Arbuscular cell invasion coincides with extracellular vesicles and membrane tubules

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Abstract:

During establishment of arbuscular mycorrhizal (AM) symbioses, fungal hyphae invade root cells, producing transient tree-like structures, the arbuscules, where transfer of photosynthates for soil minerals occurs. Arbuscule formation and collapse lead to rapid production and degradation of plant and fungal membranes whose spatiotemporal dynamics directly influence nutrient exchange. We determined ultrastructural details of both membrane surfaces and the interstitial apoplastic matrix during growth and senescence of *Rhizophagus irregularis* arbuscules in rice. Invasive growth of arbuscular hyphae was associated with abundant fungal membrane tubules (memtubs) and plant peri-arbuscular membrane evaginations. Similarly, the phylogenetically distant AM fungus, *Gigaspora rosea* and the fungal

maize pathogen, *Ustilago maydis,* developed memtubs while invading host cells, revealing novel structural commonalities independent of the mutualistic or parasitic outcome of the interaction. Additionally, extracellular vesicles formed continuously in the peri-arbuscular interface from arbuscule biogenesis to senescence, consistent with signal delivery contributing to inter-organismic communication throughout the arbuscule lifespan.

Introduction:

Arbuscular mycorrhiza (AM) symbiosis is an ancient symbiosis between roots of most plant species and fungi of the Glomeromycotina that evolved concurrent with land plants around 450 million years ago and ever since represents an integral part of plant terrestrial ecosystems (for recent review see[1]). The outcome of the symbiosis is mutualistic, manifested in a bi-directional transfer of soil minerals, such as inorganic phosphate (Pi) in exchange for host-derived carbon. Central to the symbiotic nutrient trade are specialized fungal feeding structures, called arbuscules that form inside root cortical cells. Arbuscule development starts by establishing a 'trunk' domain from where sequential dichotomous hyphal branching and tip growth produce coarse 'support' branches on which an extensive network of 'canopy' fine branches bifurcate until most of the host cell volume is filled, generating the characteristic tree-shaped structure[2]. Simultaneously, the plant plasma membrane envelops the growing fungal structure to establish the peri-arbuscular membrane (PAM). Thereby an apoplastic peri-arbuscular space (PAS) is generated between the fungal arbuscule membrane (FAM) and the plant PAM, which has been described as an 'amorphous' matrix that is continuous with the host cell wall and contains wall–related macromolecules[3]. The plant-fungal exchange of nutrients within the arbusculated cell is thought to involve release into the peri-arbuscular space (PAS), followed by uptake across either the plant PAM of the FAM.

Plant Pi and ammonium transporters with a specific role in AM symbiosis mediate transport processes at the PAM[4,5,6] whereas equivalently fungal transporters have not been reported. Plant Pi transporters of the type of the rice PT11 (*Medicago truncatula* PT4[5]) reside in PAM zones surrounding arbuscular fine branches but are absent from PAM around coarse branches or the trunk[5,7]. As these transporters mediate most if not all of the symbiotic Pi uptake[8,9], their PAM distribution pattern

indicates functionally distinct membrane subdomains. Arbuscule establishment is also essential for the organic carbon nourishment of the obligate fungal biotroph to complete its asexual life-cycle[10]. As fatty acid (FA) heterotrophs[11] AM fungi rely entirely on the supply of FA by their plant hosts[12,13,14,15]. Conceivably, plant FAs ultimately provide the building blocks for the massive *de novo* fungal plasma membrane biosynthesis associated with arbuscule, especially fine branch formation (reviewed in[16]). The mechanism by which FAs are delivered to the fungus are currently unknown. However, PAM-specific half-size ABCG transporters Stunted Arbuscule 1 and 2 (STR1, STR2) have been proposed as FA exporters in *M. truncatula*[17], suggesting that the uptake of Pi and the release of FA might be spatio-temporally linked.

Despite the seemingly huge fungal and plant investment into producing and accommodating arbuscules, these are ephemeral structures with a lifespan of only one to two days in rice[18]. Arbuscule development thus reflects an immensely vigorous cell invasion process, brought about by reiterated hyphal bifurcation and elongation that is based on polar hyphal tip growth, common to filamentous fungi. Senescence of arbuscules resembles the inverse procedure, commencing by the collapse of the fine branches that rapidly proceeds across the more basal parts of the structure until the entire arbuscule has been removed. The window for symbiotic Pi uptake (and likely FA release) appears hence restricted to the highly dynamic and short period of fine branch formation.

To capture the ultra-structural detail of the plant and fungal membrane surfaces during the successive developmental range of growing and collapsing arbuscules, we performed TEM tomography and 3-D reconstruction of high pressure frozen, freeze substituted rice roots colonized by *R. irregularis*. We found that instead of smoothly aligned parallel membranes, separated by a homogenous interfacial matrix, plant and fungal membranes adopted complex 3D structures. Invasive arbuscular growth was associated with the extensive production of paramural membraneous tubules (memtubs), a feature that occurred similarly during maize leaf cell infection by the ear smut fungus *Ustilago maydis*. PAM evaginations populated the PAS together with extracellular vesicles (EVs) of plant or fungal origin all through the different stages of arbuscule life, suggesting not only a further

significant increase in contact area between the two symbionts but also sophisticated mechanisms for inter-organismal cell-to-cell communication.

Results:

Classification of arbuscular hyphae

Images of arbuscules typically show a collection of hyphae, which in micrographs are distinguishable from the rice cortical cell cytosol by the densely stained fungal cell walls and presence of small circular vacuoles (Supplementary Fig. 1a). To ensure membrane surfaces in colonized roots were retained as close to their native state as possible, we used high-pressure freezing followed by freeze substitution (HPF-FS) to preserve rice root tissue. Indeed, in chemically fixed rice tissue we consistently observed fixation artifacts consisting of irregular membrane undulations (Supplementary Fig. 1b). Using HPF-FS of colonized rice root tissue we found excellent preservation of fungal and plant membranes, whilst membrane undulations associated with fixation artifacts were not observed (Supplementary Fig. 1c).

With the aim to characterise plant and fungal membrane details during the arbuscule lifespan, we required a strategy to accurately discriminate arbuscule fine from large hyphal branches in TEM cross sections. Trunk and low-order hyphae were visually larger in diameter than smaller fine hyphae, however, since arbuscule formation is a continuum of hyphal branches, and hyphae are often obliquely sectioned (Supplemental Fig ?), the identification of fine hyphae based on hyphal diameter alone can be challenging. During filamentous hyphal growth fungal cell walls are progressively thicker towards the older more distal part of the fungal hypha as new cell wall (CW) material is deposited just behind the growing hyphal tip[19]. The fungal CW of arbuscule fine branches are typically thinner than larger mature and arbuscule trunk hyphae[20],[21]. We, therefore, inferred that fungal CW thickness, combined with hyphal diameter, could be used to distinguish fine from large/trunk fungal hyphae in TEM, which is required to determine the relative position of a hypha within the arbuscular structure.

Hyphal diameter and CW thickness were thus measured from micrographs, whilst simultaneously visually categorizing hyphae as either fine, large or trunk. Since hyphal CW thickness is dependent on the angle of a section, the thickness of

the section and the curvature of the structure, we consistently measured CW thickness taken from the thinnest point of the fungal CW surrounding a hypha (Supplemental Fig 1?). Selecting hyphae randomly from TEM micrographs we found a good correlation between hyphal diameter and fungal CW thickness (R^2=0.88, n=61 hyphae taken from 8 micrographs across 7 independent biological replicates), which provided the training dataset and generated a simple linear regression model (y=42.2*CWT+131.3; Supplementary Fig. 2a) with an average mean absolute percentage error (MAPE) of 25% (tested 100 fold using a 80:20 ratio). We next carried out unsupervised K-means testing on our training dataset, which identified three clusters that closely matched our visual classification of hyphae as fine, large and trunk (Rand index value of 0.961, Supplementary Fig. 2a). A mean hyphal diameter corresponding to cluster centers of 866,81nm, 2058.40nm and 3506.82nm and CW thickness of 18.76nm, 47.26nm and 65.49nm were obtained for fine, large and trunk hyphae, respectively. An excellent differentiation between arbuscule fine branches and larger trunk hyphae was obtained, although the distinction between large and trunk hyphae was less clear (Supplementary Fig. 2a). We next determined if fungal CW thickness could be used to predict hyphal diameter and, together with K-means testing, to ultimately classify hyphae. A Rand index value of 0.751 (Supplementary Fig. 2b) was obtained. In spite of overlap between large and trunk hyphae, arbuscule fine hyphae were clearly discriminated, indicating that fungal CW thickness combined with K-means testing could be applied to accurately discriminate fine hyphal branches from large and trunk hyphae and this corresponded well with our visual classification of fine and thick hyphae in TEM-based analyses.

We frequently observed collapsed hyphae adjacent to viable fungal hyphae (Supplementary Fig. 1a), suggesting that arbuscule fine hyphal branching and collapse are unsynchronized with both simultaneously present in an arbuscule. Collapsed fungal hyphae appeared distorted with compressed CW, mostly entirely devoid of fungal cytoplasm (Supplementary Fig. 1a), as recorded previously[22].

Fungal plasma membrane tubules are preferentially associated with arbuscular fine branches

Unexpectedly, we observed an extensive accumulation of paramural structures in the extracellular space between the fungal plasma membrane and CW

(Fig. 1). These structures were homogeneous in diameter (49.36nm, ±14.3nm, n=80 hyphae taken from 8 micrographs across 3 independent biological replicates) and delineated by a well-defined lipid bilayer (Fig. 1b,c). While most of them appeared vesicular in cross section, transverse sections revealed that they were membrane tubules (memtubs, Fig. 1). Interestingly, memtubs were not evenly distributed in the paramural space, but were clustered together in distinct lateral pockets (Fig. 1a, e, h) or between two fungal protoplasts within a single fungal CW (Fig.1d). This created the impression of a cross-section just above the point of hyphal bifurcation, however, the possibility that memtubs were captured in the inner fold of a bending hypha cannot be excluded. Occasionally, small pockets of memtubs were also observed on the side of thick and/or trunk brunches, however, these appeared less extensive that those associated with fine branches (Fig. 1f,g). In fully collapsed hyphae, fungal protoplasts were entirely absent, however, in partially collapsed fine hyphae memtub-like structures were present (Fig 1h,i). However, it was unclear if the structures observed were memtubs or remnants of retracting fungal protoplast. The observation by Ivanov et al in the accompanying manuscript that memtubs accumulate in collapsing fine branches would suggest that once formed memtubs remain associated with fungal hyphae until their collapse. .

To determine when during arbuscule growth memtubs are most likely forming, we measured the frequency by which memtubs are observed in fine as compared with large/trunk hyphae. Our analysis showed that only 28% of all fine hyphae contained memtubs (n=258) supporting our earlier conjecture that memtubs are infrequently formed (Supplementary Table 1). Moreover, the number of memtubs associated with thick hyphal branches (large and trunk hyphae) was significantly less as compared with fine hyphae (15%, n=173, p=0.015). Together, the low abundance of memtubs at large and trunk hyphae and their preferential association with thin hyphae suggested that they are likely formed during actively growing or bifurcating arbuscular hyphae, but once formed persists until hyphal collapse.

Memtubs are continuous with the fungal plasma membrane

To determine if memtubs were continuous with the fungal plasma membrane, thereby confirming that they were of fungal origin, and also to gain further insights into their spatial organisation, semi-thin sections we generated for TEM tomography.

In tomograms, we frequently found memtubs protruding from the fungal plasma membrane into the paramural space (Fig. 2a-b; Video 1). Some memtubs remained connected to the fungal cytosol as open tubes (Fig. 2a and 2f; Video 1) while in other cases narrow constrictions between the memtub and the fungal plasma membrane (Fig. 2b and 2f, Video 1), or also ramifying tubules were observed (Fig. 2f-k, Video 1). Interestingly, memtubs appeared highly elongated, forming an interconnected network of memtubs separated by narrow membrane constrictions (Fig. 2c-e, Video 1). Although reminiscent of the budding off of plasma membrane ectosomes, distinctly separate extracellular vesicles (EVs) were not detected. We additionally found that open-type of memtubs adjoined to form cytoplasmic bridges between cytosols of early bifurcating hyphae, which were still surrounded by one fungal cell wall (Fig. 2f-l, Video 1). Collectively, we present evidence for an intricate plasma membrane tubular network and connective tubules during arbuscule fine hyphal branching.

Fungal memtubs are a conserved feature of invasive hyphal growth

To determine if memtubs are more broadly associated with arbuscule development in different AM fungal species, we examined rice roots colonized by *Gigaspora rosea*. Indeed, membrane tubules similar to those observed from *R. irregularis* were present in the paramural space of a *G. rosea* arbuscule fine hypha (Supplementary Fig. 3a), whereas they were absent from large or collapsing hyphae. Moreover, fungal memtubs were also observed in *R. irregularis* and *Glomus versiforme* colonized *Brachypodium distachyon* and *Medicago truncatula*, respectively (Ivanov et al., accompanying manuscript).

Therefore, the formation of memtubs appears to consistently coincide with arbuscular fine branch development of phylogenetically distant AM fungi.

However, we occasionally observed memtubs associated also with intra-radical hyphae in paramural pockets wedged between plant and fungal CW suggesting they are not an exclusive feature of arbuscules and AMS (Supplementary Fig. 4). We therefore investigated if memtubs were a generic feature associated with invasive growth of fungal hypha. To this end, TEM analysis was carried out with maize leaf sheath tissue colonized by the corn smut fungal pathogen *Ustilago maydis.* Tissue was collected at one to two days post inoculation (dpi) to capture the

stage of early appressorial penetration into the leaf epidermis[23]. Tubular vesicular membrane structures resembling memtubs were found in the paramural space of a young penetrating fungal hypha (Supplementary Fig. 5). The variable presence of memtub-like structures on intracellular fungal hyphae suggested that also in *U. maydis* memtubs might occur at specific stages of hyphal growth. Moreover, the presence of memtubs during the early stage of cellular infection by *U. maydis* indicated that these are formed during invasive fungal hyphal growth in both beneficial and pathogenic filamentous fungi.

PAM protrusions and membrane-bound vesicular structures occur preferentially in the PAS around arbuscular fine branches

It is largely assumed that the PAM envelopes the arbuscule like a glove, however, our TEM analysis of *R. irregularis*-colonised rice cortex cells reproducibly showed that the PAM enclosed an apoplastic PAS of variable size, often surrounding more than one fungal hypha (Fig. 3a,b). The PAS has previously been described as an amorphous, and therefore somewhat 'neutral' matrix[3]. Our TEM approach however revealed that the PAS in addition contains an extensive number of membrane-bound structures of heterogeneous shape and size (Fig. 3), adding to the membrane complexity. We distinguished complex swirl-like PAM evaginations (Fig. 3b, Video 2) from vesicular-appearing membrane-bound structures (Fig. 3c, Video 2). In contrast to the fungal memtubs, the diameter of the PAS structures varied widely, spanning two orders of magnitude from ~30nm to ~3000nm. In addition, the apoplastic vesicular structures (AVS) differed in electron density suggesting they were heterogeneous (Fig. 3c).

The symbiosis-specific rice Pi transporter PT11 uniquely localizes to the PAM subdomain surrounding expanding fine branches and would therefore be expected to be present on the observed PAM evaginations. To validate the association of the PAS-internal AVS with arbuscular fine branches, we performed immunogold labelling (IGL) on PT11-GFP expressing rice plants using the anti-GFP antibody. Immunogold particles localized to the PAM and to a few AVS within the PAS of transgenic roots only (Supplementary Fig. 6a and b), thereby indicating that at least some AVS were derived from the PAM.

Localisation of PT11-GFP with a subset of AVS suggested that they were associated with fine arbuscule branches during symbiotic Pi uptake. Therefore, to determine if AVS are preferentially associated arbuscule hyphae we determined the frequency by which AVS accumulated across all fungal hyphae. We found a significantly higher number of hyphae contain AVS as compared to memtubs (53%, n=431, $p=3.938^{e-10}$) (Supplemental Table 1). Moreover, unlike memtubs that associated more frequently with fine hyphae, no significant difference was observed in the frequency by which AVS accumulated around fine (52%, n=258) as apposed to large/trunk (54%, n=173) hyphae suggesting AVS are ubiquitously produced during arbuscule formation. Interestingly, AVS were also found in the PAS surrounding collapsing fine branches, often however appearing smaller in size and less electron-dense compared to those present around intact hyphae (Fig. 3d), which may be linked with reduced membrane deposition as the branch commences to collapse. This further suggests that AVS remain associated with arbuscule hyphal branches throughout their formation and collapse.

To determine if AVS are a widespread feature of AMS we examined the PAS of *G. rosea* colonised rice cells. An accumulation of membraneous bodies that were similarly delineated by a clear lipid bilayer and appeared heterogeneous in size, shape and electron density were also observed (Supplementary Fig. 4). The equivalent appearance of the PAS-internal structures in *R. irregularis* and *G. rosea* infected roots suggested common membrane remodeling mechanisms during cell invasion by these distantly related AM fungi. Presence of structures similar in appearance and heterogeneity to AVS are also described in the accompanying manuscript by Ivanov *et al* supporting our hypothesis that AVS occur widespread during AMS.

PAS-localised AVS consist of PAM-connected, interconnected and separate extracellular vesicles

To determine if the AVS were connected to the PAM as we had seen for the fungal memtubs, or would also include separate extracellular bodies, we performed 3-D tomography and IMOD reconstruction (Fig. 4a-l, Video 2). In the PAS around thin arbuscular branches we monitored vesicles that were either separate from the

PAM but linked with one another, containing narrow, stalk-like connections (Fig. 4a-d, Video 2) or appeared as free individual units of extracellular vesicles (EVs, Fig. 4 a-f, Video 2). Although imaging artifacts can formally not be excluded, the appearance of EVs resembled structures made in other plant-fungal interactions[24-26]. Further support for the occurrence of EVs is provided by the observation of similar structures in the PAS surrounding arbuscules of *G. versiforme* in *M. truncatula* cortex cells (Ivanov et al., accompanying paper). In addition, our tomography captured a PAM-continuous evagination in the same PAS, which also showed a noticeable constriction between the PAM and the more vesicular part (Fig. 4g-l, Video 2). In contrast to memtubs, tubular connections between different neighboring PAMs were not observed. In summary, diverse types of membrane structures populate the PAS, including PAM evaginations of variable size and shape, and single or clustered interconnected EVs, consistent with a role in intercellular communication.

Multivesicular bodies fuse with PAM

The release of EVs into the apoplast can be due either to vesicles budding off the plasma membrane (ectosomes) or by fusion of multivesicular bodies (MVB) with the plasma membrane (exosomes)[27]. Either mechanism may be involved in the generation of EVs in the PAS. On the one hand, the narrow constrictions observed on PAS vesicular bodies would suggest that pinching off might occur. On the other hand, we frequently found MVBs in the host cytoplasm in proximity to the fungal hyphae (Supplementary Fig. 7a), and also observed plant MVBs that appeared to fuse with the PAM in areas where vesicular structures were found in the adjacent PAS (Supplementary Fig. 7b), thereby suggesting that the MVB pathway may contribute to EV secretion.

Discussion:

During arbuscule formation, the switch from lower order to higher order hyphal branching generates a dramatically increased surface area where symbiotic nutrient exchange occurs. We found highly elongated fungal tubular membrane structures in spatially restricted paramural pockets on opposite sides of thin-walled, small hyphae or juxtaposed between interconnected bifurcating hyphae, indicating that these memtubs form during invasive hyphal growth. Paramural vesicles from AM fungi

have been reported as early as 1961[28] and repeatedly since for a range of different AM fungal species[29,30,31,32,33,34]. Their organisation into tubular networks, continuous with the FPM, has in the absence of 3-D tomography however been largely overlooked. Since memtubs not only occurred during cell invasion by diverging AM fungi but also in the ear smut fugus *U. maydis*, we hypothesize a generic role during cell-invasive hyphal growth. Indeed published reports of several axenically grown filamentous fungi and yeasts documented the presence of membrane tubular structures called lomasomes/plasmalemmasomes in the hyphal paramural space[29]. Lomasome-like structures were also observed in the apical zone behind the growing tip of extracellular branched absorbing mycelia of monoxenic symbiotic cultures of *R. irregularis* and thus a role for lomasomes was suggestion in cell wall deposition and hyphal growth *(Bago et al 1998)*. Intriguingly in 2-D micrographs these structures resemble memtubs described here suggesting that memtubs may occur both during filamentous growth and during intercellular tissue invasion. This would indicate a generic function for memtubs that could include a role in water or nutrient uptake or hyphal cell wall deposition as opposed to a specialized function in the context of symbiosis.

The presence of memtubs between bifurcating AM fungal hyphae and their infrequent association with larger hyphae would argue against a role for memtubs during hyphal tip growth. Conceivably, memtubs could increase the absorptive surface area for the acquisition of plant-derived compounds that would diffuse through the fungal cell wall. By analogy, in the giant algae *Chara coralline* a positive correlation was found between the rate of chloride influx and the associated increase in membrane surface area via an interconnected membrane tubular network named charasomes[35,36]. We favour however an alternative scenario where memtubs would be involved in the release of molecules that modulate their environment to facilitate hyphal growth, including perhaps also the secretion of fungal effectors to manipulate the host cell. Although we were unable to detect the shedding of microvesicles from memtubs, the delivery of 'bioactive cargo' via EVs cannot be excluded. Furthermore, the finding of memtubs interconnecting bifurcating arbuscule hyphae is perplexing as it suggests a symplastic connection between hyphae that are undergoing spatial separation while in addition sharing a common stem hypha. Memtubs are predominantly associated either with such early forking hyphae that at this stage are

still contained within the same fungal cell wall, or found in lateral pockets of single hyphae. The spatio-temporal relationship between the two patterns, for instance such that memtub production in lateral pockets would be remnants of hyphal branching is unclear.

In addition, we documented the accumulation of EVs within the PAS surrounding arbuscular hyphal branches. These were of different electron density, which might either indicate their heterogeneous cargo, or alternatively, their heterogeneous origin, being plant or fungus derived. Although we at this point cannot provide experimental evidence, it can formally not be excluded that also the fungus releases EVs into the PAS.

EVs have been described to consist of both ectosomes and exosomes that facilitate not only intercellular but also interspecies communication[26] (reviewed in[37]). The nature of the PAS-internal EVs is presently unclear as the vesicular constrictions of PAM evaginations would be reminiscent of a stage just before pinching off the vesicle. PT11-eGFP signal on vesicular structures within the PAS would be consistent with ectosomes that had budded off the PAM, however in 2-D IGL imaging the distinction between free and connected vesicles cannot be made. Our finding is in agreement with previous reports about the localization of the symbiotic H^+ATPase to the PAM and to membranous vesicles present in the PAS[38,39,40]. However, also here the documentation was limited to 2-D IGL, thereby not permitting the distinction between free or attached membrane structures. EVs that resemble ectosomes were also detected in *G. mossae* colonizing different plant species as well as in arbuscule fine hyphae from natural inoculum[41], suggesting that they are ubiquitously produced during arbuscular fine branches, which is consistent with our observation that EVs are also present during the rice-*G. rosea* association.

In addition to an accumulation of MVBs around arbuscular hyphae, we observed MVBs that appeared to fuse with the PAM, thereby confirming that exosomes may also be released during arbuscule formation. Thereby proportions of the host cytoplasm would intriguingly be transferred into the PAS that could contain nucleic acids, proteins and nutrients. In plant-pathogen interactions, EVs corresponding to exosomes accumulated at sites where early defense structures, namely CW appositions, were forming[24,25]. Exosomes were also present in the extrahaustorial matrix of the biotrophic powdery mildew pathogen *Golovinomyces*

orontii and *Arabidopsis thaliana*[42]. Moreover, proteome analysis of EV cargo from the apoplast of *Arabidopsis thaliana* and sunflower showed that EVs were enriched for a diverse range of stress and defense proteins, confirming a role for exosomes in innate immunity and intercellular communication[43,44]. In addition, only 16% of proteins present in EVs had signal peptides suggesting a role for EVs in non-canonical secretion[43].

Importantly, recent studies have shown that fungi are capable of taking up host EVs; for instance, spores of the phytopathogenic fungus *S. sclerotium* were able to take up EVs isolated from sunflower causing a suppression of fungal growth[44]. Similarly, *Arabidopsis thaliana* EVs containing small RNAs (sRNAs) are acquired at infection sites by the fungal pathogen *Botrytis cinerea*[26], demonstrating a role for plant EVs in inter-kingdom communication to combat the invading microbe, more specifically in host-induced gene silencing (HIGS). Interestingly, HIGS of the *R. irregularis Monosaccharide Transporter 2* in *Medicago truncatula* confirmed that also in AM symbiosis plants transferred small interfering RNAs to AM fungi[45], however, an involvement of EVs was not shown.

In summary, our study provides an ultrastructural 3-D reconstruction of the hitherto little understood dynamic and spatial reorganization of plant and fungal membranes as well as of the intermittent PAS during the arbuscule lifespan. Future identification of proteins and cargo of EVs during arbuscule formation and collapse will provide exciting novel insights into the host-fungal dialogue at the PAS and uncover novel mechanisms of arbuscule functioning.

Methods

Plant and fungal material

Seven day old maize inbred line Zea mays (L.) (corn) seedlings of the variety Early Golden Bantam (Olds Seeds, Madison, WI, USA) and two to three week old *Oryza sativa* ssp. japonica cv. Nipponbare wild type and transgenic rice lines carrying *PromPT11:PT11-eGFP*[7] were used in this study. For rice inoculation with beneficial fungi, de-husked seeds were surfaced sterilized in 3% hypochlorite solution and pre-germinated on 0.3% Bacto-agar plates for 4 days at 30°C in the dark. Germinated seedlings were transferred to 60mm petri-dishes containing untransformed Nipponbare rice, which were colonized with *R. irregularis* or *G. rosea* and used at 6-

weeks post inoculation (wpi), thus functioning as high inoculum strength 'nurse plants'. Rice seedling roots were harvested 10 days post inoculation (dpi). Plants were grown in a growth chamber with a 12h/12h day/night cycle at 28°C/22°C and 60% humidity. Plants were fertilized every second day with half strength Hoagland solution, containing 25μM of KH_2PO_4. Seedlings were harvested for imaging at 10 days post inoculation (dpi).

To infect maize with *Ustilago maydis*, the solopathogenic strain ULL152 (SG200Suc2AvitagHA-Pcmu1-GFP-Avitag HA) was generated. This strain expresses eGFP from the strong *cmu1* promoter. The *cmu1* promoter is induced after colonization[46] and concommitant cytoplasmic eGFP expression facilitates the detection of biotrophic hyphae. To construct the strain, plasmid pLL188 was generated by cloning eGFP into pLL181, a derivative of p123 that contains the *cmu1* promoter and the AvitagHA (L. Lo Presti, unpublished). To this end eGFP was amplified using OLL258 (BamHI site, primer sequence ttttGGATCCATGGTGAGCAAGGGCGAG) and OLL499 (XbaI site, primer sequence: ttttTCTAGACTTGTACAGCTCGTCCATGCC), digested with BamHI/XbaI and ligated to BamHI/XbaI digested pLL181. pLL188 was then linearized with Ssp1 and integrated into the *ip* locus of strain ULL152[47]. For the infection of maize seedlings the protocol of[23] was followed. Seven day-old seedlings were used and infected leaf samples for microscopy were harvested at 2 dpi, a stage where penetration had occurred and biotrophic hyphae were established.

Sample preparation for TEM and IGL

Ultra-structural analyses of arbuscule-containing cells in *R. irregularis* colonized rice roots are largely hindered by physical barriers of mature tissue such as thick cell walls, suberized schlerenchyma layer and extensive aerenchyma that slows penetration of chemical fixatives, resulting in ultra-structural artifacts (reviewed in[48]). Therefore, to overcome physical barriers to rice tissue preservation, here we used young seedlings at 10 days post germination (dpi) for TEM analysis. For chemical fixation, 1-2mm sectors of tissue were fixed in 1.5% paraformaldehyde/0.5% gluteraldehyde in 50mM cacodylate buffer (pH7.4). Samples were post-fixed in 1% OsO_4 followed by *en block* staining in 0.5% Uranyl Acetate, serial dehydration in acetone and embedded in Spurr's resin. For high pressure

freezing (HPF), samples were excised into 1mm sectors in 1-hexadecene and immediately vacuum infiltrated in a solution of 200mM sucrose, 10mM trehalose, 10mM Tris Buffer, pH6.6 used for freeze protection for approximately one minute before being transferred to aluminium planchettes (types 241 and 242, Engineering Office M. Wohlwend GmbH, Sennwald, Switzerland). For high pressure freezing was carried out in a Baltec HPM010 (Bal-Tec, Liechtenstein, http://www.chemeurope.com/en/companies/16374/bal-tec-ag.html) and stored in liquid nitrogen until further use. Freeze substitution (FS) was carried out as described previously[49].

Tomography and 3-D modeling

For electron tomography, 250-nm thick sections were placed on formvar coated copper slotgrids were counterstained and placed in a high-tilt holder (Model 2040; Fischione Instruments; Corporate Circle, PA). The area of interest was recorded on a Tecnai F20 EM (FEI, Eindhoven, The Netherlands), operating at 200kV using the SerialEM software package (Mastronarde 2005 https://doi.org/10.1016/j.jsb.2005.07.007). Images were taken at every degree over a ±60° range on an FEI Eagle 4K x 4K CCD camera at a magnification of 19000x and a binning of 2 (pixel size 1.13 nm). The tilted images were aligned by using the positions of the fiducal gold particles. The tomograms were generated using the R-weighted back-projection algorithm. Tomograms were displayed as slices one voxel thick, the vesicles modeled, and analyzed with the IMOD software package[50] (https://doi.org/10.1006/jsbi.1996.0013).

Quantitative Analysis and modeling

To compute the model for hyphal diameter and fungal CW thickness, the data were split in test and train sets with a ratio of 20:80. The operation was repeated 100 times and the average MAPE was calculated. The splitting and the prediction were performed by using caret package (https://CRAN.R-project.org/package=caret).

The first step in cluster analysis was the cluster number determination. R package factoextra (factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.5. https://CRAN.R-project.org/package=factoextra) was employed to apply the silhouette method. For cluster determination, base R

function *kmeans* was used. For the calculation of Rand index, the function *arandi* from R package mcclust (mcclust: Process an MCMC Sample of Clusterings. R package version 1.0. https://CRAN.R-project.org/package=mcclust) was applied. All the plots were drawn using ggplot2 package (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.). Statistical analysis of the frequency by which membrane structures accumulate across the different fungal hyphae tested using the Fischer Test.

Acknowledgements

We thank Anne Bates and Steffi Gold for technical assistance and Y. Kobae for kindly providing PT11-GFP transgenic rice lines. Ronelle Roth was supported by the Marie Curie FP7-PEOPLE-2013-IEF Grant number 629887 and by the Isaac Newton Trust RG74108, and the BBSRC grant BB/N008723/1 to Uta Paszkowski.

Author contribution

R.R. and U.P. conceptualized the project; R.R., S.H. and C.F. carried out the experiments; R.R. and S.H. conducted the TEM and IGL analysis; C.F. and S.H. carried out the tomography and R.R. performed the IMOD 3-D reconstruction; R.R. and M.C. did the quantitative analysis; R.R and U.P wrote the manuscript.

Competing interest.

The authors declare that no competing interests exist.

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