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**Article:**

Hunt, L. and Gray, J.E. [orcid.org/0000-0001-9972-5156](https://orcid.org/0000-0001-9972-5156) (2020) How the stomate got his pore : very long chain fatty acids and a structural cell wall protein sculpt the guard cell outer cuticular ledge. *New Phytologist*, 228 (6). pp. 1698-1700. ISSN 0028-646X

<https://doi.org/10.1111/nph.16843>

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## Commentary

# How the stomate got his pore: very long chain fatty acids and a structural cell wall protein sculpt the guard cell outer cuticular ledge

The epidermis forms a crucial barrier between a plant and its environment. It stops uncontrolled loss of water by evaporation or transpiration and protects underlying cells from solar radiation and pathogens (Lewandowska *et al.*, 2020). Overlying the leaf epidermis is the cuticle which consists of a matrix of cell wall proteins and carbohydrates, interweaved fatty acids with various chain lengths (known as cutin) and an outermost layer of wax, which varies in constituents and thickness depending on species and in response to environmental stimuli. The leaf epidermis contains trichomes (hair cells), epidermal cells and stomatal guard cells, and it is likely that the cuticular wax composition varies with cell type although this has been little studied (Hegebath & Jetter, 2017). The majority of CO<sub>2</sub> entry and water loss occurs through stomatal pores, with smaller amounts diffusing through the epidermis when stomata are closed (Boyer, 2015). The ability to quickly close these pores or in the longer-term to adapt stomatal density in response to environmental conditions such as drought is well documented, but less is known about whether plants alter their cuticle composition to regulate water loss, and if such changes occur in an epidermal cell type-specific way (Holroyd *et al.*, 2002). In the recently published article in *New Phytologist*, Tang *et al.* (2020; doi: 10.1111/nph.16741) have identified a novel *Arabidopsis thaliana* mutant *osp1* (*occluded stomatal pore 1*) that has disrupted synthesis of cuticular waxes and shows structural alterations to the stomatal cuticular ledge. This phenotype, which is reminiscent of the previously described *fused outer cuticular ledge* mutant (*focl1*; Hunt *et al.*, 2017), has effects on both stomatal and nonstomatal water losses.

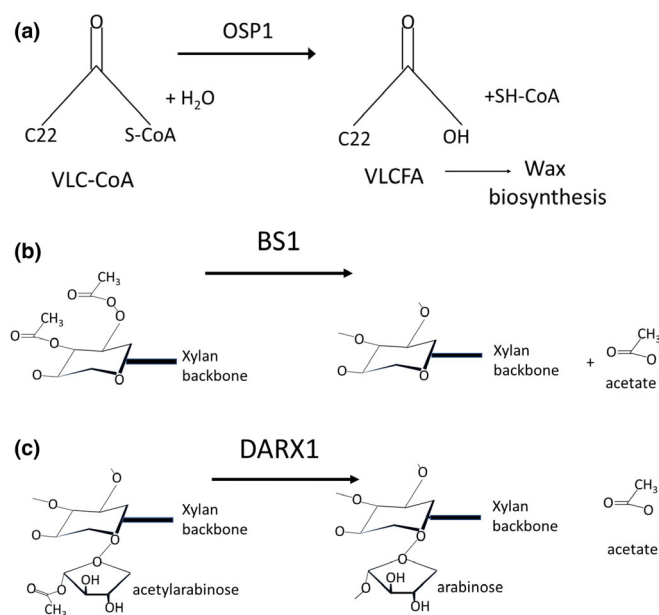
### Plants lacking OSP1 have altered wax composition and occluded stomatal pores

*OSP1* is highly expressed in guard cells and encodes a putative GDSL lipase, so called because of an arrangement of four conserved Ser-Gln-Asn-His motifs (Akoh *et al.*, 2004). These GDSL proteins form a large family in plants (Volokita *et al.*, 2011) and while annotated as lipases, few experiments have been carried out to define their likely *in vivo* substrate(s). Tang *et al.* show that *OSP1* has thioesterase activity. *OSP1* acts on C22:0-AcylCoA and C26:0-AcylCoA substrates to generate very long chain fatty acids (VLCFAs), which are major components of the cuticular waxes

and have roles as lipid signals. Like other GDSLs, *OSP1* shows broad esterase activity with artificial substrates but recent work on two rice mutants suggests that GDSLs may have functions beyond cutin and wax metabolism (Fig. 1). The *BS1* and *DEACETYLASE ON ARABINOSYL SIDECHAIN OF XYLAN1* (*DARX1*) GDSL

*We therefore propose that an interplay between a unique proline rich protein in the guard cell wall, and a specifically modified VLCFA component in the overlying wax cuticle, is required to form each stomatal pore.'*

lipases mutants both show secondary cell wall defects, which are most likely due to the lack of removal of acetyl groups from acetylated xylan and acetylated arabinosyl residues (Zhang *et al.*, 2017, 2019). Further evidence that lipids may not be the only substrate for these enzymes comes from analysis of the mucilage of *Arabidopsis* seeds which is rich in carbohydrates (rather than lipids) yet proteomic analysis suggests several GDSLs are present at high levels (Tsai *et al.*, 2017).



**Fig. 1** The known substrate specificities of plant GDSL lipases. *In vitro* experiments have shown that members of the GDSL lipase family can act on (a) *Arabidopsis thaliana* very long chain acyl-CoAs (VLC-CoA), which are precursors of very long chain fatty acids (VLCFAs) (in the recently published paper in *New Phytologist*, Tang *et al.*, 2020; doi: 10.1111/nph.16741); (b) rice acetylated xylan (Zhang *et al.*, 2017) and (c) acetylated arabinose (Zhang *et al.*, 2019).

This article is a Commentary on Tang *et al.* (2020), doi: 10.1111/nph.16741.

The *osp1* null mutant is the first *Arabidopsis* GDSL mutant to have an identifiable phenotype. The stomata of *osp1* lines are occluded, caused by an extension of the thickened cuticular ledge that surrounds the pore. This occlusion blocks transpiration and results in plants with increased temperature when observed by infrared imaging. This is an identical phenotype to that described by Hunt *et al.* (2017), in a totally unrelated proline rich protein mutant. *focl1* has stomatal pores that are blocked in a similar manner, resulting in increased leaf temperature and reduced plant size, most likely due to a reduced ability to take in CO<sub>2</sub>. Analysis of *osp1* revealed a reduction in the total wax contents, with specific reductions in the long chain C26 and C28 alcohols and C27–C33 alkanes. Cutin levels were largely unaltered. This led to *osp1* having increased epidermal permeability as shown by chlorophyll leaching experiments.

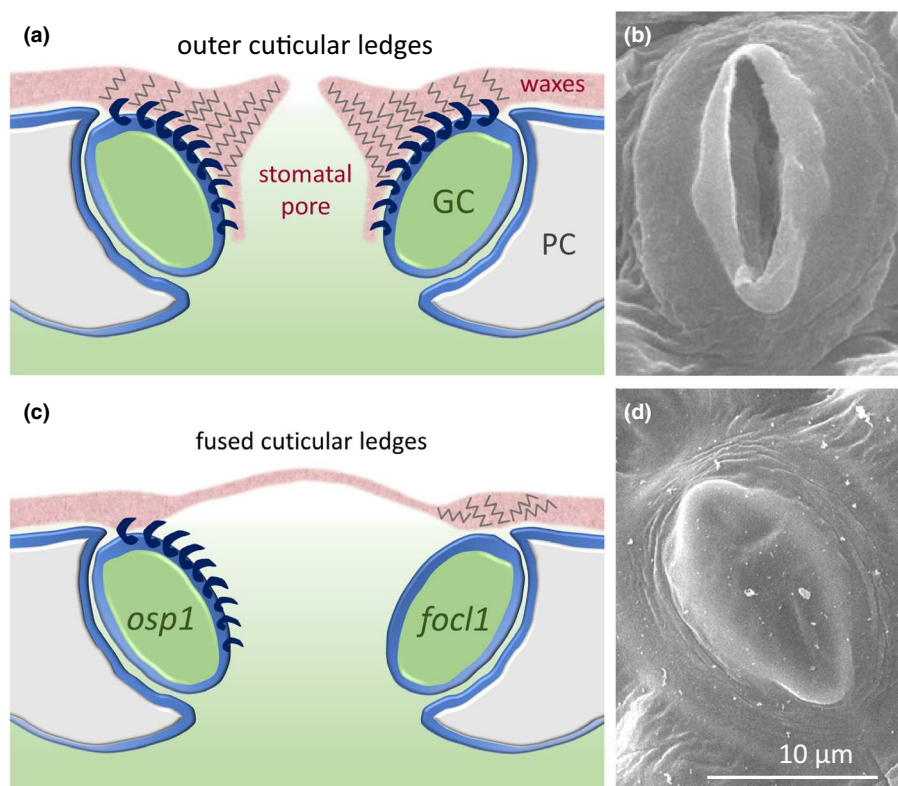
### What is the role of the cuticular ledge?

The outer cuticular ledge (OCL) is an extension of stomatal cell walls (Merced & Renzaglia, 2014). Its exact functions are

unknown, but the OCL is believed to prevent water droplets entering when the pore is open, to tilt its orientation to help open and close the stomatal pore, and to prevent water entry by sealing the pore when the stomate is closed (Fricker & Wilmer, 1996; Kozma & Jenks, 2007). The ledges are also proposed to provide a recognition point for pathogenic fungi that must locate stomatal pores to gain entry to their host (Hoch *et al.*, 1987). Absence of a cuticular ledge, as seen in the *gpta4gpta8* (*glycerol-3-phosphate acyltransferase 4/8*) double mutant reduces drought resistance but these mutants have defects in both pavement cell and guard cell cuticle structure and thereby alter both stomatal and nonstomatal water loss (Li *et al.*, 2007).

### What is the nature of the occlusion in OSP1?

There are now two reports of *Arabidopsis* mutants with cuticle-covered stomatal pores (Tang *et al.* and Hunt *et al.* (2017)) which raises the intriguing question of what links the FOCL1 and OSP1 proteins. Do they fulfil similar functions or act in the same pathway? Intriguingly, *FOCL1* and *OSP1* both show root expression, a tissue that does not have a wax layer suggesting both proteins



**Fig. 2** Proposed mechanism for stomatal cuticular ledge and pore formation. (a) Guard cells (GC) produce the extensin-like cell wall structural protein FUSED OUTER CUTICULAR LEDGE 1 (FOCL1, shown in dark blue) and the GDSL lipase OCCLUDED STOMATAL PORE 1 (OSP1) which catalyses the formation of specific very long chain fatty acid (VLCFA) components of the cuticular waxes (shown as grey zig zags). PC marks adjacent epidermal pavement cell. It is proposed that FOCL1 is anchored in the guard cell wall (blue) and interacts with the specific VLCFA OSP1 product in the waxes of the overlying cuticle layer (pink), thereby fixing it in position. (b) Scanning electron micrograph of wild-type *Arabidopsis thaliana* stomate showing structure of GC outer cuticular ledges. (c) Knockout mutants lacking either the product of OSP1 (left) or FOCL1 (right) fail to anchor the cuticle to the GC wall and do not develop the distinct shape and separation of the GC cuticular ledges. This results in occluded stomatal pores and prevents gas exchange. (d) Electron micrograph of *focl1* stomate with fused outer cuticular ledges and occluded pore.

may have wider interactions in different plant tissues. FOCL1, however, is not a GDSL lipase but a putative proline rich glycoprotein, more likely to have a structural role in the cell wall. The cuticle of *osp1* has an altered wax content, but why should this cause the two ledges to fuse together? This may be due to alterations in physicochemical properties of the wax on the ledge that normally hold the ledge apart, or the ledges may join together during development and fail to separate during pore formation. Alternatively, the wax may no longer be attached to the cell wall/cutin matrix beneath possibly through a specific wax–cell wall protein interaction.

### Very long chain fatty acids play unique and poorly understood roles in stomatal development

We have previously proposed that FOCL1 might facilitate interactions between the cell wall and the cuticle that are needed to sculpt the ledge (Hunt *et al.*, 2017). It now seems likely that OSP1 might provide the anchor points for FOCL interactions within the cuticle or cuticular wax (Fig. 2). We therefore propose that an interplay between a unique proline rich protein in the guard cell wall, and a specifically modified VLCFA component in the overlying wax cuticle, is required to form each stomatal pore. This is not the first time that VLCFA metabolism has been implicated in the regulation of stomatal development; 20 years ago *HIGH CARBON DIOXIDE (HIC)*, which encodes a putative enzyme involved in synthesis of guard cell VLCFAs, was shown to regulate stomatal development at excessive CO<sub>2</sub> levels (Gray *et al.*, 2000). Exactly how HIC modulates epidermal cell fate remains a mystery, but what is now clear is that the specific composition of guard cell cuticles is important for controlling both the number and morphology of stomatal pores.

### Acknowledgements

The authors received funding from the Biological Sciences Research Council (grant no. BB/N004167).

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**Key words:** cuticle, GDSL lipase, guard cell, stomata, very long chain fatty acid (VLCFA), wax.