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PROJECT PERIODIC REPORT

Grant Agreement number: 629887

Project acronym: ENDOSYM

Project title: 3-D Quantitative Modelling of Eukaryotic Endosymbiosis: A Pioneering Innovative Imaging Approach

Funding Scheme: MC-IEF (Intra-European Fellowships (IEF))

Date of latest version of Annex I against which the assessment will be made:

Periodic report: 1st x 2nd x 3rd x 4th x

Period covered: from 1 July 2014 to 30 June 2016

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[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.
[2] The home page of the website should contain the generic European flag and the FP7 logo which are available in electronic format at the Europa website (logo of the European flag: http://europa.eu/abc/symbols/emblem/index_en.htm logo of the 7th FP: http://ec.europa.eu/research/fp7/index_en.cfm?pg=logos). The area of activity of the project should also be mentioned.

Declaration by the scientific representative of the project coordinator

I, as scientific representative of the coordinator of this project and in line with the obligations as stated in Article II.2.3 of the Grant Agreement declare that:

- ***The attached periodic report represents an accurate description of the work carried out in this project for this reporting period;***
- ***The project (tick as appropriate)[3]:***

  x has fully achieved its objectives and technical goals for the period;
  ☐ has achieved most of its objectives and technical goals for the period with relatively minor deviations.
  ☐ has failed to achieve critical objectives and/or is not at all on schedule.

- ***The public website, if applicable***

  X is up to date
  ☐ is not up to date

- ***To my best knowledge, the financial statements which are being submitted as part of this report are in line with the actual work carried out and are consistent with the report on the resources used for the project (section 3.4) and if applicable with the certificate on financial statement.***
- ***All beneficiaries, in particular non-profit public bodies, secondary and higher education establishments, research organisations and SMEs, have declared to have verified their legal status. Any changes have been reported under section 3.2.3 (Project Management) in accordance with Article II.3.f of the Grant Agreement.***

[3] If either of these boxes below is ticked, the report should reflect these and any remedial actions taken.

Name of scientific representative of the Coordinator:

.....

Date: ...../...../.....

For most of the projects, the signature of this declaration could be done directly via the IT reporting tool through an adapted IT mechanism and in that case, no signed paper form needs to be sent

3.1 Publishable summary

A summary description of project context and objectives:

The ancient microbe-host symbiotic association between plants and arbuscular mycorrhiza fungi (AMF) presents an excellent model system to elucidate molecular processes that underpin complex invader-mediated reprogramming of eukaryotic organisms. Within the roots of most land plants beneficial AMF form complex tree-shaped feeding structures called arbuscules. Monumental cellular re-differentiation and reprogramming in the inner root cortex result in the *de novo* synthesis of a host-derived membrane that surrounds the arbuscule, the peri-arbuscular membrane (PAM)[1] (Figure 1). This functional symbiosome interface facilitates the bi-directional exchange of metabolites such as inorganic phosphate for plant-derived photosynthates. Symbiosome membrane biogenesis, dynamics and ultra-structural modifications to plant and fungal membranes to facilitate this dialogue are poorly characterized. Furthermore, the mechanisms by which PAM-specific proteins are incorporated and retained within the symbiosome interface during arbuscule development remain unclear. This is partly due to a lack of bioimaging techniques that would permit live-cell deep-tissue 4-D imaging of membrane dynamics and high-resolution ultra-structural analyses that would permit quantitative 3-D modelling of plant and fungal surfaces during arbuscule development in the inner root cortex. To this end this project aimed at pioneering the application of 1.) high resolution multi-photon confocal microscopy (MPCM) for time-lapse 4-D in planta live-cell imaging and 2) developing ultra-structural tomography and/or serial block-face SEM (SBFSEM) techniques that would permit quantitative 3-D analyses of membrane structures during arbuscule differentiation.

A description of the work performed since the beginning of the project and the main results achieved so far:

1. *Establish rice root colonization by AMF as a model system to facilitate live-cell imaging using MPCM*

Rice is an important crop species with half the world's population relying on rice for human consumption. It is also an excellent genetic model system with which to study AMF symbiosis. However, colonization of rice roots by the AMF *Rhizophagus irregularis* typically takes 6 to 7 weeks at which stage rice roots exibit high levels of auto-fluorescence, which restrict deep tissue live-cell imaging. In this study a rice colonization protocol using a nurse-plant (NP) system was established to permit colonization of rice roots as early as 12-14 days post-inoculation (dpi). The earliest colonized sectors on embryonic crown roots were consistently observed at 12dpi with colonization reaching up to 80% at 3-4wpi. In these young roots auto-fluorescence was low and roots were sufficiently transparent to permit whole mount deep tissue live-cell imaging. These young seedling roots were also amenable to hyperbaric freezing (HPF) and transmission electron microscopy (TEM) for ultra-structural analyses. Due to the success of rice colonization using *R. irregularis* there was no need to consider additional AMF species for colonization of rice roots.

2. *Pioneering the use of MPCM to image R. irregularis colonized rice roots*

Deep tissue live-cell imaging of cells in the rice root cortex using confocal laser scanning microscopy (CLSM) has been hampered by low resolution and photo-bleaching that impairs cell viability. In contrast, deep-tissue imaging at high resolution with minimal tissue damage is permitted by state-of-the-art multi-photon confocal microscopy (MPCM). Yet, at the start of this project MPCM imaging on rice roots had not been achieved. Training on a newly acquired LaVision MPCM was provided by the Cambridge Advanced Imaging Centre (CAIC). Rice roots

expressing the well characterized mycorrhiza-induced phosphate transporter, PT11 fused to the enhanced green fluorescent protein (PT11-eGFP) [2], driven by its native promoter were used to asses the feasibility of using MPCM over conventional CLSM. In this project we successfully developed MPCM live-cell imaging of mycorrhiza colonized rice roots. Excellent PT11-eGFP-specific signals were obtained (with a high signal to noise ratio) and minimal photo-bleaching. Additional fluorescent marker lines including the red fluorescent protein (RFP) were also tested using MPCM. We confirmed that both GFP and RFP marker lines were suitable for MPCM imaging. Image data generated from MPCM were denoised using the MATLAB denoising algorithm from Boulanger et al [3] and were analyzed using FIJI and Imaris image analyses software.

3. Characterize transgenic fluorescent protein rice marker lines and confirm their sub-cellular localization using CLSM and MPCM

A number of subcellular fluorescent (GFP and RFP) membrane marker lines were available for analyses at the onset of this project and were assessed for their usefulness for MPCM imaging. A comprehensive characterization of the constitutively expressed endoplasmic reticulum (ER) marker, HDEL-GFP and the plasma membrane marker lines Lti6b-eGFP ([4]) and Lti6a-CFP (unpublished) were verified in embryonic uncolonized coleoptile and root tissue using CLSM. However their expression in older colonized root tissue proved more difficult, possibly due to the non-rice promoter sequences used to drive gene expression. To overcome this limitation in future new marker lines using rice-specific gene promoter sequences will be generated.

Interestingly, the higher resolution and deep tissue live cell imaging permitted by MPCM has revealed exciting novel insights into the localization and dynamics of PT11 and potentially Pi uptake during arbuscule development. We show for the first time that PT11 localization at the PAM of developing arbuscules occur at distinct foci surrounding the tips of fine arbuscule branches resembling a 'thimble' pattern of localization (Figure 2). As arbuscules mature the PT11 thimble distribution changes to more diffused (glove-like) culminating in a punctate distribution in mature senescing regions of the arbuscule. MPCM time-lapse live cell imaging showed that PT11 'thimbles' initially start as lateral PAM foci adjacent to arbuscule tips and precedes new bifurcated arbuscule branching (Figure 2). This raises two possible hypotheses 1) that PT11 and Pi uptake at the tips of arbuscule branches provide the signals required to maintain reiterative arbuscule branching; or 2) that arbuscule branching promotes PT11 localization and polarized Pi uptake at arbuscule tips. This work lends itself to future mathematical modeling to test these scenarios.

To date the tightly co-ordinated plant-fungal dialogue mediated by signaling at the PAM remains poorly understood. In this study using MPCM we demonstrated for the first time localization of a putative signaling RLK, specifically to the PAM. This RLK was previously identified in a transcriptome analyses ([5]) as *Am14* and verified as a late marker for *Ri* colonization in rice ([6]). More recently AM14 was also identified in a universal quantitative proteomics screen in both maize and rice to isolate PAM-specific proteins (Roth & Chiapello, unpublished). MPCM deep tissue live-cell imaging and immunogold-labeling and analyses using TEM of T2 and T3 stable transgenic rice pArk1::ARK-mRFP lines confirmed the sub-cellular localization of ARK1 to the PAM fine branch domain. Crosses were generated between ARK-mRFP and PT11-eGFP and co-localization of ARK-mRFP and PT11-eGFP to the PAM was confirmed using MPCM.

4. MPCM live cell imaging of PAM-specific membrane proteins in arbuscule-defective mutants:

The extent to which PAM-specific protein dynamics are disrupted in arbuscule defective mutants will elucidate the role of respective gene products in regulating PAM-specific membrane proteins. To this end, F1 crosses were generated between various PAM-specific marker lines and available mutants *pt11, pt13* [7], *str2* [8] and *ark1* (unpublished). Segregating F2 progeny have been generated and are available for further analyses.

5. Preliminary results obtained from ultrathin-TEM and SBF-SEM:

To facilitate quantitative 3-D ultra-structural modelling of membrane structures of rice roots colonized with *R. irregularis* using SBF-SEM or tomography it is imperative that membrane structure preservation is maintained as near to the native state as possible. Therefore, membrane preservation was compared using several protocols involving chemical fixation and high pressure freezing (HPF) with freeze substitution (FS). Chemical fixation resulted in convolution of membranes, which are undesirable for 3-D analysis. In contrast, HPF and FS resulted in excellent membrane preservation. Interestingly, novel fungal tubular membrane extensions resembling nanopodia ([9]) were observed using HPF, but not when using chemical fixation (Figure 3). Similarly, the PAM also showed extensive tubular extensions into the peri-arbuscular space (PAS) suggesting the PAS is more complex than previously thought (Figure 3, [10]). 3-D analyses using SBF-SEM could not resolve these plant and fungal membrane extensions. However, 3-D analyses using TEM tilt tomography confirmed that fungal nanopodia membrane structures were extensions of the fungal plasmalemma (Figure 3, Roth, unpublished data).

References:

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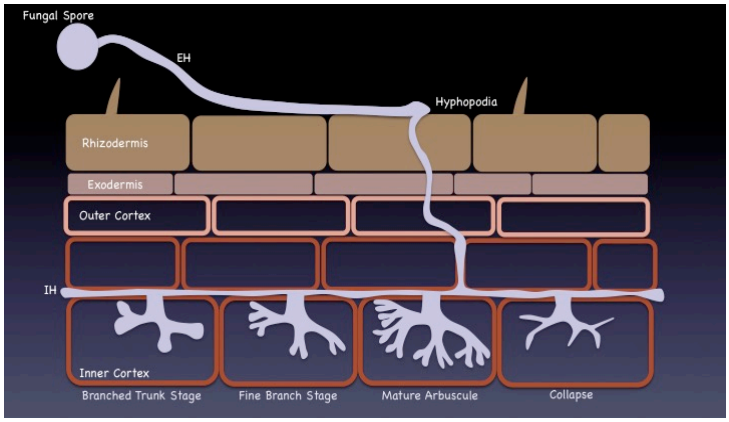

Figure 1. Schematic illustration showing the non-synchronous development of arbuscules within the inner root cortex.

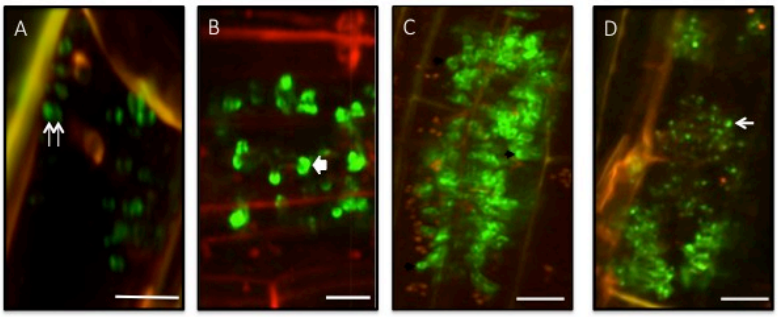

Figure 2. MPCM deep tissue live-cell imaging of PT11-eGFP at different developmental stages along the root length. A. PT11-eGFP localize to 'thimble initials' (arrows) in young developing arbuscules. B. Initials become 'thimbles' at the tips of new arbuscule branches (arrow). C. In mature arbuscules PT11-eGFP localization is evenly distributed along the PAM resembling a 'glove' D. As the arbuscule collapses PT11-eGFP becomes punctate. Scalebar, 3 um

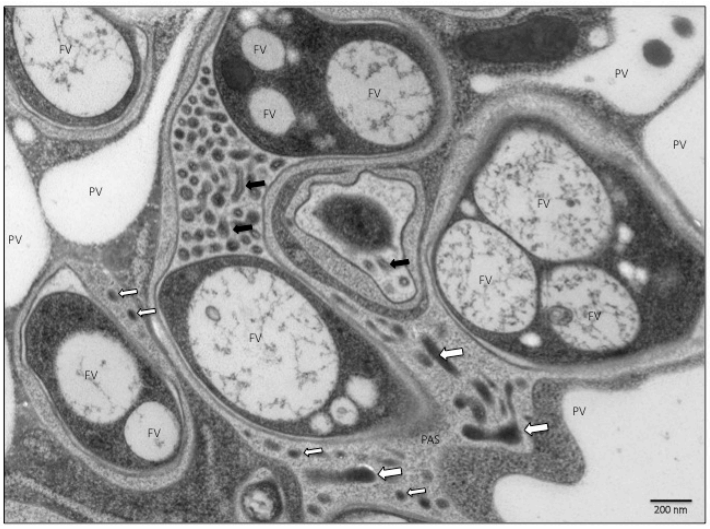

Figure 3. HPF and TEM analyses of colonized rice roots have uncovered novel PAM extensions in the PAS (red arrows) and fungal nanopodia (yellow arrows) that derive from fungal plasmalemma. PV, plant vacuole, FV, fungal vacuole, FCW, fungal cell wall, PAM, peri-arbuscular membrane, PAS, peri-arbuscular space