**Short title**: Organized smooth endoplasmic reticulum in plants

**IntEResting structures: formation and applications of organised smooth endoplasmic reticulum in plant cells**

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One-sentence summary: Novel findings in organised smooth endoplasmic reticulum uncover mechanisms of formation and potential applications for this structure.

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**Abstract**

The endoplasmic reticulum (ER) is an organelle with remarkable plasticity, capable of rapidly changing its structure to accommodate different functions based on intra- and extracellular cues. One of the ER structures observed in plants is known as ‘organised smooth endoplasmic reticulum’ (OSER), consisting of symmetrically stacked ER membrane arrays. In plants, these structures were first described in certain specialised tissues, e.g. the sieve elements of the phloem, and more recently in transgenic plants overexpressing ER membrane resident proteins. To date, much of the investigation of OSER focused on yeast and animal cells but research into plant OSER has started to grow. In this update, we give a succinct overview of research into the OSER phenomenon in plant cells with case studies highlighting both native and synthetic occurrences of OSER. We also assess the primary driving forces that trigger the formation of OSER, collating evidence from the literature to compare two competing theories for the origin of OSER: that OSER formation is initiated by oligomerizing protein accumulation in the ER membrane or that OSER is the result of ER membrane proliferation. This has long been a source of controversy in the field and here we suggest a way to integrate arguments from both sides into a single unifying theory. Finally, we discuss the potential biotechnological uses of OSER as a tool for the nascent plant synthetic biology field with possible applications as a synthetic microdomain for metabolic engineering and as an extensive membrane surface for synthetic chemistry or protein accumulation.

**Introduction**

The endoplasmic reticulum (ER) is a remarkably dynamic organelle which undergoes significant morphological changes over time. Some changes are on the scale of hours or days (e.g., during development, the proportion of cisternae and tubules changes), but local rearrangement of connections, tubule growth or shrinkage, and interconversion between cisternae and tubules occur continuously in a matter of seconds (Sparkes et al., 2011; Pain and Kriechbaumer, 2020). The ER also responds to endogenous and exogenous cues that can induce these changes (Almsherqi et al., 2009; Stefano and Brandizzi, 2018). These ultrastructural rearrangements are primarily facilitated by the actin cytoskeleton in plants (Sparkes et al., 2009; Griffing et al., 2014), with microtubules playing a minor additional role (Hamada et al., 2014; Hawes et al., 2015).
The ER is a single, spatially continuous structure, which has the largest membrane surface of all organelles. In plant cells, most of the ER is spread out around the periphery of the cell, between the tonoplast and the plasma membrane (Stefano and Brandizzi, 2018; Kriechbaumer and Brandizzi, 2020). It can be divided into the nuclear envelope and the peripheral ER, and these can be further subdivided into several morphologically and functionally different regions, most notably the smooth and rough endoplasmic reticulum (Borgese et al., 2006; Sparkes et al., 2011). This distinction is based on the extent that ribosomes are docked on the ER via the Sec61 translocon (Kalies et al., 1994).

The ER is also in connection with most other organelles, serving as an important organisational unit for the entire cellular architecture (Stefano et al., 2014), and continuously exchanging materials with many of these organelles (Stefano and Brandizzi, 2018). The ER has a wide range of other functions and plays a central role in lipid and protein biosynthesis, co- and post-translational modifications, quality control, and the secretory pathway (Borgese et al., 2006; Kriechbaumer and Brandizzi, 2020). The large surface area of the ER is also an important site for spatial organization of metabolism with a number of metabolons apparent on its cytosolic surface (Obata, 2019; Smirnoff, 2019). These different functions are often associated with the different subdomains of the ER, and this can cause specialised cells to have distinct ER morphologies: for example, cells which are expressing a high load of secreted proteins tend to have more cisternae and fewer tubules (Shibata et al., 2006; Sparkes et al., 2011).

Mechanistically, the shape of ER membranes is influenced by integral membrane proteins. The best characterised group of proteins that influence ER architecture are the reticulons, which increase local membrane curvature via their wedge-shaped membrane domains, promoting the formation of tubules (Voeltz et al., 2006; Shibata et al., 2008; Sparkes et al., 2010). Other important membrane shaping factors also exist, such as the atlastin RHD3, which is involved in membrane fusion and three-way junction generation in the ER network (Chen et al., 2011; Ueda et al., 2016) and the Lunapark protein family, which promotes cisternae formation in plants (Kriechbaumer et al., 2018).

The local ER shape is important for plant development (as deletion of two members of the RHD3 family is lethal (Zhang et al., 2013)), but the direct effect of network architecture on ER function and cellular physiology is not yet clear (Stefano and Brandizzi, 2018).

One specialised morphology of the ER observed in some cells is the arrangement of sheets of smooth ER into tightly stacked arrays. When derived from the nuclear envelope, these stacked cisternae are called karmellae. In the peripheral ER, the stacks are called lamellae, but the arrays can take different forms as well, such as large membrane whorls and crystalloid ER (regular sinusoidal arrays with cubic symmetry) (Snapp et al., 2003; Borgese et al., 2006) (Figure 1). Collectively, these have been referred to as organised smooth endoplasmic reticulum (OSER) (Snapp et al., 2003). These structures were first described using electron microscopy (EM) in a variety of highly specialised animal tissues such as pigment epithelial retina cells (Porter and Yamada, 1960; Tabor and Fisher, 1983) and neurons (Naftolin et al., 1985; Takei et al., 1994), or in reaction to infection by a variety of pathogens (Koestner et al., 1966; Ruebner et al., 1967). While the membrane stacks can be organised in different ways, transition from one phenotype into another (most commonly stacks to whorls) has been observed, and a recent study found that in yeast the endosomal sorting complexes required for transport (ESCRT) machinery is closely involved in this transition (Schafer et al., 2020).

With the introduction of modern tools for recombinant protein expression, additional examples of synthetic OSER have been described in mammalian (Yamamoto et al., 1996; Snapp et al., 2003; Korkhov et al., 2008; Korkhov and Zuber, 2009; Volkova et al., 2012) and yeast...
cells (Federovitch et al., 2008; Emmerstorfer et al., 2015; Schäfer et al., 2020), often as accidental by-products following overexpression of an ER membrane protein that induced drastic changes in ER morphology (Yamamoto et al., 1996; Snapp et al., 2003; Emmerstorfer et al., 2015). Most of these examples utilised similar protein constructs: an oligomerizing enzyme or fluorescent marker (most commonly GFP) genetically fused to the cytoplasmic terminus of an ER-targeted membrane domain (Yamamoto et al., 1996; Snapp et al., 2003; Korkhov et al., 2008; Lingwood et al., 2009; Costantini et al., 2012). There remains significant controversy in the field about the relative importance of the oligomerization and transmembrane domains to trigger OSER formation (Fukuda et al., 2001; Snapp et al., 2003; Lingwood et al., 2009; Schäfer et al., 2020), and the general function of OSER for cellular physiology (Fukuda et al., 2001; Korkhov and Zuber, 2009).

Research into OSER has primarily focused on mammalian and yeast cells, and the frequency and effects of OSER in plants have been understudied until recently. To the best of our knowledge, there has been no previous attempt to collectively describe the observations of plant OSER nor to provide an overarching explanation for the mechanism or significance of OSER formation. Plant cells differ from other eukaryotes in a few key properties with respect to OSER, most notably the positioning of the ER, which is pushed to the edges of the cell by the vacuole, which could impact OSER formation or phenotype. On the other hand, many characteristics of the ER are conserved across the eukaryotes, and valuable lessons can be learned from plant OSER studies that could be applied generally. In this update, we aim to give a comprehensive overview of OSER in plant cells and the current understanding of the triggers and mechanisms involved in OSER formation. Finally, we wish to draw attention to another underappreciated aspect of OSER: its potential as a powerful tool for plant synthetic biology.

Occurrence of natural OSER in plants

Similar to the discoveries in yeast and animal cells, the first observations of stacked ER membranes in plants came with the advent of EM. Organised ER structures were usually observed in specialised tissues, most commonly in the higher plant vasculature, where OSER was described repeatedly in the sieve elements of the phloem across the plant kingdom, in palms (Parthasarathy, 1974), maples (Wooding, 1967), elm (Evert and Deshpande, 1969), water lilies (Oparka and Johnson, 1978), beans (Esau and Gill, 1971), cotton (Thorsch and Esau, 1981), sugarbeet, spinach (Esau and Hoefert, 1980), squash (Esau and Cronshaw, 1968) and several species of the buttercup family (Behnke, 1981) (Figure 1). Smooth ER membrane whorls were observed following dormancy in winter in the primary and secondary parenchyma cells of willows. These whorls were hypothesised to be involved in maintaining a metabolically inactive state for the ER with reduced lipid synthesis (Sennerby-Forsse et al., 1987; Sennerby-Forsse and von Fircks, 1987). A similar function was suggested for the tightly stacked rough ER membranes which play an important role in pollen germination and early tube formation in Nicotiana alata, when the ER is released into its commonly observed dispersed form after the pollen grain is activated by hydration (Cresti et al., 1977; Cresti et al., 1985).

Another highly specialised cell type with OSER is the indentation cells of the trigger hairs of the Venus flytrap. These cells are implicated in the signalling process that springs the trap, either purely as a transmission device, or possibly as the source of the action potential (Benolken and Jacobson, 1970; Volkov et al., 2008). The characteristic ER whorls in the basal part of the cell were suggested to be a candidate for the pressure transducer system turning the mechanical stimuli of the prey into an electric signal (Williams and Mozingo, 1971).

More recently, a novel native OSER structure was discovered in the petals of honeysuckle that hints at an important potential function for OSER. A thick layer of multiply folded ER membranes was
described exclusively around tannin-containing vacuoles, suggesting a functional connection between tannin production/storage and OSER (Qu et al., 2017). Condensed tannins are a group of phenolic-rich polymers belonging to the flavonoid family, primarily serving as defence molecules. The biosynthesis of the flavonoid monomer precursors takes place on the cytosolic surface of the ER (Zhao et al., 2010), but due to their toxicity, polymerisation of tannins does not occur in the cytosol (Hagerman and Butler, 1981). The OSER could serve as a detoxifying structure and shield the proteins of the bulk cytoplasm from precipitation by sequestering any tannin leakage during their transport into the vacuoles. It has also been hypothesised that the increased membrane surface area could increase the carrying capacity of the ER for the enzymes involved in tannin synthesis and transport (Qu et al., 2017). Determining the exact native functions of OSER in plants is impeded by the fact that in none of the examples discussed above were the OSER inducing forces (i.e., the responsible proteins) identified.

Interestingly, to our knowledge, no examples of OSER have been found in algae. This is possibly due to the fact that the amount of smooth ER is quite low in algae (Broadwater and Scott, 1994), and native OSER in plants is usually found in specialised tissues that algae do not possess. However, unicellular yeast is able to form synthetic OSER, thus even if algae do not form native OSER, they might be capable of forming synthetic OSER. To test if algae shares the capacity for OSER formation with higher plants, animals and yeast, overexpression of OSER-inducing recombinant proteins would be informative.

**Formation of synthetic OSER in plants**

Most of our understanding of synthetic OSER in plants has been gained by investigations into the cellular secretory pathway. The first example of recombinant protein expression inducing the formation of OSER in plants was discovered when a β-glucoronidase enzyme was attached to the cytoplasmic domain of the integral membrane M protein of the coronavirus avian infectious bronchitis virus to observe trafficking through the Golgi of tobacco (Gong et al., 1996). Unexpectedly, the heterologous proteins accumulated exclusively in large membranous whorls that were identified as part of the ER and remained stable, without obvious signs of degradation (Figure 1). When only cytoplasmic β-glucoronidase was expressed, no OSER formed, and the authors hypothesised that the head-to-head tetramerization of the β-glucoronidase domains anchored into the ER caused the stacking of the membranes and the formation of the OSER. However, it must be noted that no experiment was carried out where only the integral membrane protein M was expressed, to see if that alone was sufficient for triggering OSER formation (Gong et al., 1996).

Another case of synthetic OSER was observed after overexpression of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (HMGR) in several plant species, including tobacco, Arabidopsis, leek and onion (Leivar et al., 2005; Merret et al., 2007; Ferrero et al., 2015) (Figure 1). HMGR overexpression has been shown previously to induce OSER in yeast and mammalian cells, usually as karmellae around the nucleus (Almsherqi et al., 2009), and the proper folding of both the transmembrane domains and the C-terminal cytosolic facing tetrameric catalytic domain were required for OSER induction (Parrish et al., 1995; Profant et al., 1999). However, only the catalytic domains of HMGR are strongly conserved across the Eukaryotic kingdoms, and the transmembrane domains show considerable variability (Ferrero et al., 2015). Expression of a GFP-tagged HMGR in plants led to a clear OSER phenotype, even when the HGMR catalytic domain was removed (Leivar et al., 2005; Merret et al., 2007; Ferrero et al., 2015). In fact, the expression of the plant HMGR transmembrane domain alone was sufficient for OSER biogenesis, although at a reduced level, suggesting that in plants, the oligomerizing catalytic domain is not essential for OSER formation (Ferrero et al., 2015).
A third notable example of synthetic plant OSER formation came from a mutation screen investigating disruptions to the ER export pathway. A single missense mutation of the modified vacuolar phenotype 1 (MVP1), a vacuolar GDSL lipase-like protein caused it to be retained in the ER, which consequently exhibited large globular aggregate structures under fluorescent microscopy (Agee et al., 2010; Marti et al., 2010; Nakano et al., 2012). When imaged using EM, striated membranous structures and whorls resembling OSER were observed. Different missense and splicing mutations of the same protein caused similar phenotypes (Jancowski et al., 2014). Further investigation into this phenomenon showed that MVP1 is indirectly required for proper protein transport between the ER and the Golgi, and numerous other proteins and Golgi markers were incorrectly retained in the ER when MVP1 was mutated or knocked out (Agee et al., 2010; Nakano et al., 2012; Jancowski et al., 2014). Notably, when MVP1 knock-outs were complemented (by transformation with MVP1 expressed from a strong 35S promoter), the OSER phenotype was abolished, suggesting that the phenotype is caused by the lack of functional MVP1, and not by the activity of the mutated versions in the ER (Marti et al., 2010). Interestingly, MVP1 is not predicted to be a membrane protein or to be oligomerizing, so it is not a usual candidate for inducing OSER formation. One possible explanation is that MVP1 triggers the formation of OSER indirectly, by disrupting ER export, causing the aberrant retention of large quantities of other OSER-inducing membrane proteins in the ER.

Current understanding of the mechanism of OSER formation

Since the first observations of OSER, establishing the key forces triggering this ER rearrangement has been a point of great interest. Early papers emphasised the role of overexpressing proteins that anchor opposing membranes into organised stacks via their cytoplasmic-facing oligomerizing domains (Yamamoto et al., 1996; Snapp et al., 2003; Volkova et al., 2012) (Figure 2A). It was repeatedly shown that when proteins which trigger OSER have their head-to-head dimerizing capacity abolished (e.g., by switching from a dimerizing GFP to a monomeric fluorophore), the ER returns to its native state (Yamamoto et al., 1996; Fukuda et al., 2001; Snapp et al., 2003; Barbante et al., 2008; Federovitch et al., 2008; Schäfer et al., 2020). This was shown to be completely inducible in HeLa cells in an elegant experiment using membrane bound Fv domains: these form oligomers when a membrane permeable dimerizer (AP20187) is added but remain monomeric in absence of it. OSER whorls appeared shortly after the addition of AP20187 and disappeared quickly when removed (Lingwood et al., 2009). The oligomerizing proteins also must be anchored to the ER surface by a transmembrane domain, as cytoplasmic or ER luminal variants do not form OSER (Yamamoto et al., 1996; Almsherqi et al., 2009). The expression level of the OSER inducing recombinant proteins is also a key factor, as low quantities of these proteins failed to consistently produce OSER (Snapp et al., 2003; Federovitch et al., 2008). These facts taken together gave rise to the “threshold model” of OSER formation: when a certain level of OSER-triggering protein abundance is reached in the ER, membrane stacks and whorls start to form as the membrane bound proteins oligomerize across opposing membrane layers, anchoring them together (Snapp et al., 2003). (Figure 2A) Indeed it is possible that highly specialised plant cells with native OSER might have a dedicated protein to generate and maintain OSER, but to our knowledge, no such examples have been described to date. However, this theory has been challenged by the observation that not all head-to-head-dimerizing ER membrane bound proteins cause OSER formation, even when expressed at high levels (Li et al., 2003; Federovitch et al., 2008; Schäfer et al., 2020). Other new findings were even more difficult to reconcile with the “threshold model”: for example, certain ER-anchored monomeric proteins appear to be capable of inducing OSER (Vergères et al., 1993; Korkhov and Zuber, 2009; Ferrero et al., 2015; Schäfer et al., 2020). Notably, the transmembrane domain of HMGR alone was able to trigger the
formation of membrane whorls and stacks (Ferrero et al., 2015). These studies also noted that in response to the expression of these proteins, the ER undergoes significant membrane proliferation, followed by OSER formation (Vergères et al., 1993; Sandig et al., 1999; Korkhov and Zuber, 2009; Ferrero et al., 2015).

The combination of these findings led to the development of an alternate hypothesis of OSER formation: that overexpression of integral membrane proteins in the ER leads to membrane proliferation and this excess membrane is organised into OSER (the “membrane proliferation model”) (Ferrero et al., 2015) (Figure 2B). Indeed, it was suggested that there might be a surveillance system that senses the lipid-protein ratio in membranes and responds to the sudden increase in protein abundance by increasing lipid content to restore the original lipid-protein ratio (Vergères et al., 1993; Machettira et al., 2011). However, this could lead to the dilution of ER shaping proteins (e.g., reticulons, atlastins, or members of the Lunapark family) if the expression of those are not upregulated in tandem. In a study using mouse fibroblasts, OSER-induction did not increase endogenous ER protein levels, also suggesting that this dilution of ER shaping proteins is likely (Biehn et al., 2004). The effects of reducing the relative concentrations of these ER shaping proteins have been observed in Arabidopsis following de-repression of ER membrane biosynthesis, which caused massive membrane proliferation, and transformed most of the ER into sheets (Eastmond et al., 2010). Overexpression of reticulons rescued this phenotype (Tolley et al., 2010). Modelling of curvature stabilising proteins of the ER suggested that low concentrations of factors promoting edge formation would energetically favour the remodelling of the ER to sheets and helicoidal stacks and reduce tubule abundance (Shemesh et al., 2014). These findings were validated by manipulating the expression levels of reticulons and Lunapark proteins in animal cell lines (Shemesh et al., 2014).

Thus, in the case of OSER a sudden increase in membrane lipid content could dilute the native ER shaping proteins and cause alternative membrane organisational forces to take over (Sprocati et al., 2006), and shifting the system into a different part of the phase space (Shemesh et al., 2014). These could include the overexpressed transmembrane domains, which tend to stabilise lamellar membranes (Marsh, 2008), or other natively ER-resident proteins that could facilitate membrane re-organisations. There is some evidence of the latter, as molecular arrays connecting adjacent membranes in OSER structures have been observed when a non-dimerising Calnexin-mCherry construct was overexpressed in HEK293 cells (Korkhov and Zuber, 2009). The effect of oligomerizing proteins of the “threshold model” was also assumed to be a membrane structuring force (Ferrero et al., 2015), but the primary determinant of OSER formation in the “membrane proliferation model” is the capacity of overexpressed proteins to induce the expansion of ER membranes (Figure 2B).

In parallel, evidence has been accumulating of another method for triggering OSER formation. As discussed above, disrupting ER-to-Golgi transport by mutating MVP1 induced OSER in Arabidopsis, even though MVP1 is not predicted to be membrane-bound or oligomerizing (Agee et al., 2010; Marti et al., 2010; Nakano et al., 2012; Jancowski et al., 2014). The inhibition of ER export as a driver for OSER formation has been implicated by other studies as well. HeLa cells treated with 1-Phenyl-2-decanoyl-amino-3-morpholino-1-propanol hydrochloride (PDMP), a potent ER-export inhibiting drug caused rapid formation of OSER membrane structures. This was reversible, as clearance of PDMP quickly led to the recovery of normal ER phenotype. Cells expressing b5-GFP (an ER membrane resident, oligomerizing, OSER inducing protein) were more susceptible to PDMP-induced OSER formation, suggesting an additive effect (Sprocati et al., 2006). Triggering ER stress in yeast using dithiothreitol also produced membrane whorls and stacked cisternae with identical phenotype to the ones produced by dimerizing membrane bound proteins (Schäfer et al., 2020). Together this suggests an alternative pathway for triggering OSER via inducing ER stress and inhibiting export of
proteins (the “export inhibition pathway”). This could lead to an accumulation of proteins in the ER, which if dimerizing and membrane-resident could trigger the formation of OSER, via mechanisms consistent with either the “threshold model” or the “membrane proliferation model”. As ER stress is usually also associated with ER membrane proliferation activated by the unfolded protein response (UPR) (Pichler and Emmerstorfer-Augustin, 2018), an alternative possibility is that the “export inhibition pathway” feeds directly into the “membrane proliferation model” for OSER formation. The fact that when overexpressing OSER inducing proteins, ER-to-Golgi export is not usually disrupted (as seen by the correct localisation of exported markers (Yamamoto et al., 1996)) suggests that ER export inhibition is sufficient, but not necessary for OSER formation.

The involvement of UPR in OSER related membrane proliferation is also supported by experimental evidence in mammalian systems: when b5-GFP was expressed at physiological levels in HeLa cells, it elicited a twofold increase in ER membrane surface area (Maiuolo et al., 2011). This was directly linked to the activating transcription factor 6 (ATF6) protein (Maiuolo et al., 2011) which initiates UPR by serving as a transcription factor after translocation to the Golgi and activation via proteolytic cleavage (Bennett et al., 2019). Further experiments showed that b5-GFP induces ATF6 via sphingolipids binding to the transmembrane domain of ATF6, causing the expression of a range of lipid-related genes, and that b5-GFP does not induce other UPR pathways, or causes proteotoxic stress due to an accumulation of unfolded proteins (Tam et al., 2018). Since this serves as a direct link between OSER and membrane proliferation, ATF6 inhibitors would serve as valuable tools to determine if membrane proliferation is essential for OSER formation. In plants, two UPR-inducing transcription factors (bZIP17 and bZIP28) have been identified which are functional homologues of ATF6. However, it is currently unclear if these can directly induce expression of UPR related genes involved in membrane proliferation (Chakraborty et al., 2016; Wan and Jiang, 2016).

While the three models have previously been discussed as competing, incompatible explanations of OSER formation, as all three have experimental support it seems likely that triggering OSER could be more complex than previously imagined. Instead of a single pathway to induce OSER, there might be redundant systems in play. We propose that it is possible to integrate all three models into a single unifying theory of OSER formation (Figure 2C). This would allow OSER formation to be triggered either by blockage of ER export (the “export inhibition pathway”) or by overexpressing integral membrane proteins that induce membrane proliferation (the “membrane proliferation model”). The expression of oligomerizing membrane proteins is likely a special case of the latter, although it cannot be ruled out that it might also be capable of inducing OSER without membrane proliferation if expressed at a high enough level, just by overwhelming local native ER organisational forces (the “threshold model”). However, further investigation into the redundancy of this system would be required to validate this hypothesis.

**Potential applications of OSER for synthetic biology**

As an inducible membranous structure, OSER has great potential for a variety of applications in the nascent plant synthetic biology field that seeks to exploit plants as a safe, scalable, high-yielding, and relatively cheap system for the production of high-value biochemicals or proteins (Holtz et al., 2015; O’Neill and Kelly, 2017; Reed and Osbourn, 2018; Maeda, 2019; Smirnoff, 2019). Moreover, as Eukaryotes, plants have an innate capacity for the proper folding of proteins with secondary (or more complex) structures and addition of post-translational modifications. There is growing interest in the bioproduction of commercially valuable membrane-bound proteins (Pedrazzini, 2009; Pichler and Emmerstorfer-Augustin, 2018). These are often difficult to express, and since the ratio of proteins-to-lipids in biological membranes could be as high as 1:1, one bottleneck to membrane
protein production was suggested to be the amount of membrane available to accommodate these
(Pichler and Emmerstorfer-Augustin, 2018). Thus, a controllable way to induce membrane
proliferation could alleviate this problem and increase yield. There is some evidence in favour of this
idea: in a study, yeast mutant strains with derepressed ER membrane biosynthesis had massive ER
proliferation. When the researchers tested the yields of eight different membrane proteins, they
found that all of them expressed to a higher level than in their wild-type counterparts with regular
ER phenotypes (Guérfaï et al., 2013).

Attaching soluble proteins to a membrane domain also has been shown to increase stability of these
recombinant proteins, possibly by protecting them from proteolysis (e.g., by reducing the likelihood
of entry to proteolytic vacuoles) (Gong et al., 1996; Barbante et al., 2008; Ferrero et al., 2015;
Smirnoff, 2019), and this can directly lead to higher yields. For example, anchoring the potential
antiviral factor Nef to the ER in tobacco resulted in a significant improvement in stability, and a
roughly 250% increase in protein yield (Barbante et al., 2008). OSER also allows the localisation of
the membrane-bound proteins to a discrete position inside the cells, which could improve the
expression rate of potentially toxic proteins or their reaction products, by sequestering them from
the rest of the cell. Furthermore, ER-anchored membrane proteins that do not enter the Golgi have
reduced plant-specific glycosylation patterns, which may be beneficial for the production of certain
classes of valuable proteins, such as antibodies (Kallolimath and Steinkellner, 2015).

Beyond improving the production of recombinant proteins, the increased surface area and
compartmentalisation of OSER could be also used for metabolic engineering. Since OSER self-
assembles after expression of certain membrane proteins, if other enzymes could be attached to the
OSER-inducing elements, these would accumulate to a high local concentration inside the OSER
membrane whorls or stacks. In turn, this could enable probabilistic metabolic channelling: that is,
the greater probability of transfer of a metabolic intermediate from one enzyme to the subsequent
one in a metabolic pathway, without equilibration with the bulk aqueous solution (Sweetlove and
Fernie, 2018) (Figure 3). When enzymes of a pathway are concentrated, the probability that an
intermediate diffuses away without encountering the subsequent enzyme is reduced (Sweetlove and
Fernie, 2018). This would be expected to increase metabolic flux, protect the intermediate from
competing pathways, and reduce the chance of degradation of an unstable intermediate. Metabolic
channelling also protects the cell from toxic intermediates, and when introducing a novel pathway,
reduces the chance of interaction with the endogenous metabolism of the host (Polka et al., 2016;
Sweetlove and Fernie, 2018; Smirnoff, 2019) (Figure 3). Inducing probabilistic channelling is expected
to be easier than other types of direct metabolic channelling (e.g., molecular tunnelling (Weeks et
al., 2006), swing-arms (Smolle and Lindsay, 2006) or electrostatic guidance (Bulutoglu et al., 2016)),
since these require a significantly deeper understanding of the molecular structures involved and an
ability to construct such structures in a rational fashion. Direct fusion of enzymes is also expected to
be less effective, as these are unlikely to possess inherently complementary structures to enable
metabolic channelling (Smirnoff, 2019), and many of the most attractive target pathways involve
enzymes that are membrane bound (Behrendorff et al., 2019) (e.g., cytochrome P450s of the
terpenoid pathway (O’Neill and Kelly, 2017)).

It has already been speculated that some OSERs might have a similar native function. Indeed, most
native OSER structures are strongly associated with cells that have highly specialised functions
(Almsherqi et al., 2009). For example, the production of high amounts of toxic tannins in
honesuckle petals might occur inside OSERs specifically to protect the proteins of the bulk
cytoplasm from precipitation (Qu et al., 2017). Another example was recently found in
phytopathogen Fusarium graminearum, where the biosynthetic pathway of trichotecene mycotoxins
is localised to an OSER structure, aptly named ‘toxisomes’, possibly to improve pathway efficiency
and spatially sequester the toxic intermediates of the trichotecene pathway (Boenisch et al., 2019).
When OSER formation was abolished (without directly disturbing the production of the enzymes),
trichotecene production was significantly reduced as well (Tang et al., 2018). Thus, OSERs might be
even more amenable to be repurposed for metabolic channelling than initially expected.

Concluding remarks
Initially OSER was thought to be an aberrant ER membrane phenotype. However, our understanding
of OSER has greatly increased in the past few years. Naturally occurring OSER seems likely to have a
functional role in certain specialised plant cell types, whilst with the rapid proliferation of
recombinant protein overexpression experiments focusing on the ER, synthetic OSER might be more
common than previously anticipated. In fact, OSER observations could be vastly underreported, as
they are often assumed to be a membrane artefact (Maggio et al., 2007; Gidda et al., 2009) perhaps
due to lack of awareness of OSER, or because of the absence of a current standardised method of
detecting and classifying OSER. EM is required to confirm the presence of stacked ER membranes
and absence of ribosomes. However, experiments using fluorescent probes and light microscopy
currently dominate experimental cell biology. This presents two problems: light microscopy might
not be sufficient for an accurate description of the phenotype due to the aggregated signal of
fluorophores in the OSER structures. Furthermore, fluorescent protein tagging significantly limits the
number of observed species and tissue types investigated, with experiments heavily focusing on
certain tissues in Arabidopsis and tobacco. In contrast, natural OSER is often associated with highly
specialised cell types, thus this lack of specimen diversity could mean that we are not observing
more OSER structures simply because we are looking in the wrong place.

Light microscopy also cannot reliably distinguish between smooth and rough ER. OSER is assumed to
be exclusively composed of smooth ER stacks, but ER sheets usually accommodate a higher density
of polysomes for protein synthesis on their large surface area (Sparkes et al., 2011). Furthermore, in
Nicotiana alata pollen, rough ER structures similar in both phenotype and theorised function to
OSER have been observed (Cresti et al., 1985). This raises the question if some of these ER
membrane structures might house ribosomes. Ribosome attachment to the ER is mediated by Sec61
translocon, and in HeLa cells, one study found that these were only partially excluded from OSER
structures (Fasana et al., 2010). However, further investigation into this question is required for
plant OSERs either by assessing Sec61 location in a wider scale of structures, or more in-depth
investigation of a range of OSER structures using higher resolution techniques, such as 3D EM
tomography.

The unexpected formation of membrane stacks due to dimerizing GFP attached to ER-resident
membrane domains also highlighted an important lesson from OSER: that using oligomerizing
fluorescent probes for labelling experiments can lead to erroneous conclusions about subcellular
localisation of a protein (Snapp et al., 2003; Costantini et al., 2012). However, the use of monomeric
probes (while highly recommended in general (Denay et al., 2019)) might not be a sufficient solution
either for tagging membrane proteins, since it is now clear that those can also trigger the formation
of OSER (Ferrero et al., 2015). Thus, the appearance of membrane stacks in membrane protein
localisation experiments should always prompt a careful examination of the results with OSER in
mind. These lessons can be applied to unexpected membrane aberrations of other organelles as
well, such as the chloroplast (Machettira et al., 2011).

Importantly, our understanding of OSER formation has grown considerably in recent years.
Elucidating the exact pathways for triggering OSER will be critical for continued control over this
phenotype (see Outstanding Questions). Engineering OSER would enable a wide range of exciting opportunities for applications of this phenomena, both for producing difficult-to-express membrane proteins and for metabolic control, giving a valuable and versatile new tool for the plant synthetic biology community.

**Figure Legends**

Figure 1: Examples of OSER phenotypes in plant cells. A: Native crystalloid ER in phloem parenchyma cells of *Helleborus lividus* (figure from (Behnke, 1981)). B: OSER whorl induced by overexpression of integral membrane M protein of the coronavirus avian infectious bronchitis virus with an oligomerizing 8-glucuronidase attached to the cytoplasmic domain (figure from (Gong et al., 1996)). C&D: Crystalloid, whorl and lamellae OSERs in *Nicotiana benthamiana* leaves agro-infected with HMG1 membrane domain with a cytoplasmically fused dimerizing GFP. (figure from (Ferrero et al., 2015)). Cr: crystalloid ER; La: ER lamellae; W: ER whorl; N: nucleus; n: nucleolus. Scale bars: 2.5 μm (A), 0.5 μm (B-D).

Figure 2: OSER formation models. A: In the “threshold model” (blue), expression of ER membrane anchored head-to-head oligomerizing proteins accumulate to a high concentration inducing OSER by stacking of opposing membranes. B: In the “membrane proliferation model” (orange), the primary driving force of OSER formation is membrane proteins inducing ER proliferation, diluting out the native organising factors (e.g., reticulons). Other organising factors (which could include the head-to-head oligomerizing membrane anchored proteins of the “threshold model”) are responsible for the stacking of membranes in OSER. C) These two models can be integrated together with the third, emerging new model (the “export inhibition pathway” – pink). The “threshold model” is likely a special case of the “membrane proliferation model”, although the possibility of an independent secondary pathway (dashed arrow) cannot be completely excluded. The “export inhibition pathway” has a separate trigger, but it is unclear if it uses the same mechanism as the “membrane proliferation model” or is completely independent.

Figure 3: OSER as a microdomain. A: Schematic representation of a hypothetical metabolic pathway, where enzyme A (yellow circle) produces an intermediate product (black star) which is used by either enzyme B (red square) or enzyme C (blue triangle) as a substrate. B: OSER can be used to increase flux towards enzyme B by increasing local concentrations of enzyme A and enzyme B inside the OSER whorl (purple). This enables probabilistic channelling, increasing the probability of the intermediate encountering enzyme B compared to the cytoplasmic enzyme C. Furthermore, unstable intermediates are more protected from degradation, and toxic intermediates are sequestered away from the bulk cellular environment.

**Acknowledgements**

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Naftolin F, Bruhlmann-Papazyan M, Baetens D, Garcia-Segura LM (1985) Neurons with whorl bodies have increased numbers of synapses. Brain Res 329: 289–293


ADVANCES

- ER stress and blockage of export pathways can trigger organised smooth endoplasmic reticulum (OSER) formation
- ER membrane proliferation is the main driving force that initiates OSER formation
- OSER is likely involved in the sequestration of toxic metabolic intermediates in specialised cells
- Increased membrane surface area allows accumulation of a greater quantity of ER membrane proteins and could improve the stability of recombinantly expressed proteins
OUTSTANDING QUESTIONS

- What is the phylogenetic distribution of OSER across algae and plants?
- Are specific ER proteins involved in generating and maintaining native OSER?
- How can the physiological functions of native OSER be conclusively identified, and what are these functions?
- Are all OSERs composed exclusively of smooth ER?
- Are the different OSER formation mechanisms independent?
- Is it possible to trigger OSER without ER membrane proliferation?
- Can overexpression of membrane shaping proteins block or reduce OSER formation?
- Are the ATF6 plant homologues (bZIP17 and bZIP28) connected to OSER-induced membrane proliferation?
- What are the effects of OSER on linked organelles and plant viability and productivity?
- Can OSER be standardized as a tool for plant synthetic biology by developing a simple and reliable OSER-inducing construct?
- Are the applications of OSER viable and reproducible?
Figure 1: Examples of OSER phenotypes in plant cells. A: Native crystalloid ER in phloem parenchyma cells of *Helleborus lividus* (figure from (Behnke, 1981)). B: OSER whorl induced by overexpression of integral membrane M protein of the coronavirus avian infectious bronchitis virus with an oligomerizing β-glucoronidase attached to the cytoplasmic domain (figure from (Gong et al., 1996)). C&D: Crystalloid, whorl and lamellae OSERs in *N. benthamiana* leaves agro-infected with HMGR membrane domain with a cytoplasmically fused dimerizing GFP. (figure from (Ferrero et al., 2015)). Cr: crystalloid ER; La: ER lamellae; W: ER whorl; N: nucleus; n: nucleolus. Scale bars: 2.5 µm (top), 10 µm (left), 0.5 µm (right & bottom).
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