Short-Term scientific Mission Report- Daniela Bulgari

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Host**:**	**Saskia Hogenhout, The John Innes Centre (JIC) Norwich research Park, Norwich, NR4 7UH, UK.**
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STSM topic	**Detection of phytoplasma proteins by confocal microscopy**

Background

Recently it was demonstrated that phytoplasmas secrete effector proteins that manipulate host processes. SAP11 (11.8 kDa), a protein secreted by Aster Yellows phytoplasma strain Witches Broom (AY-WB), localizes in the nuclei of mesophyl and trichome cells in young tissue of AY-WB-infected China aster plants (Bai et al., 2009. MPMI 22: 18-30) indicating that SAP11 unloads from the phloem and can migrate to other cells. This finding is consistent with published scientific literature that proteins smaller than 40kDa can unload from the plant phloem in sink tissues, but from the phloem in fully matured tissues.

To determine whether SAP11 can unload from the phloem by itself, the Hogenhout lab generated AtSuc2:SAP11 transgenic lines that express SAP11 in the phloem. Furthermore, as a control they generated AtSuc2:GFP-GUS lines that produce GFP-GUS, which is larger than 40 kDa, in the phloem. They generated new antibodies to SAP11 (the anti-SAP11 used in Bai et al., 2009 ran out), and showed that the antibody detected SAP11 in dissected salivary gland cells of AY-WB-infected *Macrosteles quadrilineatus* leafhoppers but not healthy leafhoppers (Landi L., 2009. PhD thesis, Chapter4, Polytechnic University of the Marche, Ancona, Italy) indicating that the antibody can be used for by immunofluorescence Confocal Laser Scanning Microscopy (iCLSM). The Hogenhout lab also purchased antibodies to GFP for immunolocalization studies in the AtSuc2 lines. Finally, they received antibodies to the phytoplasma membrane proteins SecA and Antigenic membrane protein (Amp) (kindly provided by Professor Shigetou Namba, Tokyo University, Japan) to study the localization of SAP11 relatively to AY-WB phytoplasmas in infected plants.

Purpose of the visit

The goals of this STSM were to learn iCLSM and western blot analyses, foster new collaborations, to investigate the possibility of unloading of SAP11 from the phloem to adjacent cells, and to study the localization of SAP11 relatively to AY-WB phytoplasmas in infected plants by iCLSM.

Work carried out

To determine the sensitivities of the SAP11, SecA and Amp antibodies, corresponding proteins were produced in *Escherichia coli*, purified and used for western blot analyses. Different amounts of each purified protein were size-separated by Sodium Dodecyl Sulphate - PolyAcrylamide Gel

Electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and incubated with the antibodies. This showed that all antibodies, except anti-Amp, detected less than 10 ng of purified protein. Thus, anti-SecA and anti-SAP11 were considered of sufficient sensitivity and quality for the iCLSM experiments.

Previously, the iCLSM studies on fixed and wax-embedded tissues were conducted in China aster plants (Bai et al., 2009). Thus, first I conducted iCLSM on Arabidopsis Col-0 (control) and 35S:SAP11 and AtSuc2:SAP11 transgenic plants to determine whether the same fixation and wax-embedding technique would work for detection of SAP11 in Arabidopsis leaves. The wax embedded tissues were cut into 6-8 µm sections and then incubated with SAP11 antibody (raised in rabbits) and goat anti-rabbit Alexa Fluor 488 for iCLSM. Results revealed that tissues were severely damaged and showed high levels of autofluorescence. In an attempt to improve the preservation of the Arabidopsis leaf tissues, a slightly modified protocol was used to generate another set of fixed and wax-embedded Arabidopsis leaf tissues. To this end, several 35S:SAP11, AtSuc2:SAP11, and AtSuc:GFP lines were planted in MS-BASTA media to select homozygous lines. The homozygous plants were transferred into soil and grown in the greenhouse for two weeks. Leaf tissues, roots and meristems were fixed in 4% formaldehyde and vacuum infiltrated with paraffin at 60 °C using VIP machine and then properly orientated. The immunolocalization of SAP11 was carried out using the method described before (Bai et al., 2009). I found that root tissues were the least damaged and had the lowest autofluorescence levels. I detected faint fluorescent green signals in nuclei of root tissues of both Col-0 and 35S:SAP11 plants incubated with SAP11 and goat anti-rabbit Alexa Fluor 488 indicating that the SAP11 antibody may not specifically bind SAP11 but also plant proteins. I also used another antibody, 4G3 antibody (kindly provided by Prof. Peter Shaw, JIC) as a positive control. 4G3 is a monoclonal antibody that targets a RNA-binding protein in plant cell nuclei. Incubation of Col-0 root tissues with 4G3 antibodies showed a weak green signal in nuclei of root tissues. It is possible that the weak signal is due to vacuum infiltration at 60°C that could affect protein epitopes or change protein folding thereby hindering or reducing binding efficiency of the antibodies.

Because SAP11 was demonstrated to target plant cell nuclei (Bai et al., 2009), I tested whether the SAP11 antibody can detect SAP11 in isolated nuclei of Col-0 and 35S:SAP11 plants. To isolate the nuclei, leaf tissues was sliced thinly in nuclei isolation buffer and then the isolated nuclei were fixed on the slides and incubated with SAP11 antibody, and goat anti-rabbit FITC, and DAPI, which stains nuclei fluorescent blue. The green signal was detected both in Col-0 nuclei and 35S:SAP11 nuclei. This result indicated that the SAP11 antibody may aspecifically bind plant proteins. Discussions with several researchers at the JIC revealed that this can happen with antibodies generated in rabbits, because rabbits eat plants and hence can have background levels of antibodies to plant proteins. If this is the case, then one would expect high background labeling of plant samples, but not tissues of other organisms, such as insects.

To increase specificity, the SAP11 antibody was cross-absorbed with plant extracts. Briefly, the antibodies were incubated with plant extract over night, centrifuged and then the supernatant was used for immunolabeling experiments. The sensitivity and the specificity of cross-absorbed SAP11 antibody (anti-XSAP11) was tested on purified SAP11 protein and on the total protein extracts from Col-0, 35S:SAP11 and AtSuc:SAP11 seedlings. This showed that Anti-XSAP11 antibody can detect < 10 ng of purified protein and detected a protein corresponding to the size of SAP11 and no other proteins on western blots containing 35S:SAP11 and AtSuc:SAP11 plant extracts.

Anti-XSAP11 was used to determine whether SAP11 can unload from the phloem. For this purpose AtSuc2:SAP11, AtSuc2:GFP-GUS and Col-0 were grown vertically on Paul's media for 4 days in constant light. In addition, 35S:SAP11 plants were included to determine whether the XSAP11 antibodies can detect SAP11. Whole mount Arabidopsis roots were treated with cell-wall digestion enzyme and detergents to help antibody penetration. The iCLSM experiment was carried out as described before. As expected, the iCLSM results showed a green signal in 35S:SAP11 phloem and

in the root tip consistent with SAP11 being produced in all tissues under control of the 35S promoter. No signals were detected in anti-SAP11-labeled Col-0 and AtSuc2:GFP-GUS root tips. In AtSuc:SAP11 roots SAP11 was visualized not only in the phloem tissue but also in the root tip indicating that SAP11 can unload from the phloem. Roots of AtSuc2:GFP-GUS plants immunolabeled with GFP antibody showed that the GFP-GUS protein was localized only in the phloem confirming that this protein can not unload from the phloem consistent with GFP-GUS being larger than 40 kDa.

Another aim of the project was to determine the localization of SAP11 relatively to AY-WB phytoplasmas in infected Arabidopsis plants. For this, Arabidopsis seeds were planted in Paul's media and after 10 days, inoculated with phytoplasmas by exposure to AY-WB-carrying *Macrosteles quadrilineatus*. Seven days after inoculation, AYWB infected roots were analyzed by iCLSM. As expected, SecA was detected in the phloem tissues consistent with the phloem-restricted localization of phytoplasmas in infected plants. Due to lack of time, experiments to detect SAP11 in AY-WB-infected plants will be conducted by members of the Hogenhout lab.

In summary, I have optimized a protocol to detect proteins by iCLSM in whole-mount Arabidopsis roots. In addition, I have been able to show that SAP11 can unload from the phloem without the presence of AY-WB phytoplasmas.

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