Knocking on the heaven's wall: pathogenesis of and resistance to biotrophic fungi at the cell wall

Paul Schulze-Lefert

New findings challenge the traditional view of the plant cell wall as passive structural barrier to invasion by fungal microorganisms. A surveillance system for cell wall integrity appears to sense perturbation of the cell wall structure upon fungal attack and is interconnected with known plant defence signalling pathways. Biotrophic fungi might manipulate this surveillance system for the establishment of biotrophy. The attempts of fungi to invade also induce a sub-cellular polarisation in attacked cells, which activates an ancient vesicle-associated resistance response that possibly enables the focal transport of regulatory cargo and the secretion of toxic cargo. The underlying resistance machinery might have been subverted by biotrophic fungi for pathogenesis.

Addresses
Max-Planck-Institut für Züchtungsforschung, Department of Plant–Microbe Interactions, Carl-von-Linné-Weg 10, D-50829 Cologne, Germany
e-mail: schlef@mpiz-koeln.mpg.de

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Abbreviations

Bgh	*Blumeria graminis* f. sp. *hordei*
CWA	cell wall apposition
GSL5	GLUCAN SYNTHASE-LIKE5
MLO	powdery mildew resistance protein o
MtPT4	*Medicago truncatula* PHOSPHATE TRANSPORTER4
PEN1	*PENETRATION1*
PMR4	POWDERY MILDEW RESISTANCE4
ROR2	REQUIRED FOR MLO RESISTANCE2
SA	salicylic acid
SNAP	soluble *N*-ethylmaleimide-sensitive-factor-association protein
SNARE	SNAP receptor

Introduction

Penetration through the plant cell wall represents an Achilles heel in the pathogenesis of most biotrophic fungi and marks a lifestyle transition from extra-cellular to invasive growth. Modification of the plant cell wall was recognised for the first time as potential resistance mechanism almost 80 years ago following work in which 78 plant species and varieties were challenged with *Alternaria* spp. and other leaf-spotting fungi [1]. The termination of fungal pathogenesis at the cell wall was commonly associated with wall 'thickenings' and the formation of local additions or 'callosities' in the paramural space (i.e. the space between the cell wall and the plasma membrane). Formation of these cell wall appositions (CWAs) or papillae is usually accompanied by a co-localised accumulation of phenolics and reactive oxygen species [2–6]. The complex process of sub-cellular cell wall remodelling is tightly linked to the rapid disassembly and subsequent focal reassembly of the plant cytoskeleton at fungal entry sites, which is indicative of a pathogen-triggered cell polarisation [6–9]. There has been a long-standing controversy, however, over whether CWAs function in disease resistance or facilitate the entry of fungal pathogens into host cells by providing a structural collar for the intruder. New molecular genetic data from *Arabidopsis* and barley have indeed revealed that molecular processes at and in CWAs have Janus-faced functions, that is, functions for fungal pathogenesis and in resistance responses. Focal vesicle transport and vesicle fusion events that are dependent on SNAP (soluble *N*-ethylmaleimide-sensitive-factor-association protein) receptor (SNARE) proteins at the plasma membrane emerge as potential common underlying mechanisms that might be interconnected with a poorly understood cell wall integrity surveillance system. In this review, I discuss the seemingly paradoxical functions of these processes in establishing the biotrophic lifestyle and in disease resistance at the cell periphery.

Linking cell wall structure to biotic stress signalling

Callose, a (1→3)-β-D-glucan, has long been known to be synthesised and deposited rapidly at CWAs upon microbial attack. This polymer was thought to contribute to a physical barrier that slowed the invading microorganism and enabled the plant to focus anti-microbial compounds, such as wall-degrading enzymes, phytoalexins and active oxygen species, upon them [10]. Recently, it has been shown that a single glucan synthase-like isoform in *Arabidopsis*, GLUCAN SYNTHASE-LIKE5 (GSL5)/POWDERY MILDEW RESISTANCE4 (PMR4), is essential to synthesise papillary callose [11••,12•]. Mutants in which the *GSL5*/*PMR4* gene is disrupted exhibit broad-spectrum enhanced disease resistance to tested virulent powdery mildew fungi, including *Erysiphe cruciferarum*, *Golovinomyces orontii* and the oomycete *Peronospora parasitica* (but not to the bacterial pathogen *Pseudomonas syringae*). This indicates a role for the wildtype gene in the fungal colonisation of host plants rather than in

disease resistance. GSL5 callose also accumulates after the successful entry of fungal pathogens into host cells at haustorial complexes [12•]. One possibility is that the GSL5 callose synthase assists in the containment of pathogen-derived elicitors at infection sites, thereby preventing the perception of fungus-derived elicitors by the plant [13]. In addition, the (1→3)-β-D-glucan might have a role in protecting the invading fungus against plant-derived antimicrobial compounds.

Interestingly, disruption of the salicylic acid (SA) defence pathway (by mutation of *PHYTOALEXIN DEFICIENT4* [*PAD4*] or by expression of salicylate hydroxylase) in *gsl5*/*pmr4* mutants fully restored susceptibility to the powdery mildew pathogen, whereas impairment of the ethylene or jasmonate signalling pathways did not change the resistant phenotype [11••]. These findings exclude the possibility that GSL5/PMR4 functions as compatibility factor, and suggest that the callose synthase isoform is specifically interconnected with the SA defence pathway.

Why and how should an enzyme that is involved in cell wall biosynthesis be interconnected with SA-dependent resistance responses? In this context, it is conspicuous that each of nine identified ethyl methane sulfonate-induced *gsl5*/*pmr4* resistance alleles is a predicted null mutation [11••]. Thus, it is possible that GSL5/PMR4 exerts an indirect regulatory role through interactions with other proteins rather than through its catalytic activity. In *Saccharomyces cerevisiae*, a complex surveillance system monitors cell wall integrity. This system includes (1→3)-β-D-glucan synthase FK506 sensitivity (*FKS*) genes, cell-surface proteins that act as mechanosensors of changes in wall shape and a signalling cascade [14,15]. If surveillance of cell wall integrity is conserved in plants, one would expect that biotrophic fungi will have evolved means to manipulate components of cell wall integrity in order to suppress SA-dependent resistance. Consistent with this, inefficient SA-dependent resistance (often designated 'basal defence') limits the extent of powdery mildew growth in compatible interactions [16–18]. Further evidence of a connection between cell wall structure and stress signalling comes from the finding that a mutation in *Arabidopsis CONSTITUTIVE EXPRESSION OF VSP1* (*CEV1*), which encodes the cellulose synthase isoform CesA3, or treatment with inhibitors of cellulose biosynthesis leads to enhanced production of jasmonate and ethylene, to constitutive expression of jasmonate/ethylene stress-response genes, and to enhanced resistance to a broad range of pathogens [19•,20].

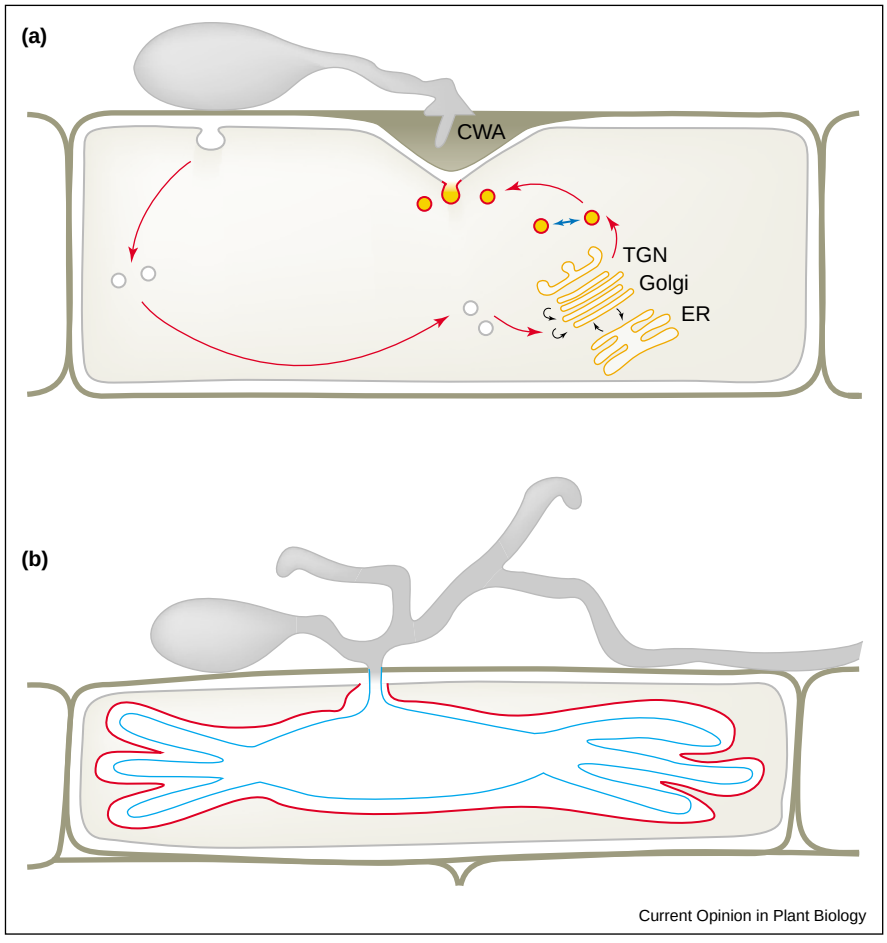
SNARE proteins and the first line of defence against fungal intruders

The other half of the Janus-faced biological functions at CWAs was revealed by the isolation of a novel class of *Arabidopsis* mutants and a previously described barley mutant [21•,22]. This work links processes at the cell wall with nonhost resistance to powdery mildew fungi. In wildtype *Arabidopsis*, nonhost resistance to the grass powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) is tightly associated with CWA formation and the failure of fungal sporelings to enter attacked leaf epidermal cells at most interaction sites [21•]. Recessive mutations in *PENETRATION1* (*PEN1*), *PEN2* or *PEN3* result in cell wall penetration rates of *Bgh* sporelings that are enhanced by up to seven-fold and allow the subsequent differentiation of haustorial complexes in leaf epidermal cells. However, the further growth of the inappropriate fungus is terminated in each *pen* mutant coincident with a cell death response of attacked epidermal cells. This cell death is reminiscent of the hypersensitive reaction (HR), which is triggered upon recognition of strain-specific pathogen effectors by race-specific resistance genes [23]. It remains to be tested whether the cell death in *pen* mutants responding to *Bgh* is mechanistically similar or dissimilar to the HR. *PEN1* encodes a plasma-membrane-resident syntaxin and represents one of 24 sequence-related family members in the *Arabidopsis* genome [24]. Syntaxins are conserved in plants and animals, and contain a heptad repeat 'SNARE motif' of 60–70 amino acids that can participate in coiled-coil formation. Syntaxins play a crucial role in vesicle trafficking by cycling in target membranes between a monomeric form and hetero-complexes that include various other SNARE-motif-containing proteins [25].

A similar, if not identical, SNARE-dependent resistance mechanism operates in barley, the natural host of *Bgh*. In compatible interactions, the plasma-membrane-resident barley syntaxin REQUIRED FOR MLO RESISTANCE2 (ROR2) accounts for a proportion of the penetration failures of fungal germlings at CWAs (designated 'basal penetration resistance') [21•,22]. This is consistent with an earlier hypothesis that plants limit pathogen growth even in compatible interactions by mounting inefficient resistance responses [26]. Of the 24 *Arabidopsis* syntaxins, PEN1 has the greatest resemblance to barley ROR2 [21•]. *PEN1* can complement the barley *ror2* mutant phenotype, hence the dicot *PEN1* and monocot *ROR2* syntaxin genes appear to be functionally synonymous. The apparent conservation of an isoform-specific syntaxin function in barley and *Arabidopsis* demonstrates that basal penetration resistance and nonhost resistance to *Bgh* share at least one common component. Further evidence for the involvement of SNARE-dependent resistance at the cell periphery came from the observation that a barley SNAP25 homologue, designated HvSNAP34, is required for basal penetration resistance to *Bgh*, contains two SNARE domains, and is capable of forming a binary SNARE complex together with ROR2 [21•].

In animals, binary SNARE complexes that consist of a syntaxin and a SNAP25 protein usually bind a third

Figure 1



Disease resistance at the cell wall and its potential subversion for the accommodation of intracellular fungal feeding structures. Schematic diagram of a **(a)** failed and **(b)** successful fungal invasion of a plant cell. (a) Plant cells normally respond to attempted fungal invasion by forming a cell wall apposition (CWA) at the site of attempted entry. Genetic evidence suggests the existence of an ancient SNARE-protein-dependent resistance reaction that is thought to involve focal transport of vesicles (small yellow circles) to and fusion of vesicles with the plasma membrane beneath an incipient penetration site. This exocytosis must be compensated by endocytosis (empty circles) to prevent net growth of the plasma membrane. Yellow colour indicates potentially toxic vesicle cargo. The blue double-headed arrow denotes the possibility of additional homotypic vesicle fusion events. Black arrows indicate constitutive vesicle trafficking between the endoplasmic reticulum (ER), Golgi, and trans-Golgi network (TGN). (b) Biotrophic fungi such as powdery mildews might exploit the SNARE-dependent resistance machinery at the cell periphery for the accommodation of intracellular feeding structures such as haustoria. An increased rate of exocytosis relative to endocytosis is thought to lead to invagination of the plasma membrane. The extra-haustorial membrane (red line) follows the contours of the haustorial membrane (blue line) and is physically continuous with the plasma membrane. The intracellular accommodation of feeding structures is essential for nutrient uptake and fungal growth (denoted by hyphal growth on the leaf surface).

partner, a membrane-anchored v-SNARE that resides in vesicles (also called an R-SNARE) [25,27]. These three complex partners are sufficient to overcome the energy barrier presented by the fusion of vesicle membranes with the syntaxin-containing target membrane and to help ensure the specificity of membrane fusion [28]. Thus, plasma-membrane located ROR2/PEN1 and HvSNAP34 may be components of an exocytosis pathway that prevents powdery mildew invasion at CWAs. However, genetic evidence for a contribution of one or several of the many plant v-SNARE-type genes to penetration resistance is lacking. Vesicles that accumulate reactive oxygen intermediates have been shown to congregate at CWAs, and the incidence of their congregation is associated with penetration resistance in barley [21•,29]. It remains unclear whether these vesicles contain v-SNARE proteins that associate directly with ROR2 and HvSNAP34. Although focal vesicle transport appears to be an attractive mechanism for concentrating the delivery and release of toxic cargo directly beneath fungal penetration sites (Figure 1a), future systematic characterisation of the vesicle cargo will be important to rule out alternative functions, such as the transport of regulatory proteins.

Previous work in a range of other plant–microbe interactions suggests that a stereotypic cellular polarisation and secretion process occurs at the cell periphery. For example, the epidermal cells of *Sorghum* leaves respond to attempted penetration of the hemibiotroph *Colletotrichum graminicola* with focal accumulation of coloured vesicles containing the antifungal 3-deoxcyanthocyanidin flavonoid phytoalexins apigeninidin and luteolinidin [30,31]. Shikonin, a red naphthoquinone derivative, is a secondary metabolite that has antimicrobial activity that specifically occurs in boraginaceous plants such as *Lithospermum erythrorhizon* [32]. Upon fungal elicitation, shikonin is secreted into the apoplast. The key enzyme in shikonin biosynthesis, geranyl diphosphate:4-hydroxybenzoate 3-geranyltransferase, is localised in 0.5–1.0 μm vesicles that are thought to be derived from the endoplasmic reticulum and to mediate shikonin secretion [33,34]. Likewise, onion epidermal cells respond to attempted invasion by the necrotising fungus *Botrytis allii* by forming CWAs and by focal secretion of free hydroxycinnamoyl amides, including feruloyl-3′methoxytyramine and feruloyltyramine [6]. In this case, however, it seems likely that the free hydroxycinnamoyl provides a pool of precursors for peroxidative cross-linking into the cell wall. Hence, the free hydroxycinnamoyl seems to prevent fungal degradation of the plant cell wall rather than to exert a direct fungi-toxic activity [6]. In cultured parsley cells, treatment with a fungal elicitor or exposure to UV-containing white light stimulates partly overlapping phenylpropanoid biosynthetic pathways [35]. Elicitor treatment induces a branch pathway that leads to the secretion of antimicrobial furanocoumarins, whereas UV light stimulates a different branch pathway that leads to the accumulation of flavonoids in vacuoles [35]. It should be interesting to find out whether these examples of pathogen-triggered secretion events are based on a common molecular framework, which might include components that are the same or similar to those in SNARE-dependent resistance to powdery mildews at the cell periphery [21•]. For example, the SNARE-dependent secretion machinery might have been conserved during evolution while the cargo became subject to diversification, enabling different plant species to present a diverse chemical bouquet to fungal invaders.

Suppression of disease resistance at the cell wall

The detection of an ancient SNARE-dependent and vesicle-associated process that mediates effective non-host resistance to *Bgh* in *Arabidopsis* and inefficient basal penetration resistance to this pathogen in the host barley raises the question of how biotrophic fungi bypass this 'first line of defence' in compatible interactions. Recent data suggest that the fungus might suppress the SNARE-dependent resistance layer by misuse of another host protein, MLO. Homozygous loss-of-function alleles of *MLO* (*mlo*) confer resistance to all known *Bgh* isolates, leading to the cessation of growth of fungal sporelings during cell wall penetration [36]. Barley *MLO* encodes the prototype of a plant-specific family of seven transmembrane domain proteins, accumulates in the plasma membrane, and interacts with the Ca^{2+} sensor calmodulin via a calmodulin-binding site in its carboxy-terminal cytoplasmic tail [37–40]. The *ror2* mutation was originally isolated as suppressor of *mlo* resistance (in a *mlo* null mutant background) and partially restores the susceptibility of *mlo* mutants to *Bgh* [41], suggesting that *MLO* might directly or indirectly antagonise *ROR2* function. The finding that overexpression of the wildtype *MLO* gene leads to super-susceptibility, rendering essentially all attacked epidermal cells susceptible to the invading *Bgh* fungus, is consistent with a negative regulatory role for MLO in disease resistance [39].

Arabidopsis MLO isoforms were found to interact directly with the PEN1 syntaxin in the split ubiquitin membrane yeast two-hybrid system (C Consonni, R Panstruga, P Schulze-Lefert, unpublished; [42]). In living barley leaf epidermal cells, the transiently expressed fluorescent reporter fusion proteins MLO::yellow fluorescent protein (YFP) and ROR2:: cyan fluorescent protein (CFP) produce a strong fluorescence resonance energy transfer (FRET) signal, indicating that a direct MLO–ROR2 interaction occurs *in vivo* (R Bhat, R Panstruga, P Schulze-Lefert, unpublished; [43]). A mutant ROR2 protein, which lacked 31 amino acids in the amino-terminal autoinhibitory domain, accumulated like the wildtype protein in the plasma membrane but failed to generate a FRET signal upon co-expression with MLO–YFP (R Bhat, R Panstruga, P Schulze-Lefert, unpublished; [21•,44]). Thus, MLO might sequester monomeric ROR2 in an inactive complex, thereby inhibiting/delaying productive cycling between free ROR2 and a SNARE complex that involves both HvSNAP34 and a putative v-SNARE. If this is true, there is an intriguing possibility that the fungal pathogen has evolved specific means to manipulate MLO activity during cell wall penetration in order to suppress/delay the SNARE-dependent fusion of vesicles with the plasma membrane. This may provide time needed for detoxification of vesicle cargo.

It is noticeable that despite exhaustive screening by breeders for *Bgh*-resistant mutants in barley, only one locus, *mlo*, has been identified. In contrast, several *Arabidopsis* mutants that have enhanced resistance to various virulent powdery mildew species have been found, including the *gsl5*/*pmr4* mutant mentioned above [45–47]. Interestingly, T-DNA insertions in *AtMLO2*, one of three *Arabidopsis AtMLO* homologues that share greatest sequence relatedness to barley *MLO* [48], are sufficient to render *Arabidopsis* resistant to the dicot powdery mildew, *G. orontii*. In contrast, T-DNA insertions in the other two *AtMLO* genes (*AtMLO6* and *AtMLO12*) do not affect the outcome of the interaction.

It remains to be tested whether *AtMLO2*-mediated resistance to *G. orontii* is impaired in a *pen1* background. If so, the suppression of SNARE-dependent resistance through particular MLO isoforms is an ancient evolutionarily conserved mechanism.

One would expect that different fungi have invented different molecular strategies to subvert SNARE-dependent resistance. In fact, the cowpea rust fungus *Uromyces vignae* elicits essentially no wall-associated defence upon invasion of its host *Vigna unguiculata*, whereas challenge by the same fungus on nonhost pea plants results in typical CWA formation [49•]. The lack of wall-associated defence responses in compatible interactions with rust fungi is thought to involve an active suppression mechanism that is linked to a disruption of cell wall–plasma membrane connectivity (as indicated by a reduction in the frequency of Hecht threads beneath attempted penetration sites) [49•]. Hecht threads are known to bridge the paramural space by physically connecting (unknown) sites in the cell wall and the plasma membrane [50]. The reduction in Hecht thread density appears to be a specific feature that is provoked by the rust fungus; the density of these threads was increased rather than decreased in both host and nonhost interactions with powdery mildews [49•,51]. If Hecht threads serve a role in cell wall integrity surveillance, then biotrophic fungi might target these structures to manipulate cell wall–plasma membrane connectivity to their advantage.

Intracellular accommodation of fungal infection structures

The invasive growth of biotrophs after cell wall penetration leads to the invagination of the plasma membrane and creates an interface between host and fungus that consists of the haustorial membrane, an extra-haustorial matrix, and the extra-haustorial membrane, which follows the contours of the haustorial membrane (Figure 1b; [52]). The presumed role of the haustorium in nutrient absorption has been supported experimentally by the identification and functional characterisation of proton-symport-driven transporters for hexose or amino acids that are resident in the haustorial membrane of the rust fungus *U. vignae* [53–55]. In contrast, the origin and function(s) of the extra-haustorial membrane of pathogenic biotrophs is still poorly understood.

Arabidopsis transgenic lines that express green fluorescent protein (GFP)::cDNA fusions of plasma-membrane-resident proteins are being used as tools in fluorescent-imaging studies to visualise the subcellular distribution of various plasma-membrane proteins during the invasive growth of *Erysiphe cichoracearum* (S Koh, S Somerville, unpublished; [56]). Fluorescent signals were absent from haustorial complexes, suggesting that the extra-haustorial membrane may either lack any protein or contain proteins that are unique to this membrane. Interestingly, in symbiotic interactions between plants and arbuscular mycorrhizal fungi, elegant work points to an accumulation of specific proteins at the periarbuscular membrane, a probable structural analogue of the extra-haustorial membrane [57••]. The *Medicago truncatula* phosphate transporter MtPT4 was shown, by complementation of yeast phosphate transport mutants, to function in phosphate uptake. Immunolocalisation analysis revealed that MtPT4 co-localises with the arbuscules, consistent with a localisation in the periarbuscular membrane. It is tempting to speculate that MtPT4 acquire the phosphate released by the fungal symbiont. Although the periarbuscular membrane of symbiotic fungi, like the extra-haustorial membrane of pathogenic biotrophs, is believed to be physically continuous with the plasma membrane, the absence of MtPT4 in the plasma membrane of cells that contain arbuscules is a striking observation. It suggests the existence of a specific targeting mechanism for periarbuscular membrane proteins.

Conceptually, the formation of a periarbuscular membrane or an extra-haustorial membrane could be explained by a net growth of the plasma membrane (Figure 1b). Exocytosis is typically balanced by endocytosis events to prevent net growth of the plasma membrane [58]. Indeed, clathrin-coated pits and coated vesicles, which serve as endocytosis markers, were detected by immunolabelling in high numbers at *U. vignae* penetration sites in broad bean epidermal cells [59,60]. An excess rate of exocytosis compared to endocytosis would inevitably result in the invagination of the plasma membrane.

Conclusions

There is an intriguing possibility that pathogenic and symbiotic biotrophic fungi have evolved similar strategies to manipulate normally coupled exo- and endocytosis pathways such that exocytosis prevails. It remains to be shown whether the same or different vesicle-trafficking pathways become activated in response to attempted cellular invasion by both classes of fungal microorganisms. In this context, it might be relevant that the expression of the *MtPT4* gene occurs exclusively in cells containing arbuscules, suggesting the existence of a specific gene-induction mechanism [57••]. Thus, one possibility is that different gene-induction pathways contribute to generate vesicle-cargo diversity in pathogenic and symbiotic interactions. In one scenario, biotrophs could have exploited the ancient vesicle-associated and SNARE-dependent resistance machinery at the cell periphery for the generation of extra-haustorial or periarbuscular membranes (Figure 1a,b). This could explain why the protein make-up of these specialised membranes and the plasma membrane is different.

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