resolution microscopy, modelling and biophysical 506
experiments, the authors decipher a molecular mechanism regulating 507
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