STSM Completion Scientific Report

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STSM Topic: Study of secreted AYWB phytoplasma proteins

Background

Phytoplasmas are plant pathogenic bacteria causing disease in over 200 commercially important crops. Despite the fact that phytoplasmas possess very small genomes and lost several basic metabolic pathways (Bai at al, 2006; Oshima et al., 2004), they are able to survive in different environments of their plant and insect host cells by apparent manipulation of them (Hogenhout et al, 2008). However, until recently the mechanisms involved in host adaptation of phytoplasmas remained largely unknown. The publication of 4 phytoplasma genomes facilitated the study of this non-cultivable pathogen, and work from the groups of Dr. Hogenhout and Prof. Namba significantly contributed to the understanding of phytoplasma biology.

Indeed, phytoplasma genomes contain genes encoding virulence proteins (also named effectors) that appear to manipulate insect and plant hosts. The sequences of 56 candidate effectors have been identified in Aster yellows phytoplasma strain Witches' broom (AY-WB) of which one (SAP11) was further functionally characterized (Bai et al., 2009). The goal of this study was to functionally characterize more AY-WB effectors.

In a preliminary gene expression study of AY-WB genes for the 56 candidate effectors in plants (*Arabidopsis thaliana*) and insects (*Macrosteles quadrilineatus*), I found that some effector genes were upregulated in plants relatively to insects whereas others were upregulated in insects relatively to plants. Our hypothesis was that the effector genes upregulated in plants may play a role in AY-WB adaptation to the plant host and symptom development. One of these effectors named EP (Effector Protein) was selected for further analysis. In addition, the Hogenhout group found that stable overproduction of this effector in *Arabidopsis thaliana* changed the plant phenotype. We subjected EP to Y2H screening against an *Arabidopsis* cDNA library and several possibly interacting proteins were identified.

Purpose of the visit

The purpose of my visit was to confirm the Y2H results to identify plant proteins that interact with EP. In addition, I wanted to learn new molecular biology techniques, improve my protein work skills. Access to the host's institute facilities, such as the insectary would enable to study how EP protein influences AYWB phytoplasma fitness. Finally, this visit would give an opportunity for professional networking and strengthening of the collaboration between the two laboratories.

Work carried out and the main results obtained during the visit

The first step was to clone the coding sequence of EP without the region for the signal peptide sequence into *in planta* expression vectors. We selected two vectors that allowed transient *Agrobacterium tumefaciens*-mediated production of GFP-EP and FLAG-EP in *Nicotiana benthamiana* leaves. The GFP and FLAG tags would allow detection of the protein fusions and co-immunoprecipitation (pull down) assays with commercially available GFP and FLAG

antibodies. I was able to detect GFP-EP and FLAG-EP on western blot analyses (following SDS-PAGE gel separation) with GFP and FLAG antibodies upon transient infiltration of constructs into *N. benthamiana* leaves. s. Thus, GFP-EP and FLAG-EP were successfully produced in plants.

Y2H analyses revealed interaction with 9 proteins that belong to the same transcription factor (TF) family that has a total of 12 members in *Arabidopsis.* We decided to clone coding sequences of all 12 TF genes into plasmids that would generate myc-TF fusions. I cloned 8 myc-tagged TFs and found that 3 showed detectable *in planta* expression upon infiltration of *N. benthamiana* leaves and western blot analyses with myc antibodies.

To investigate whether EP destabilizes the 3 TF proteins, I conducted co-expression analyses. This provided preliminary evidence that EP destabilizes multmeric forms of the TFs. Thus, EP seems to interact with the TFs.

Next, I continued co-expression experiments to check whether it is possible to pull down the TFs using FLAG-EP as a bait. In these experiments infiltrated leaves may be treated with cross-linking reagents to preserve any interactions that may occur between proteins *in planta*. However, whereas FLAG-EP and myc-TFs were present in the input samples, both proteins were not detected after pull down suggesting that the proteins may aggregate into large complexes that may not bind to the FLAG antibody pull down resin. Experiments were repeated without cross-linking and this revealed a band corresponding to the size of one myc-TF on western blots. I intend to confirm this result. In addition, I will repeat the cross-linking experiment using a modified protocol in which I include a reverse cross-linking step. Finally, I would like to attempt to use FLAG-TFs as bait for pull down of myc-EP.

I also conducted a morphological analysis of the 35S:EP plants and of insertion mutant lines for many of the *Arabidopsis* TF genes. Compared to wild type Col-0 plants, the 35S:EP plants develop slightly faster, tend to bolt relatively quickly in short day conditions and possess heart-shaped pointy leaves. Preliminary phenotypic analysis of the TF gene insertion mutant lines did not yield any mutant that resembled the morphology of 35S:EP plants. This can be due to several issues. Firstly, the Arabidopsis TF gene mutant stocks purchased were segregating, thus genotyping to relate phenotype to genotype is necessary. Secondly, TF family members are highly redundant in function. Hence, null mutants of single TF genes do not manifest any morphological changes as other TF family members may take over. Thus, we may have to explore possibilities for knock down of multiple TF family members.

Because AY-WB-infected plants are highly attractive to the AY-WB leafhopper vector *Macrosteles quadrilineatus* and maize specialist *Dalbulus maidis*, I intended to investigate if the 35S:EP plants are also attractive to these insects. Unfortunately, I didn't have enough time to conduct these experiments, but colleagues in the Hogenhout lab included the 35S:EP plants in other ongoing experiments. Preliminary leafhopper survival and fecundity analyses showed that the EP may have a positive influence on *M. quadrilineatus* fecundity and allows slightly longer survival of *D. maidis* on *Arabidopsis*.

In summary, I generated constructs for FLAG-EP, GFP-EP and 8 myc-TFs, and detected FLAG-EP, GFP-EP and 3 myc-TFs *in planta*. Co-expression of FLAG- EP with myc-TFs seems to affect the stability of the latter, and I may have pulled down myc-TF with FLAG-EP. Phenotypic analysis of 35S:EP Arabidopsis lines revealed changes in development and morphology. Experiments to continue these investigations are being planned. Also I have mastered new molecular biology techniques, and had an opportunity to be introduced to the cutting edge inter-disciplinary research being carried out at The John Innes Centre.

Future collaboration with host institution

This STSM led to a close collaboration between the Hogenhout group at The John Innes Center, UK and the Nicolaisen group at Aarhus University, Denmark. We plan to validate the results from Y2H screen and find additional evidence for the interactions of the AY-WB EP protein with *Arabidopsis* TFs by repeating co-expression and pull-down experiments and developing other methods for studying protein-protein interactions, such as bimolecular fluorescence complementation (BiFC). We also intend to continue our *Arabidopsis* TF mutant analyses and insect survival and fecundity assays.

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