RESEARCH PAPER

Ethylene and hydrogen peroxide regulate formation of a sterol-enriched domain essential for wall labyrinth assembly in transfer cells

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Abstract

Transfer cells (TCs) facilitate high rates of nutrient transport into, and within, the plant body. Their transport function is conferred by polarized wall ingrowth papillae, deposited upon a specialized uniform wall layer, that form a scaffold supporting an amplified area of plasma membrane enriched in nutrient transporters. We explored the question of whether lipid-enriched domains of the TC plasma membrane could serve as organizational platforms for proteins regulating the construction of the intricate TC wall labyrinth using developing Vicia faba cotyledons. When these cotyledons are placed in culture, their adaxial epidermal cells trans-differentiate to a TC phenotype regulated by auxin, ethylene, extracellular hydrogen peroxide (apoH₂O₂), and cytosolic Ca²⁺ ([Ca²⁺]cyt) arranged in series. Staining cultured cotyledons with the sterol-specific dye, Filipin III, detected a polarized sterol-enriched domain in the plasma membrane of their trans-differentiating epidermal transfer cells (ETCs). Ethylene activated sterol biosynthesis while extracellular apoH₂O₂ directed sterol-enriched vesicles to fuse with the outer periclinal region of the ETC plasma membrane. The sterol-enriched domain was essential for generating the [Ca²⁺]cyt signal and orchestrating construction of both the uniform wall layer and wall ingrowth papillae. A model is presented outlining how the sterol-enriched plasma membrane domain forms and functions to regulate wall labyrinth assembly.

Keywords: Ethylene, hydrogen peroxide, Ca²⁺ signal, plasma membrane, sterol-enriched domain, trans-differentiation, transfer cell, wall labyrinth.

Introduction

Transfer cells (TCs) exhibit an enhanced capacity for nutrient transport conferred by an amplified plasma membrane (PM) surface area, enriched in membrane nutrient transporters, supported on an intricate wall labyrinth (Offler et al., 2003). Consistent with this functional capacity, TCs are located at bottlenecks for nutrient transport between apo-symplasmic compartments. For example, TCs can occur at interfaces between soil/root, host/biotroph, and maternal/filial tissues of developing seeds or at sites of nutrient loading/unloading of vascular pipelines (Offler et al., 2003; Andriunas et al., 2013). The tight functional relationship between the presence of a wall labyrinth (i.e. PM amplification) and enhanced nutrient transport is essential for nutrient acquisition and allocation within the plant body. This review focuses on the sterol-enriched plasma membrane domain and its role in the construction of the wall labyrinth.
transport capacity is graphically illustrated by compromised filling of endocytic and monocot seed mutants in which wall labyrinth formation of their TCs is attenuated. That the shrivelled seed phenotype of these TC mutants are displayed in a number of major crop species, including cereals and grain legumes, underscores the important role TCs play in determining crop yields (Andriunas et al., 2013).

Walls labyrinths of TCs are one of two principal architectural types—reticulate or flange, or, in a few instances, both wall architectures occur in the same cell with the reticulate type deposited on flanges (Andriunas et al., 2013). The reticulate architecture provides the greatest plasticity for amplifying PM surface areas. The degree of reticulation ranges from a single layer of discrete or branched wall ingrowth (WI) papillae to repeating fenestrated layers of WI papillae that branch and fuse (Andriunas et al., 2013). The selective advantage of the reticulate wall labyrinth design is illustrated by its presence in species of all plant taxa from algae to angiosperms (Offler et al., 2003). As a consequence, most studies have focused on reticulate TCs with their wall labyrinths comprised of a uniform wall layer (UWL) from which WI papillae arise (Xia et al., 2017).

To understand the mechanisms and regulatory signals responsible for constructing reticulate wall labyrinths, our investigations have focused on the assembly of the UWL and the first round of construction of WI papillae using cultured cotyledons of Vicia faba. On being transferred to culture, the adaxial epidermal cells of the V. faba cotyledons undergo rapid (hours) transition-differentiation to a TC morphology and function comparable with their in planta abaxial epidermal TC (ETC) counterparts (Andriunas et al., 2013). In both adaxial and abaxial ETCs, wall labyrinths are polarized to their outer periclinal walls. An inductive signalling cascade, driving the trans-differentiation process, is initiated by an auxin spike in the adaxial epidermal cells (Dibley et al., 2009), triggering ethylene production (Zhou et al., 2010). The ethylene signal elicits a polarized burst in extracellular hydrogen peroxide (H$_2$O$_2$) production that switches on, and provides the positional cue for, the localized deposition of the UWL to the outer periclinal wall (Andriunas et al., 2012; Xia et al., 2012). Acting co-operatively, ethylene and H$_2$O$_2$ induce the formation of a polarized cytosolic Ca$^{2+}$ ([Ca$^{2+}$]$_{cyt}$) signal (Zhang et al., 2015b) shaped into plumes of elevated [Ca$^{2+}$]$_{cyt}$ by enhanced fluxes of Ca$^{2+}$ through PM-localized clusters of Ca$^{2+}$-permeable channels surrounded by Ca$^{2+}$-ATPases (Zhang et al., 2015a). The inward-directed [Ca$^{2+}$]$_{cyt}$ plumes cause re-modelling of the actin cytoskeleton into a spatial configuration that trafficks vesicle cargoes, containing wall building materials, to defined loci on the outer surface of the UWL for assembly of WI papillae (Zhang et al., 2017a).

As described above, assembly of reticulate wall labyrinths is dependent upon mechanism(s) that localize apoH$_2$O$_2$ and [Ca$^{2+}$]$_{cyt}$ signals, together with cell wall deposition, to the outer periclinal wall of the trans-differentiating ETCs. It is well established that sterol- and sphingolipid–enriched PM domains selectively compartment proteins participating in signalling pathways and cell wall biosynthesis (e.g. Schrick et al., 2012; Tapken and Murphy, 2015; Iswanto and Kim, 2017; Han et al., 2018). In this context, transcriptomic analyses have found that genes encoding sterol and sphingolipid biosynthetic enzymes, along with a microdomain marker protein, remorin, were up-regulated during trans-differentiation of both flange and reticulate wall labyrinths (Thiel 2012; Zhang et al., 2015c). However, until now, there is no direct evidence for the presence of lipid–enriched PM domains organizing signalling pathways or cell wall biosynthesis underpinning assembly of TC wall labyrinths. Nevertheless, precedents for such domains are evident in analogous systems. For instance, sterol–enriched macrodomains are found in the PM lining tips of developing pollen tubes and root hairs (Liu et al., 2009; Overchak et al., 2010). Significantly, sterol sequestration to the cytosol disrupted polarization of these macrodomains, and depressed activities of tip-located respiratory burst oxidase homologues (RBOHs) producing a reactive oxygen species (ROS) signal that in turn dissipated the apically orientated [Ca$^{2+}$]$_{cyt}$ gradient. The consequence was arrested tip growth of elongating pollen tubes (Liu et al., 2009). Similarly, precedents for localizing callose syntheses to deposit callose sheaths of WI papillae (Vaughn et al., 2007) are PM domains that regulate callose deposition to build defence papillae and control gating of plasmodesmal pores (Faulkner et al., 2015; Iswanto and Kim, 2017).

Given these precedents, we explored the proposition that lipid–enriched domains in the PM of ETCs of V. faba cotyledons contribute to assembling their reticulate wall labyrinths. To this end, we show that the PM lining the outer periclinal region of developing ETCs was selectively and rapidly enriched in sterols during the early phases of trans-differentiation to a TC morphology. Ethylene switched on sterol biosynthesis and apoH$_2$O$_2$ directed trafficking of sterol–enriched membrane vesicles to fuse with the PM lining the outer periclinal ETC region. In turn, the sterol-enriched domain (SED) within the PM directly regulated assembly of the UWL. Influence over formation of WI papillae was mediated through the SED functioning as an organizing platform for Ca$^{2+}$-permeable channels to generate the polarized plumes of elevated [Ca$^{2+}$]$_{cyt}$ that direct construction of WI papillae.

Materials and methods

Plant growth conditions

Vicia faba L. cv. Fiord plants were raised under controlled environmental conditions according to Zhou et al. (2010).

Cotyledon culture

Vicia faba cotyledons were aseptically cultured on a modified Murashige and Skoog (MS) liquid medium (Murashige and Skoog, 1962; see Andriunas et al., 2012). Sister cotyledons were divided between MS medium with or without pharmacological agents and held in the dark at 4 °C for 4 h to allow their penetration before being cultured at 26 °C for a further 15 h unless stated otherwise. Each pharmacological agent was applied at a concentration that did not negatively impact cell viability as verified by staining cotyledon sections with 0.1% (w/v) tetrazolium blue.

Visualization of sterol-enriched membrane domains

Sterol–enriched membrane domains in ETCs were visualized by staining freshly prepared tissue sections of cotyledons with the UV fluorescent probe, Filipin III (hereafter referred to as Filipin), that binds to the 3'-β-OH group of sterols, following a protocol for live cell imaging described by Botté et al. (2011) modified as follows. Cotyledons, sampled directly from plants or following culture with or without pharmacological treatments, were hand sectioned along their transverse axis to provide ready
access of all cells to the Filipin stain (Boutté et al., 2011). Tissue sections (~100 μm in thickness) were immediately incubated in 30 μM Filipin dissolved in MES-buffered MS medium (pH 5.8) containing 10 mM sucrose for 30 min at 4 °C in the dark to prevent wound responses, Filipin photobleaching, and sterol-mediated endocytosis. Holding the sectioned tissues at 4 °C with ice was employed to minimize confounding effects on sterol abundance and distribution in the ETCs that may have been introduced by sectioning-induced wound responses and by Filipin impacting endocytosis and sterol sequestration, while darkness reduced photobleaching of Filipin fluorescence (Boutté et al., 2011). The stained sections were washed in the MS/sucrose solution for 3 min at 4 °C, changing the wash solution at 1 min intervals, to remove unbound Filipin. Thereafter, the stained sections were mounted in pre-chilled MS/sucrose solution and observed with an Olympus Fluoview FV1000 confocal laser-scanning microscope (CLSM) using a 60× oil-immersion objective (NA 1.25). Filipin was excited with a 405 nm UV laser source (50 mW, laser power set to 15%) and emitted fluorescence captured at 425–485 nm using a photomultiplier gain of 700 V. Total fluorescence of Filipin, measured as pixels in specified regions of the PM of ETCs and storage parenchyma cells (SPCs), was estimated using the freehand selection tool in ImageJ (http://rsbweb.nih.gov/ij/), and corrected for background fluorescence measured in unstained cells.

To determine the subcellular localization of Filipin fluorescence, whole cotyledons were stained with 20 μM RH-414, a PM marker (Hulte et al., 2007), for 30 min prior to hand sectioning and Filipin staining. To visualize RH-414-stained PMs, the co-stained tissue sections were exposed to an excitation wavelength of 559 nm (15 mW, laser power set to 25%) and emitted fluorescence captured at 625–725 nm using a photomultiplier gain of 700 V. Total fluorescence of Filipin, measured as pixels in specified regions of the PM of ETCs and storage parenchyma cells (SPCs), was estimated using the freehand selection tool in ImageJ (http://rsbweb.nih.gov/ij/), and corrected for background fluorescence measured in unstained cells.

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Transmission and scanning electron microscopy

Ultrathin transverse sections of ETCs were visualized with a JEOL 1200 EX II transmission electron microscope (JOEL, Japan) as previously described (Zhang et al., 2015a). Average UWL thicknesses were estimated from measures of UWL cross-sectional areas divided by their corresponding widths (i.e. nm² nm⁻¹=nm) using ImageJ software (http://rsbweb.nih.gov/ij/). A Zeiss VP field emission scanning electron microscope (FESEM; Zeiss, Germany) was used to visualize WI papillae on the cytoplasmic faces of the outer periclinal walls of fractured ETCs, prepared as described in Zhang et al. (2015a). To determine the subcellular localization of Filipin fluorescence, whole cotyledons were stained with 20 μM RH-414, a PM marker (Hulte et al., 2007), for 30 min prior to hand sectioning and Filipin staining. To visualize RH-414-stained PMs, the co-stained tissue sections were exposed to an excitation wavelength of 559 nm (15 mW, laser power set to 25%) and emitted fluorescence captured at 625–725 nm using a photomultiplier gain of 700 V. Total fluorescence of Filipin, measured as pixels in specified regions of the PM of ETCs and storage parenchyma cells (SPCs), was estimated using the freehand selection tool in ImageJ (http://rsbweb.nih.gov/ij/), and corrected for background fluorescence measured in unstained cells.

Monitoring vesicle trafficking

To assess the impact of SEDs on vesicle trafficking during different phases of wall labyrinth assembly, transverse free-hand sections of cultured cotyledons were stained with FM4-64FX (Molecular Probes, Eugene, OR, USA; see Zhang et al., 2017a). Total fluorescence of specified ETC regions was measured using ImageJ software and corrected against PM fluorescence, as described in Zhang et al. (2017a).

Measurement of apoH₂O₂ flux and distribution

Flux (pmol H₂O₂ min⁻¹ mm⁻² of the adaxial cotyledon surface) of apoH₂O₂ generated in the outer periclinal wall of ETCs was measured using Amplex Red reagent (10-acetyl-3,7-dihydrophenoxazine; Invitrogen, Australia) as described by Andriunas et al. (2012). Fresh cotyledon sections (~100 μm in thickness) were viewed under bright light with a Zeiss Axioshot microscope and ETC images were recorded with a Zeiss AxioCam HRc camera using Axiovision software. Images were processed through Photoshop CS6 level command with input levels adjusted to 156–237 in both the negative control and DAB-stained sections to an identical setting so that the image of the brown DAB stain was intensified. Absolute pixel numbers of the DAB stain in each cell wall region, corrected for background, were determined using Image J in RawIntDen under Integrated Density measure (http://imagej.nih.gov/ij/docs/menus/analyze.html).

Visualization of cytosolic calcium and fluorescently labelled Ca²⁺-permeable channels

Estimates of [Ca²⁺]cyt were obtained by loading cultured cotyledons with a membrane-permeable Ca²⁺-sensitive dye, Oregon Green BAPTA-1 acetoxy-methyl ester (OGB-1), while the cellular distribution of Ca²⁺-permeable channels relied on staining cultured cotyledons with DM-BODIPY(–)- dihydropropyridine (fl-DHP; Invitrogen, USA; see Zhang et al., 2015a).

Thereafter transverse hand-cut sections were stained with tetrazoil blue to identify viable cells for microscope observations and counterstained with 0.1% (w/v) Calcofluor White to outline the ETC walls. An Olympus FV1000 CLSM set with a 405 nm laser and a 440–490 nm emission filter set was used to visualize Calcofluor White fluorescence, while a 473 nm laser and a 510–580 nm emission filter set was used to visualize OGB-1 and fl-DHP fluorescence. Fluorescence densities (pixels per unit area) of OGB-1 in the outer periclinal cytosol of ETCs were measured using ImageJ and converted to estimates of [Ca²⁺]cyt, using the calibration curve presented in Zhang et al. (2015a). The utility of the pre-existing calibration curve was verified by finding that it yielded identical [Ca²⁺]cyt estimates to those reported by Zhang et al. (2015a, b). Total fluorescence of fl-DHP in specified regions in the ETCs was measured using ImageJ.

RINseq expression analysis

A transcriptomic database for trans-differentiating ETCs and their underlying SPCs of cultured V. faba cotyledons, annotated in Mapman Mercurator and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Zhang et al., 2017b), was used to investigate expression profiles of transcripts encoding proteins participating in phytosterol and sphingolipid biosynthesis and metabolism (Michaelson et al., 2016; Sonawane et al., 2016; Valitova et al., 2016), the cytosolic ethylene insensitive2 (EIN2) pathway (Salchin and Estelle, 2015; Zheng and Zhu, 2016), and vesicle trafficking (Kim and Brandizzi, 2012; Paez-Valencia et al., 2016). Identified transcripts with RPKM values (reads per kilobase of transcript per million mapped reads) >1 at 3 h or 12 h of cotyledon culture were selected for subsequent analysis. Functions of the encoded proteins were inferred by best-fit percentage amino acid alignment with their closest Arabidopsis homologue using TAIR10 and Araport 11 databases. The impact of ethylene and apoH₂O₂ on expression profiles of these transcripts was assessed as described in Zhang et al. (2017b).

Results

The outer periclinal region of the plasma membrane of trans-differentiating epidermal transfer cells is sterol enriched

By 15 h of cotyledon culture, the trans-differentiating ETCs had deposited a wall labyrinth across the outer periclinal region of their original primary wall that is thicker than the anticlinal and inner periclinal walls (Fig. 1A). The wall labyrinth is comprised of a UWL from which WI papillae arise (Fig. 1B). Filipin-stained ETCs of the 15 h cultured cotyledons exhibited a strong fluorescent band located in their outer periclinal region (Fig. 1C). Co-localization of fluorescence emanating from bound Filipin with the PM marker, RH-414, in both turgid and plasmolysed ETCs pointed to Filipin binding to the PM of trans-differentiating ETCs (Fig. 1D; Supplementary Fig. S1 at JXB online). Consistent with this conclusion was finding that the intracellular distribution of Filipin fluorescence levels was unaffected by treating cotyledons with the vesicle trafficking inhibitor, brefeldin A (BFA; Supplementary Table S1). This result excludes localization of bound Filipin to vesicles that would be distributed evenly throughout the ETC cytosol by cytoplasmic streaming.
The 43-fold enhanced fluorescence levels of bound Filipin located in the ETC outer periclinal region of their PM (Fig. 2A versus Fig. 2B, E) was absent from the underlying SPCs in which very low Filipin fluorescence levels were spread evenly across their entire PM (Fig. 2F). The slightly higher fluorescence values recorded in the anticlinal and inner periclinal PM regions of SPCs compared with those of the ETCs was proportionate to their larger size (Fig. 2E versus Fig. 2F; for more details, see Supplementary Table S2).

Blocking formation of the polarized band of Filipin fluorescence by culturing cotyledons on media containing biosynthesis inhibitors of sterol (fenpropimorph, Yang et al., 2013) or sphingolipids (myriocin, Michaelson et al., 2016) indicated that the fluorescence emanated from Filipin bound to an SED located in the outer periclinal region of the ETC PM (Fig. 2B versus Fig. 2C–E); a conclusion confirmed using the PM sterol stripping drug, methyl-β-cyclodextrin, and the sphingolipid biosynthesis inhibitor, fumonsin B1 (Supplementary Table S3; Yang et al., 2013). The possibility that Filipin stained brassinosteroids was eliminated as inhibiting their biosynthesis with triadimefon or propiconazole (Yang et al., 2013) exerted no impact on Filipin fluorescence (Supplementary Table S3).

Temporal accumulation of the polarized SED in the PM of ETCs (Fig. 2E) was monitored at specified times across 15 h of cotyledon culture by recording percentages of cells exhibiting the band of intense Filipin fluorescence and mean fluorescence levels per ETC (Supplementary Fig. S2A and B, respectively). Both parameters increased from the onset of cotyledon culture to asymptote at 6 h (Supplementary Fig. S2).

**Ethylene regulates biosynthesis, while apoH$_2$O$_2$ determines the intracellular distribution, of the sterol-enriched domain**

To test if any of the known signals (auxin, ethylene, apoH$_2$O$_2$, or calcium) regulating wall labyrinth assembly was responsible for switching on sterol biosynthesis or determining SED polarization (Fig. 2), cotyledons were cultured on media containing blockers of auxin [p-chlorophenoxyisobutyric acid (PCIB),

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**Fig. 1.** Micrographs illustrating the morphological characteristics of trans-differentiating epidermal transfer cells (ETCs) of cultured *V. faba* cotyledons and co-localization of Filipin staining with their plasma membrane. (A and B) Light (A) and transmission electron (B) micrographs of transverse sections of 15 h cultured ETCs. Note that the wall labyrinth is polarized to the outer periclinal region (A), is deposited on the original primary wall (OW) in (B), and is composed of a uniform wall layer (UWL, bracketed) from which wall ingrowth (WI) papillae arise. Identified ETC regions referred to in this study are marked on (A), namely outer periclinal (blue line), anticlinal (rusty brown line), and inner periclinal (green line). (C and D) CLSM images of transverse sections of ETCs stained with Filipin to detect sterol-enriched domains (fluorescence indicated by arrowheads in C), co-stained with the plasma membrane tracker RH-414, and presented as an overlay (D) with brackets delineating the outer periclinal wall. Scale bar=10 µm in (A), 500 nm in (B), and 5 µm in (C) and (D).

**Fig. 2.** Impact of inhibitors of sterol and sphingolipid biosynthesis on the intracellular distribution of Filipin fluorescence in trans-differentiating epidermal transfer cells (ETCs) and storage parenchyma cells (SPCs) of cultured *V. faba* cotyledons. (A–D) CLSM images of transverse sections of ETCs prepared from cotyledons that were (A) freshly harvested or (B–D) cultured on MS medium for 15 h in the (B) absence or (C) presence of 10 µM fenpropimorph or (D) 1 µM myriocin. The ETCs are bracketed, and arrowheads indicate the position of their outer periclinal region. Scale bar=5 µm. (E, F) Filipin fluorescence measured as total pixels detected in outer periclinal, anticlinal, and inner periclinal PM regions of (E) ETCs and (F) SPCs treated with ±10 µM fenpropimorph or ±1 µM myriocin. Data are means ±SEs from four replicate cotyledons; 20 cells per cotyledon.
Sterol-enriched plasma membrane domain in transfer cells

Dibley et al., 2009], ethylene [aminoethoxyvinylglycine (AVG), Zhou et al., 2010], apoH2O2 [ascorbic acid (AA), Andriunas et al., 2012], and [Ca2+]cyt (nifedipine, Zhang et al., 2015a). Filipin fluorescence levels were measured in outer pericinal, anticlinal, and inner pericinal PM regions to assess impacts on SED polarization. The summed fluorescence levels in these regions provided estimates of sterol biosynthesis per ETC.

Only AVG caused a 96% depression in total Filipin fluorescence per ETC (Table 1), indicating that ethylene up-regulated sterol biosynthesis, a conclusion consistent with elevated ETC sterol levels in response to a 54% enhanced ethylene production in the presence of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Table 1; Zhou et al., 2010). Our data do not provide any insight as to why the increase in sterol levels was confined to the anticlinal and inner periclinal PM domains. In comparison, while Filipin biosynthesis per ETC was not attenuated, the Filipin fluorescence band was depolarized in ETCs of cotyledons cultured without ethylene in the absence/presence of the RNA inhibitor, 6-methylpurine, or the protein inhibitor, cycloheximide, and 6-β-VfEpsin1, 2012). However, the ETC level, and polarity, of the Filipin fluorescence was retained when the spike in [Ca2+]cyt was blocked on cotyledon exposure to the Ca2+ channel inhibitor, nifedipine (Table 1; Zhang et al., 2015a).

Ethylene regulates biosynthesis of the sterol-enriched domain

To better understand how ethylene regulated ETC sterol biosynthesis, an experiment was designed to distinguish ethylene action at the various levels of molecular control. Here cotyledons were cultured under conditions with or without ethylene in the absence/presence of the RNA inhibitor, 6-β-methylpurine, or the protein inhibitor, cycloheximide, and treatment effects on Filipin fluorescence were determined (Supplementary Table S4). These data were then used to distil out actions of ethylene at the transcriptional, translational, and post-translational levels (for details, see Table 2 footnotes). The analysis demonstrated that ethylene acted primarily on translation of mRNA encoding sterol biosynthetic enzymes, with decreasing impacts at the transcriptional and post-transcriptional levels (Table 2).

Our RNA sequencing (RNAseq) data set was interrogated for ETC-specific differentially expressed genes (DEGs) encoding enzymes known to participate in the biosynthetic pathways for phytosterols (Sonawane et al., 2016; Valitova et al., 2016) and sphingolipids (Michaelson et al., 2016). This analysis yielded two phytosterol and seven sphingolipid biosynthetic ETC-specific DEGs (Supplementary Table S5A). Ethylene had no impact on expression of the phytosterol biosynthetic DEGs, VfβHSD and VfHMGS1 (Sonawane et al., 2016). In the case of the seven sphingolipid biosynthetic ETC-specific DEGs, ethylene up-regulated expression of Vfinositol phosphoceramide synthase1 (VfIPCS1) operating in the pathway generating the largest group of plant sphingolipids, glycosyl inositol phosphoceramides (Michaelson et al., 2016). The remaining sphingolipid DEGs were unresponsive to ethylene. Four DEGs encoded VfCB2SPT1, VfSPT2, VfTSC10B, and VfNCER, all of which could regulate sphingolipid levels. Amongst them, VfCB2SPT catalyses the first step in the sphingolipid biosynthetic pathway, VfTSC10B reduces 3-ketodihydrosphinganine into dihydrosphinganine, while VfNCER maintains homeostasis of the ceramide pool. The other two DEGs encoded VfSBH and VfA3-DS. These enzymes modify the long chain bases of sphingolipids by hydroxylation and desaturation, respectively, that in turn can alter their biological properties (Michaelson et al., 2016).

EIN2 was recently shown also to act in the cytosol to mediate ethylene control of translation (Salehin and Estelle, 2015; Zheng and Zhu, 2016). Of downstream proteins associated with this pathway, expression of genes encoding ethylene-binding F-box proteins, VfEBF1 and VfEBF2, were found to be ethylene sensitive between 3 h and 12 h of cotyledon culture (Supplementary Table S5B).

In the absence of ethylene, the SED was disassembled to background levels (Table 1). This response was associated with AVG increasing expression of two ETC-specific down-regulated DEGs, VfPIP5K6 and VfEpin1 (Supplementary Table S5C). These respectively encode proteins that participate in

Table 1. Effect of auxin, ethylene, apoH2O2, and Ca2+ signalling on the plasma membrane (PM) sterol-enriched domain formation and distribution in trans-differentiating epidermal transfer cells (ETCs) of cultured V. faba cotyledons

| Cotyledon treatment | Active signal(s) | Inhibited signal(s) | Filipin fluorescence (arbitrary units) in ETC: | Outer pericinal PM | Anticlinal PM | Inner pericinal PM | Total |
|---------------------|-----------------|---------------------|-----------------------------------------------|--------------------|--------------|-------------------|-------|
| Control             | IAA, C2H4, apoH2O2, Ca2+ | None                | 131±5                                         | 2±1                | 0±0          | 132±5             |       |
| POIB (100 μM)+ACC (100 μM) | C2H4, apoH2O2, Ca2+ | IAA                 | 136±6                                         | 39±3               | 33±2         | 208±8             |       |
| AVG (100 μM)+H2O2 (10 μM) | IAA, apoH2O2, Ca2+ | C2H4                | 0±0                                          | 4±1                | 1±0          | 5±1               |       |
| AVG (100 μM)+ACC (100 μM) | IAA, C2H4, apoH2O2, Ca2+ | None                | 142±7                                         | 38±2               | 28±3         | 208±10            |       |
| Ascorbic acid (10 mM) | IAA, C2H4, apoH2O2 | apoH2O2, Ca2+       | 48±4                                         | 43±3               | 49±4         | 140±9             |       |
| Nifedipine (100 μM) | IAA, C2H4, apoH2O2 | Ca2+                | 128±9                                         | 5±1                | 0±0          | 133±10            |       |

Cotyledons were cultured for 15 h on MS medium in the absence or presence of various combinations of the auxin action inhibitor, p-chlorophenoxyisobutyric acid (POIB), ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG), ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), apoH2O2 scavenger, ascorbic acid, and the DHP-receptor Ca2+ channel blocker, nifedipine. Thereafter, transverse sections of treated cotyledons were stained with Filipin. Fluorescence was measured as total pixels in specified regions of the ETC PM. Data are means ±SEs from four replicate cotyledons; 20 cells per cotyledon.
Table 2. Regulatory role of ethylene on sterol biosynthesis at the transcriptional, translational, and post-translational levels in trans-differentiating epidermal transfer cells (ETCs) of cultured V. faba cotyledons

| Effect of ethylene on: | Filipin fluorescence/ETC (arbitrary units) |
|-----------------------|-------------------------------------------|
| Transcription         | 33±13 (16±6%)                             |
| Translation           | 159±10 (77±5%)                            |
| Post-translational    | 14±4 (7±2%)                               |

Data are derived from Supplementary Table S4 as follows. Fluorescence levels in the presence of ethylene were corrected by subtracting fluorescence in its absence. Effects of ethylene at the transcriptional, translational, and post-translational levels were estimated, respectively, from differences in total Filipin fluorescence levels detected in ETCs between cotyledons cultured on: (i) MS medium alone versus 6-methylpurine; (ii) 6-methylpurine versus cycloheximide; and (iii) cyclohexamide alone. The percentage contribution to sterol biosynthesis by each level of biological control is presented in parentheses.

Extracellular hydrogen peroxide regulates intracellular distribution of the sterol-enriched domain

The time course kinetics of apoH2O2-regulated assembly and disassembly of the SED were examined once Filipin fluorescence had acquired steady-state levels in ETCs at 9 h of cotyledon culture (Supplementary Fig. S2). For SED assembly, cotyledons were cultured on MS medium containing the apoH2O2 scavenger, AA, for 9 h. This treatment resulted in a depolarized intercellular distribution of Filipin fluorescence (Fig. 3A). Thereafter, on transfer to an AA-free MS medium, the polarity of Filipin fluorescence distribution was restored within 2 h (Fig. 3A). The polarity of the SED achieved by 9 h of cotyledon culture was depolarized completely within 1.5 h of transferring cotyledons to a MS medium containing AA (Fig. 3B). Together, these findings demonstrate that the apoH2O2 signal not only acts to polarize SED assembly, but is also essential in maintaining their polarized state by blocking disassembly.

The reliance of an apoH2O2-dependent polarized assembly/disassembly of the SED on the ETC cytoskeleton was tested by culturing cotyledons in the absence/presence of microtubule- and actin-depolymerizing drugs, oryzalin (Zhang et al., 2015a) and latrunculin B, respectively (Zhang et al., 2017a). The experimental design was identical to that described for Fig. 3. The presence/absence of an intact cytoskeleton did not exert any influence over either apoH2O2-dependent assembly or disassembly of the polarized SED (Table 3).

Whether apoH2O2-regulated assembly and disassembly of the SED depended upon vesicle trafficking and endocytosis of sterol-enriched membrane vesicles was explored using the experimental plan described for Fig. 3 augmented by exposing cotyledons during 9–15 h of culture to media also containing dynasore (endocytosis inhibitor; Zhang et al., 2017a). The resulting distribution of Filipin fluorescence is consistent with endocytosis blocking both apoH2O2-driven SED assembly (Supplementary Table S6A) and SED disassembly in the absence of apoH2O2 (Supplementary Table S6B).

The above findings point to apoH2O2 activating vesicle docking and inactivating endocytosis. Our RNAseq data set (see Supplementary Table S7) identified apoH2O2-sensitive expression of genes encoding proteins that function in the endomembrane secretory pathway from vesicle biogenesis in the Golgi/trans-Golgi network (VfSYP32, VfSEC22, Vfβ-COP, Vfγ-COP, and VfAGD5; Kim and Brandizzi, 2012; Arakel and Schwappach, 2018; Inada et al., 2014) to vesicle docking and fusion to the PM (VfEXO70H7, VfSYP43, and VfSYP121; Vukasnovic and Zárský, 2016; Wang et al., 2016; Supplementary Table S7A). At a post-translational level, apoH2O2 could influence vesicle docking through switching on formation of a [Ca2+]cyt signal (Zhang et al., 2015b) to activate the Ca2+-sensitive docking proteins, VfSYT1, VfSYT2, and VfSYT3 (Pérez-Sancho et al., 2016; Supplementary Table
S7B). The identified apoH$_2$O$_2$-sensitive docking/fusion proteins are the most probable candidates conferring SED polarity. In the absence of apoH$_2$O$_2$, up-regulated expression of genes encoding endocytotic proteins (VfHS3P1 and VfPIP5K2 with apoH$_2$O$_2$ depressing endocytosis. Valencia et al., 2016; Supplementary Table S7C) is consistent with apoH$_2$O$_2$ depressing endocytosis.

The sterol-enriched domain is essential for Ca$^{2+}$ but not apoH$_2$O$_2$ signalling

Lipid-enriched PM domains play key roles in determining the activity and cellular localization of RBOHs across a range of physiological contexts including tip growth of pollen tubes (Liu et al., 2009) and plant–biotroph interactions (Ott, 2017). To this end, we tested whether the apoH$_2$O$_2$ signal of trans-differentiating ETCs (Table 1; Fig. 3) was subjected to SED feedback regulation through impacting RBOH activity and/or its intracellular distribution (Andriunas et al., 2012). Pharmacological blockade of SED formation affected neither apoH$_2$O$_2$ biosynthesis nor its intracellular distribution (Table 4; Supplementary Fig. S3).

The SED could facilitate plumes of elevated [Ca$^{2+}$]$_{cyt}$ being generated in the ETC outer periclinal cytosol by influencing the activity/oranization of Ca$^{2+}$-permeable channels and/or Ca$^{2+}$-ATPases in their PM (Zhang et al., 2015a). This hypothesis was evaluated by determining whether the magnitude of the [Ca$^{2+}$]$_{cyt}$ signal and/or polarized distribution of DHP-receptor Ca$^{2+}$ channels were sensitive to a pharmacological blockade of SED formation. The blockade was imposed after 9 h of cotyledon culture to ensure that [Ca$^{2+}$]$_{cyt}$ levels had reached a steady state (Table 5; Zhang et al., 2015b).

The levels of the polarized [Ca$^{2+}$]$_{cyt}$ signal in ETCs of cotyledons exposed to fenpropimorph or myriocin over the ensuing 6 h were reduced by 37-fold to match those in epidermal cells prior to their entry into the trans-differentiation pathway (Table 5; Supplementary Fig. S4). In ETCs, [Ca$^{2+}$]$_{cyt}$ levels are dynamically balanced by Ca$^{2+}$ influx through PM Ca$^{2+}$-permeable channels and efflux through PM Ca$^{2+}$-ATPases (Zhang et al., 2015a). In this context, Eosin Yellow inhibition of the Ca$^{2+}$-ATPases (Zhang et al., 2015a) did not recover [Ca$^{2+}$]$_{cyt}$ levels in the presence of fenpropimorph or myriocin (Table 5). This finding removes the possibility that an absence of the SED increased Ca$^{2+}$-ATPase activity to account for the reduction in [Ca$^{2+}$]$_{cyt}$ levels. Thus, we concluded that transport activities of PM Ca$^{2+}$-permeable channels were substantially enhanced by being embedded in the SED. This regulatory influence could result from a post-translational activation mechanism and/or regulating their PM residence. The latter possibility was explored as outlined below.

The relative abundance of Ca$^{2+}$-permeable channels was estimated using the fluorescent-tagged nifedipine analogue fl-DHP on the grounds outlined in Zhang et al., (2015a). An experimental design identical to that used to obtain estimates of [Ca$^{2+}$]$_{cyt}$ was employed. While total ETC levels of fl-DHP fluorescence remained unchanged, SED deletion between 9 h and 15 h of cotyledon culture led to the intracellular distribution of the nifedipine-sensitive Ca$^{2+}$ channels being depolarized (Table 6; Supplementary Fig. S4). Significantly, channel redistribution was inhibited by BFA, pointing to their endocytosis into the endosome vesicle pathway. Withdrawal of Ca$^{2+}$...
Cotyledons were freshly harvested or cultured on MS medium for 9 h to ensure that \( [\text{Ca}^{2+}]_{\text{cyt}} \) had reached a steady state (Zhang et al., 2015b), before being transferred to media ±fenpropimorph or ±myriocin overlaid with ±Eosin Yellow, an inhibitor of \( \text{Ca}^{2+}\)-ATPases, for 4 h at 4 °C. Thereafter, cotyledon culture was continued for a further 6 h at 26 °C. \( [\text{Ca}^{2+}]_{\text{cyt}} \) levels were then estimated from detected fluorescence levels of OGB-1 loaded into the ETCs. Data are means ±SEs from four replicate cotyledons; 20 cells per cotyledon.

### Table 6. Effect of the plasma membrane (PM) sterol-enriched domain on the distribution of DHP receptor \( \text{Ca}^{2+} \)-permeable channels in trans-differentiating epidermal transfer cells (ETCs) of cultured \( V. \text{faba} \) cotyledons

| Cotyledon treatment | \( \text{fl-DHP fluorescence (arbitrary units) in ETC:} \) |
|---------------------|--------------------------------------------------|
|                     | Outer periclinal PM | Anticlinal PM | Inner periclinal PM | Total |
| 0 h Control         | 52±4               | 49±5         | 51±5               | 152±8 |
| 9 h Control         | 577±14             | 52±6         | 55±6               | 684±19 |
| 9+6 h Control       | 585±18             | 54±9         | 63±6               | 702±21 |
| 9 h Control+6 h fenpropimorph (10 µM) | 235±9 | 226±11 | 231±12 | 692±19 |
| 9 h Control+6 h myriocin (1 µM) | 240±10 | 227±12 | 223±12 | 690±20 |
| 9 h Control+6 h myriocin (10 µM) and BFA (357 µM) | 564±16 | 47±5 | 53±5 | 669±17 |
| 9 h Control+6 h myriocin (1 µM) and BFA (357 µM) | 592±17 | 52±7 | 56±7 | 700±20 |

Cotyledons were cultured on MS medium for 9 h to ensure that the abundance of \( \text{Ca}^{2+} \)-permeable channels had reached a steady state (Zhang et al., 2015b), before being transferred to MS medium ±fenpropimorph or ±myriocin with the fenpropimorph and myriocin media containing ±brefeldin (BFA) for 4 h at 4 °C and thereafter cultured for a further 6 h at 26 °C. At the conclusion of culture, the cotyledons were immediately stained with 600 nM DM-BODIPY(–)-dihydropyridine (fl-DHP). Relative abundance of \( \text{Ca}^{2+} \) channels was estimated from fluorescence levels detected as total pixels in specified regions of the ETC PM. Data at each culture time are means ±SEs from four replicate cotyledons; 20 cells per cotyledon.

channels from the PM would negate any capacity for \( \text{Ca}^{2+} \) influx into the ETC cytosol from the cell wall compartment to replenish \( \text{Ca}^{2+} \) pumped in the opposite direction by the \( \text{Ca}^{2+}\)-ATPases and, hence, accounts for the precipitous decline in \( [\text{Ca}^{2+}]_{\text{cyt}} \) (Tables 6 and 5, respectively).

The sterol-enriched domain is essential for wall labyrinth assembly

Comparability of the spatiotemporal enrichment of the SED in the outer periclinal PM region (Figs 1, 2) with that of wall labyrinth assembly in trans-differentiating ETCs (Wardini et al., 2007; Xia et al., 2017) suggested that these events may be causally related. This proposition was tested by blocking SED formation on assembly of the UWL and WI papillae.

For epidermal cells of cotyledons cultured on fenpropimorph or myriocin, where Filipin fluorescence was reduced 39-fold, ETCs forming a UWL (Fig. 4A–C) decreased from 100% to 75%, while UWL thickness was reduced by 77% (Fig. 4D). To avoid confounding effects of quenching SED formation on UWL assembly (Fig. 4A–D) on the subsequent deposition of WI papillae, exposure of cotyledons to media containing fenpropimorph or myriocin was delayed for the first 9 h of cotyledon culture to ensure that the UWL was fully formed (Xia et al., 2017). Across the intervening 6 h of cotyledon culture on MS medium alone, Filipin fluorescence remained unchanged while percentages of trans-differentiating ETCs exhibiting WI papillae increased by 58% (Fig. 4I). In contrast, a Filipin fluorescence decrease by 8- to 9-fold on exposure to fenpropimorph or myriocin was accompanied by cessation of deposition of WI papillae or, for those formed, their bending (Fig. 4F versus Fig. 4G, H versus Fig. 4E) and recruitment of additional epidermal cells to commence assembling WI papillae (Fig. 4I).

Construction of the UWL and WI papillae depends upon polarized vesicle docking to the outer periclinal PM region of trans-differentiating ETCs (Dibley et al., 2009; Zhang et al., 2017a). In this context, SEDs have been conjectured to facilitate vesicle docking to PMs carrying cargoes of cell wall machinery such as glycosylphosphatidylinositol (GPI)-anchored proteins and enzymes contributing to callose homeostasis in plasmodesmal pores (Iswanto and Kim, 2017). This idea was tested by blocking SED biosynthesis with fenpropimorph or myriocin during construction of the UWL (0–3 h of cotyledon
culture; see Xia et al., 2017) or WI papillae (9–12 h of cotyledon culture) alone and monitoring the distribution of vesicles labelled with the membrane fluorescent dye, FM-64FX, at the conclusion of these treatment periods. The findings show that a polarized distribution of FM-64FX fluorescence to the outer periclinal region of the trans-differentiating ETCs was dissipated when SED formation was inhibited during formation of the UWL or WI papillae without any impact on total FM-64FX fluorescence per cell (Table 7). The re-distribution of FM-64FX in the absence of the SED can be reversed after
removing SED biosynthesis inhibitors. As shown by treating with the endocytosis inhibitor, dynasore, this does not require FM4-64 endocytosed from the PM, consistent with the SED facilitating vesicle docking to the outer periclinal PM domain of the trans-differentiating ETCs.

Discussion

Upon transfer of V. faba cotyledons to culture, the PM in the outer periclinal portion of their adaxial trans-differentiating ETCs progressively became enriched in sterols that asymptote to reach an ongoing steady-state level within 9 h (Fig. 2; Supplementary Fig. S2). The polarized ETC SEDs corresponded to those formed in the outer planar domain of the PM of root epidermal cells preceding root hair initiation (e.g. Stanislas et al., 2015). While localized SEDs have been observed in a number of physiological contexts including tips of elongating root hairs and pollen tubes (Ovecka et al., 2010; Liu et al., 2009), sites of defence papillae (Faulkner, 2015), and plasmodesmal construction (Iswanto and Kim 2017), little is known about the upstream mechanisms regulating their formation and maintenance (Han et al., 2018). Our ETC cotyledon induction system presented the opportunity of exploring this question as well as unravelling how SEDs may function as a PM-located platform to organize signals regulating machinery constructing the ETC wall labyrinth.

Ethylene and hydrogen peroxide signals co-regulate assembly of a polarized sterol-enriched domain

The spatial and temporal formation of the SED in the trans-differentiating ETCs coincided with construction of their wall labyrinth (Wardini et al., 2007; Xia et al., 2017). Therefore, it was not surprising to discover that these two developmental events were orchestrated by some of the same regulatory signals, specifically ethylene and apoH2O2 (Table 1; Andriunas et al., 2013). In contrast to inhibiting transcription of genes encoding enzymes in the sphingolipid biosynthesis pathway of Arabidopsis seedlings (Wu et al., 2015), ethylene activated phytosterol biosynthesis in the ETCs (Table 1). This was mediated by ethylene acting at the transcriptional (16%) and translational (77%) levels (Table 2). Translational control by ethylene (Fig. 5A) could occur through the recently described cytosolic action of EIN2 via EBF1 and EBF2 (Salehin and Estelle, 2015; Zheng and Zhu, 2016) as indicated by their up-regulated expression in the absence of ethylene. A downstream target for cytosolic EIN2 action could be the translation of VfHMG-CoA synthase1 (Supplementary Table S5A), the second enzyme in the mevalonate pathway, known to be regulated at the transcriptional, translational, and post-translational levels (Liao et al., 2018). Transcriptional regulation by ethylene of wall labyrinth assembly is considered to occur through an ETC-specific ethylene signalling pathway under control of the nuclear-located transcription factors ein3 and its homologue EIN3-like (Zhou et al., 2010). Surprisingly, ethylene-regulated expression of sterol biosynthetic genes was not detected. Rather, ethylene up-regulated expression of VfIPCS1 (Fig. 5A), encoding a key enzyme in the biosynthetic pathway generating the largest group of plant sphingolipids, glycosyl inositol phosphoceramides (Michaelson et al., 2016). A tight relationship existed between phytosterol and sphingolipid biosynthesis, as illustrated by the pharmacological blockade of sphingolipid biosynthesis resulting in lowered ETC levels of phytosterols (Fig. 2). This linkage could be mediated through HMG-CoA reductase that is known to be a site for co-ordinated post-translational regulation between sterol and sphingolipid biosynthesis (Nieto et al., 2009).

Table 7. Effect of the plasma membrane (PM) sterol-enriched domain on vesicle distribution during construction of the (A) uniform wall layer and (B) wall ingrowth papillae in trans-differentiating epidermal transfer cells (ETCs) of cultured V. faba cotyledons

| Cotyledon treatment | FM4-64FX fluorescence (arbitrary units) in ETC: |
|---------------------|-----------------------------------------------|
|                     | Outer periclinal cytosol | Anticlinal cytosol | Inner periclinal cytosol | Total |
| (A) 3 h MS          | 622±16                       | 29±3              | 32±4              | 683±17 |
| 3 h Fenpropimorph (10 µM) | 231±8                       | 234±4             | 223±4             | 688±15 |
| 3 h Fenpropimorph (10 µM)+3 h MS | 627±13                      | 31±6              | 32±4              | 690±15 |
| 3 h Fenpropimorph (10 µM)+3 h Dynasore (100 µM) | 614±12                      | 30±4              | 33±5              | 677±15 |
| (B) 12 h MS         | 414±14                       | 34±6              | 31±5              | 479±16 |
| 9 h MS+3 h Fenpropimorph (10 µM) | 166±10                      | 159±8             | 162±8             | 487±16 |
| 9 h MS+3 h Fenpropimorph (10 µM)+3 h MS | 407±14                      | 34±5              | 35±4              | 476±16 |
| 9 h MS+3 h Fenpropimorph (10 µM)+3 h Dynasore (100 µM) | 411±12                      | 28±4              | 34±4              | 473±14 |

(A) Cotyledons were cultured on MS medium containing the sterol biosynthesis inhibitor, fenpropimorph, for 3 h at 26 °C, then washed 3×5 min in dH2O before being transferred to MS medium in the absence/presence of the endocytosis inhibitor, dynasore, for 4 h at 4 °C and subsequently cultured for a further 3 h at 26 °C.

(B) Cotyledons were cultured on MS medium for 9 h to ensure deposition of the uniform wall layer (Xia et al., 2017), before being transferred to MS medium containing fenpropimorph for 3 h at 26 °C, then washed 3×5 min in dH2O before being transferred to MS medium in the absence/presence of dynasore for 4 h at 4 °C and subsequently cultured for a further 3 h at 26 °C. Immediately before and following exposure to dynasore, transverse sections of treated cotyledons were stained with the membrane dye FM4-64FX. Fluorescence was measured as total pixels in specified ETC regions. These values were adjusted for fluorescence from FM4-64FX located in the PM using the BFA values (Zhang et al., 2017a) to provide estimates of cytoplasmic fluorescence. Data are the mean differences ±SE between mean pixel levels of four replicate cotyledons; 20 cells per cotyledon.
The polarized flow of phytosterols from their sites of synthesis in the endoplasmic reticulum (ER)/Golgi to the outer periclinal portion of the ETC PM was directed by an apoH$_2$O$_2$ signal (Table 1; Fig. 3) localized to the outer periclinal wall of ETCs (Fig. 5A; Andriunas et al., 2012). The SED polarization was independent of the microtubule and actin cytoskeletons (Table 3), a feature that departs from the cytoskeleton-dependent polarity of sterol-enriched membrane domains in animal cells (Byrum and Rodgers, 2015). The cytoskeletal independence points to apoH$_2$O$_2$ activating a sterol-specific docking/fusion mechanism in the ETC PM. Possibilities include docking/fusion of sterol-enriched secretory vesicles or sterol-specific lipid transfer proteins that ferry sterols across membrane contact sites between the ER and PM (Pérez-Sancho et al., 2016; Wang et al., 2017). Some hints as to the mechanism were deduced from profiling the ETC-specific transcriptome for apoH$_2$O$_2$-dependent expression of DEGs encoding candidate proteins.

In support of secretory vesicle docking/fusion, expression of genes encoding the PM-localized Qa-SNARE homologues, ViSYP43 and ViSYP121, and an exocyst subunit, ViEXO70H7, was found to be apoH$_2$O$_2$ dependent (Fig. 5A). SYP43 plays a number of roles at the trans-Golgi network that include directing vesicles decorated with the R-SNARE, VAMP721/722, to the PM for docking/fusion mediated by PM-localized SYP121 (Wang et al., 2016). Significantly, SYP121 interactions direct tip growth of root hairs (Ichikawa et al., 2014) and localized formation of defence papillae at contact sites with biotrophic pathogens (Faulkner, 2015). Subunits of EXO70 play similar roles in polarized growth systems (Vukašinovic and Zárský, 2016). At a post-translational level, apoH$_2$O$_2$ could contribute to polarized vesicle docking through switching on a polarized [Ca$^{2+}$]$_{cyt}$ signal (Zhang et al., 2015b) that in turn activated Ca$^{2+}$-sensitive tethering synaptotagmin (SYT) proteins, ViSYT1, ViSYT2, and ViSYT3 (Pérez-Sancho et al., 2016; see Fig. 5A), a function linked with tip growth of pollen tubes (Wang et al., 2015). Expression of DEGs encoding sterol-specific lipid transfer proteins was found to be insensitive to apoH$_2$O$_2$, leaving open whether the ER to PM pathway is operative in trans-differentiating ETCs.

The rapid apoH$_2$O$_2$-dependent assembly and disassembly of SEDs in the ETC PM (Fig. 3) is suggestive of endocytosis and vesicle recycling through the early endosome pathway, a role proposed in establishing pollen tube polarity (Guo et al.,...
2012). Consistent with this suggestion, absence of \( \text{apoH}_2\text{O}_2 \) caused up-regulated expression of genes encoding homologues of the endocytotic proteins, VfPIP5K2 and VfSH3P1 (Fig. 5A). Recruitment of factors to the PM driving clathrin-mediated endocytosis depends upon the synthesis of phosphatidylinoisitol-4,5-bisphosphate catalyzed by PIP5K2, while H53P1 facilitates disassembly of the clathrin coat as a prelude to endocytosed vesicle fusion with endosomes (Paez Valencia et al., 2016). Thus, similar to auxin regulation of PIN proteins at the PM (Paez Valencia et al., 2016), \( \text{apoH}_2\text{O}_2 \) exerted a positive effect on SED vesicle docking/fusion with the ETC PM and acted negatively to prevent SED endocytosis to stabilize the SED at the PM.

In contrast to the AA-induced endocytotic withdrawal of SEDs from the PM into early endosomal vesicles that are redistributed equally around the ETC cytosol, the SEDs were completely dismantled in the absence of ethylene (Fig. 5A; Supplementary Table S4). The response to an absence of ethylene has two components. First, AVG is known to inhibit apoH2O2 production by \( \approx 40\% \) (Andriunas et al., 2012) with a consequent enhanced endocytosis of SED-containing vesicles from the PM (see above). This effect would be further amplified by the AVG-induced de-repression of Vf\(\text{PIP5K6} \) expression that functions in clathrin-mediated endocytosis (Zhao et al., 2010). The enhanced endocytosis in the absence of ethylene was coupled with a strong up-regulated expression of VfEpsin1 known to direct vesicle trafficking to vacuolar lysis (Fig. 5A; Song et al., 2006).

**Generation of calcium, but not hydrogen peroxide, signals is dependent on the sterol-enriched domain**

In contrast to other plant cell systems (e.g. Liu et al., 2009; Hao et al., 2014), the presence of SEDs in the ETC PM did not influence the formation, or intracellular distribution, of their polarized \( \text{apoH}_2\text{O}_2 \) signal (Table 4; Supplementary Fig. S3), putatively generated by PM-localized RBOHs (Andriunas et al., 2012). An explanation for \( \text{apoH}_2\text{O}_2 \) signal formation being independent of the PM SED could be that the signal is generated by diphenyleenidoindium chloride-sensitive flavin-containing enzymes located in the cell wall, such as class III peroxidases found in elongating root hairs (Mangano et al., 2017).

Using responses of the polarized plumes of elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) formed in the \textit{trans}-differentiating ETCs as a proxy to interpret SED regulation of the PM-located \( \text{Ca}^{2+} \)-permeable channels and \( \text{Ca}^{2+} \)-ATPase activities (Zhang et al., 2015a), we concluded that the SEDs selectively regulated the activity of the co-localized \( \text{Ca}^{2+} \)-permeable channels (Tables 5, 6; Fig. 5C). To our knowledge, this is the first reported presence of \( \text{Ca}^{2+} \) channels in a PM SED of a plant cell. However, such associations are well established for animal cells (Pani and Singh, 2009). Nevertheless, proteomic analyses of sterol-dependent proteins in detergent-resistant membranes of mature pollen grains detected a suite of \( \text{Ca}^{2+} \) signalling proteins including \( \text{Ca}^{2+} \)-ATPases (Han et al., 2018).

The sterol-dependent activity of the ETC \( \text{Ca}^{2+} \)-permeable channels appeared to be an all or none response achieved by their endocytosis into the endosome vesicle pathway in the absence of the SED in the ETC PM (Table 6). The sterol independence of \( \text{Ca}^{2+} \)-ATPase activity, that also occupies the outer periclinal region of the ETC PM, could be explained by their spatial inter-relationship with the \( \text{Ca}^{2+} \)-permeable channels. Within this ETC region, the \( \text{Ca}^{2+} \)-permeable channels are envisaged to be organized in clusters at the tips of developing WI papillae located between the WI papillae (Zhang et al., 2015a). This predicts that the SEDs are localized to WI papillae tips (Fig. 5D) where exo- and endocytosis are considered to be most active (Zhang et al., 2017a). Thus, blocking SED formation, by inhibiting sterol/sphingolipid biosynthesis, prevents vesicles carrying the \( \text{Ca}^{2+} \)-permeable channel docking with the tips of developing WI papillae (Fig. 5C). As a consequence, endocytosis predominates, with endocytosed vesicles carrying the \( \text{Ca}^{2+} \)-permeable channels being re-distributed equally around the ETC cytosol (Table 6).

**The sterol-enriched domain is essential for wall labyrinth formation**

The SED-dependent assembly of the polarized ETC wall labyrinth (Fig. 5B, D) joins that found for maintenance of polarized tip growth of root hairs and pollen tubes (Liu et al., 2009; Ovecka et al., 2010) and assembly of defence papillae (Faulkner, 2015). However, in the case of ETC wall labyrinth formation, the very disparate assembly patterns of the UWL and WI papillae (Xia et al., 2017) suggest that SED composition/spatial organization must undergo substantive shifts as wall labyrinth formation progresses from UWL to WI papillae construction. In the case of SED composition, there is a shift in the expression of genes encoding sterol biosynthesis between the phases of wall labyrinth formation (see Supplementary Table S5B) that could impact vesicle trafficking and PM properties (Pook et al., 2017; Sena et al., 2017). Moreover, UWL formation is characterized by an even deposition of matrix polysaccharides across the outer periclinal original wall and is independent of cellulose biosynthesis. The reverse applies to the cellulose-dominated assembly of WI papillae arising from loci on the cytoplasmic face of the UWL (Xia et al., 2017). Thus, the cytoskeleton-independent assembly of the UWL (Xia et al., 2017; Zhang et al., 2017a) must rely on ethylene/\( \text{apoH}_2\text{O}_2 \)-regulated formation of the SED (as described earlier) to provide a platform for docking and exocytosis of vesicles carrying cargoes of matrix polysaccharides (Fig. 5B). In contrast, the SED responsible for WI papillae formation must be localized in clusters, \( \approx 500 \) nm in diameter (Zhang et al., 2017a), in the ETC PM (Fig. 5D). While the \( \text{apoH}_2\text{O}_2 \) signal continues to define the outer periclinal domain for SED incorporation during WI papillae assembly (Fig. 4), whether the SED micro-pattern in the outer periclinal region is solely governed by \( \text{apoH}_2\text{O}_2 \) remains to be determined. The SED clusters define sites for insertion of the \( \text{Ca}^{2+} \)-permeable channels (Fig. 5C). These channels create cytosolic plumes of elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) that remodel the actin network to deliver vesicles, carrying cargoes of cell wall materials, to the \( \text{Ca}^{2+} \)-defined loci for WI papillae assembly (Fig. 5D; Zhang et al., 2017a). In addition, the predicted differing sterol composition of the SED between...
the UWL and WI papillae phases of wall labyrinth formation (Supplementary Table S5B) could impact cellulose biosynthesis activities (Schrick et al., 2012). The proposed sterol regulation might contribute to the lowered cellulose content of the UWL (Xia et al., 2017) and to the dense whorls of cellulose microfibrils essential for WI papillae formation (Talbot et al., 2007).

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Filipin staining co-localizes with the PM of ETCs.
Fig. S2. Temporal pattern of SED formation.
Fig. S3. Effect of the SED on $\Delta p$H$_2$O$_2$ distribution.
Fig. S4. Effect of the SED on Ca$^{2+}$ signalling and Ca$^{2+}$ channel distribution.

Table S1. Lengths of specified PM domains in ETCs and SPCs.

Table S2. Effect of blocking vesicle trafficking on SED distribution.

Table S3. Effect of sphingolipid, sterol, and brassinosteroid inhibitors on SED formation.

Table S4. Levels of biological control exercised by ethylene on SED formation.

Table S5. Impact of AVG on transcript abundance of sphingolipid- and phytosterol-related genes.

Table S6. Effect of vesicle trafficking on the regulation of sterol distribution by $\Delta p$H$_2$O$_2$ signalling.

Table S7. Impact of ascorbic acid on transcript abundance of vesicle trafficking genes.

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