Short Term Scientific Mission Report

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STSM Topic: The involvement of U-box E3 ubiquitin ligases from *Lotus japonicus* in the interaction of the plant with pathogens, symbionts and endophytes

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1. Purpose of the STSM

E3 ubiquitin ligases are involved in protein ubiquitination, a post-translational regulatory process essential for eukaryotic growth and adaptation to environment. In plants, the E3s are regulated by a very large number of genes and participate in many biological processes: hormone signaling, cell cycling, abiotic stress responses and plant-microbe interactions. A particular family of E3s, the U-box family (PUB genes), seem to have specific roles at the early stages of plant-microbe interactions participating in microbe recognition and penetration. For instance, the *Medicago truncatula LIN* (Kiss *et al.*, 2009) and the ortholog gene to *Lotus japonicus*, *CERBERUS* (Yano *et al.*, 2009), encode E3s containing U-box, ARM repeat and WD-40 repeats and have been reported to present specific roles in the nodulation processes. Moreover, a PUB-ARM E3 ubiquitin ligase in *M. truncatula*, the *Mt*PUB1, has been reported to be a negative regulator of nodulation by direct interaction to the receptor-like kinase LYK3 (Mbengue *et al.*, 2010).

The purpose of the STSM was to investigate the role of a U-box E3 ligase from *Lotus japonicus* (*Lj*PUB13) in plant-microbe interactions. *Lj*PUB13 is a typical Plant U-box (PUB) E3 ubiquitin ligase with a U-box domain and a C-terminal ARMADILLO (ARM) repeat domain. In *Arabidopsis thaliana*, PUB13 is well characterized and known to implicate in plant defense against pathogens. It is involved in plant response to bacterial flagellin through the direct ubiquitination of the flagellin receptor FLS2 (Lu *et al.*, 2011). Previous results of the research group in Greece have shown that *Lj*PUB13 is a functional E3 ubiquitin ligase possessing auto-ubiquitination activity. Moreover, expression analyses by qRT-PCR showed that *Lj*PUB13 is up-regulated after *L. japonicus* roots infection by either a pathogen (*Pseudomonas syringae*) or a symbiont (*Mesorhizobium loti*), suggesting a possible role of this gene in both pathogenic and symbiotic interactions (Tsikou *et al.*, unpublished data).

Lotus japonicus (a model legume plant) ability to interact not only with pathogens but also with beneficial microorganisms gives us the opportunity to study different kinds of interaction on the same plant material. Taking advantage of this characteristic, the main goal of my stay to Aarhus University was to study the role of PUB13 during interaction with (a) *Pseudomonas syringae* (a Lotus pathogen), (b) *Mesorhizobium loti* (the Lotus microsymbiont), (c) *Azospirillum brasillence* (a Lotus endophyte) and (d) mycorrhiza. For this purpose we used a plant line carrying a mutation at the *PUB13* gene. The research group in Aarhus maintains a huge LORE1 null mutant population, generated through an endogenous retrotransposon (Urbanski *et al.*, 2012). The scope of this work was to record the *pub13* mutant responses in the presence of the microbes, through direct comparison of the *pub13* mutants phenotype to wild-type (wt) plants.

The second goal of my visit to Aarhus University was to analyse the temporal and spatial expression of *LjPUB13* during Lotus-microbe interactions. For the temporal expression analyses, *LjPUB13* gene expression was tested in Lotus roots inoculated with (a) *Mesorhizobium loti*, (b) *Azospirillum brasillence* and (c) mycorrhiza, at different time points. For the spatial expression analyses, a promoter-GUS approach was conducted and the scope of this work was to investigate differential expression pattern of *LjPUB13* at uninoculated *vs M. loti*-inoculated roots and nodules.

2. Description of the work carried out during the STSM

For the *pub13* mutant analyses, a number of *pub13* LORE1 mutants were genotyped and an homozygous *pub13* mutant, carrying a mutation at the 4th exon of the gene, was detected. Plants grown from seeds of the homozygous plant were either infected with the pathogen or inoculated with beneficial microbes and the phenotype of the mutants was compared to the phenotype of wt plants.

(a) Infection with the pathogen: *pub13* and wt plants were infected with *Pseudomonas syringae* pv. *glycinea* race 4 and subsequently the plants were observed for the development of symptoms in leaves (chlorosis) and roots (brown-black spots).

(b) Inoculation with the rhizobium: *pub13* and wt plants were inoculated with *M. loti* (R7a) and subsequently, the nodule development was recorded, the number of nodules was counted in 7, 14, 21 and 28 days post inoculation (dpi) and the number of infection threads was counted at 14 dpi. The *M. loti* strain used carry the DsRed/GFP genes, allowing the observation of rhizobia and infection threads under the UV.

(c) Inoculation with the endophyte: *pub13* and wt plants were inoculated with *Azospirillum brasillence* and subsequently, the root of the plants was observed for the formation of special structures, described by rearrangement of the root hairs on the whole root length, and the number of these structures on roots was counted at 7, 14 and 21 dpi.

(d) Inoculation with mycorrhiza: *pub13* and wt plants were inoculated with mycorrhiza and grown for 3 weeks. The roots were harvested and the endomycorrhizal hyphae was stained with black ink (Vierheilig *et al.*, 1998). The mycorrhizal intensity was evaluated according to Trouvelot *et al*. (1986).

For the *LjPUB13* temporal expression analysis during Lotus-microbe interactions, quantitative real-time PCR (qRT-PCR) was carried out in cDNA samples produced by wt *L. japonicus* roots under different treatments and in different time points. For this experiment

we took advantage of the huge collection of cDNA samples available at the host lab. *LjPUB13* expression was tested in uninoculated *vs* inoculated roots by

(a) *Mesorhizobium loti* at 8h, 16h, 24h, 2d and 3d post inoculation (and moreover, 14 and 21 day-old nodules)

(b) *Azospirillum brasillence* at 12h, 24h, 3d and 14d post inoculation at 2 different temperatures of plant growth (21 and 28 °C)

(c) mycorrhiza at 2d, 7d, 14d, 21d and 28d post inoculation.

In order to analyse the spatial expression of *LjPUB13* during Lotus-microbe interactions, a PUB13 promoter/GUS/PUB13 terminator cassette was constructed using the newly developed cloning strategy "Golden Gate" (Weber *et al.*, 2011). *Agrobacterium rhizogenes* was transformed with this construct by mating using an *E. coli* helper plasmid. Wild-type *L. japonicus* plants were infected with the transformed *A. rhizogenes* in order to produce transgenic hairy-roots expressing the GUS gene under the control of the PUB13 promoter.

3. Description of the main results obtained

Comparison of the homozygous *pub13* plants with wt plants showed that the *pub13* mutants have a growth defect. Under normal conditions, the *pub13* mutants grow slower than the wt plants.

Infection of *pub13* and wt plants with the pathogen *Pseudomonas syringae* did not detect any differentiation in the development of the symptoms. The number of symptoms and the frequency of the plants carrying symptoms are almost the same between *pub13* and wt plants.

In contrast to that, a distinctive phenotype was observed for the mutant during the interaction with *M. loti*. Our observations showed that *pub13* plants can be well infected by rhizobium but they are defective in nodules number and appearance. *PUB13* expression analysis by qRT-PCR showed that *PUB13* expression is higher in *M. loti*-infected roots than nodules. Moreover, *PUB13* expression was found 2,5-fold up-regulated in inoculated *vs* uninoculated roots at 3 dpi. Taken together these results, it seems that PUB13 has an important role at the early stages of plant-rhizobium interaction.

Inoculation of *pub13* plants with the endophyte *Azospirillum brasillence* showed that this mutant is able to be colonized by the endophyte. The special structures formed by the endophytes on wt plants could also be observed on *pub13* roots. However, the number of these structures per roots is decreased in *pub13* compared to wt plants at all developmental stages tested (7, 14 and 21 dpi). This effect can be attributed to the growth defect of these mutants. It is possible that the decreased number of structures per root is due to the decreased root length. *PUB13* expression analysis by qRT-PCR at wt *Azospirillum brasillence*-inoculated plants did not show any differentiation between inoculated *vs* uninoculated roots at all treatments and time points tested (12h, 24h, 3d and 14d post inoculation at 21 and 28 °C of plant growth).

Inoculation of *pub13* plants with endomyccorhiza showed that this mutant can be well colonized by the endomyccorhiza. *PUB13* expression analysis by qRT-PCR at mycorrhiza-inoculated wt plants shows the same expression pattern at both inoculated and uninoculated plants at all developmental stages tested (2, 7, 14, 21, 28 dpi). However, a

distinctive phenotype was observed for the mutant during the interaction with mycorrhiza with respect to the percentage of arbuscule abundance in the whole roots. According to these results, it seems that PUB13 plays a role during mycorrhizal infection.

4. Future collaboration with the host institution

The research group at the home institution (in Greece) will continue collaboration with the research group at the host institution (in Denmark). The huge collection of LORE1 null mutants maintained at the host institution is a valuable tool for gene characterization, so, future collaboration may arise on the characterization of genes of interest to both groups.

In the context of the current project, the *LjPUB13* spatial expression analysis was not completed because of time limitation, so, a PhD student of the host institution is going to continue working on this experiment. The student is going to harvest both uninfected and *M. loti*-infected transgenic hairy-roots expressing the GUS gene under the control of the *PUB13* promoter. GUS staining of the harvested tissue will detect any differentiation at the expression pattern of *LjPUB13* in uninfected *vs* infected roots and nodules at 1d, 3d, 7d, 10d and 14d post infection. Thus, I am going to stay in close contact with the group at the host institution, so that the analyses can be completed.

Moreover, we discussed on further experiments that have to be done in order to complete this project, and we decided that both groups are going to work towards this direction. The two groups will stay in close collaboration the next months, until this project is finished.

5. Foreseen publications

The results obtained during the STSM and future results that are going to be produced by the joint work of the two groups will be included in a publication. We envisage to publish a paper which will describe the role of *Lj*PUB13 in plant-microbe interactions.

6. Confirmation by the host institution of the successful execution of the STSM

Daniela Tsikou had a very fruitful and successful stay in Denmark. She obtained a large number of results which are very promising and which will be followed up towards a publication and presentation at an important meeting next year. Besides the scientific results, this STSM gave Daniela the opportunity to get acquainted and learn a number of assays and techniques used in our group and which she can use further in her own research in Greece. Daniela is a very pleasant person who integrated very well in the group and we have been very happy to have her here.

We appreciate that her stay in Denmark, which was financially supported by the COST action FA-1103 was a very successful fellowship.

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