REPORT of the SHORT TERM SCIENTIFIC MISSION, COST FA1208-ID n° 010314-041371

Title: UBIQUITINATION AS A TARGET OF VIRULENCE PROTEINS DURING THE BARLEY POWDERY MILDEW INFECTION

Start date: 31.MAR.2014 End date: 14.APR.2014

Applicant: GEZIEL BARBOSA AGUILAR

Host: GARY LOAKE'S LAB (EDINBURGH UNIVERSITY)

- **Background:**

The collaboration work proposed here is motivated by two recent overlapping discoveries between the two research groups involved. The two groups are consolidated laboratories in the study of defense-related components and mechanisms in plant-pathogen interactions. Using nuclear Yeast two-hybrid, the laboratory of Hans Thordal-Christensen has recently identified a Candidate Secreted Effector Protein (CSEP) from *Blumeria graminis* f.sp. *hordei* (*Bgh*) that interacts with a cytosolic host component named CSEP Interactor 1 (HvCSI1). HvCSI1 is potentially involved in ubiquitination activity in barley and preliminary results suggest that this protein is also involved in resistance towards *Bgh*. In parallel, the laboratory of Gary Loake found, also by nuclear Y2H, that the closest homologue of CSI1 in *Arabidopsis thaliana*, AtCSI1 interacts with a tertiary component (Putative AtCSI1 Substrate, PCS) already known for its involvement in defense-related mechanisms. In vitro assays have shown that AtPCS can be ubiquitinated by total protein extracts from *A. thaliana.* Due to its positive interaction with AtCSI1 in Y2H experiments, it is likely that AtPCS is the target substrate of AtCSI1. Taken together, these two independent findings indicate that the ubiquitination activity of CSI1 is a potential host process targeted directly by effectors from virulent strains of *Bgh*.

- **Objectives/ Purpose of the visit:**

The primary objective of the visit was to determine whether recombinant expression of Maltose Binding Protein (MBP) N-terminal fusion with HvPCS could be poly-ubiquitinated *in vitro* in the presence of HvCSI1. As a secondary objective we wanted to establish if the *Bgh* effector could interfere with the *in vitro* activity of HvCSI1.

- **Description of the work:**

Cloning: Full length HvPCS was cloned into the pEGT-40A vector, which confers N-terminal MBP-tag fusion. The clone was checked by sequence with a primer sitting upstream the MBP fusion site. Full length HvCSI1 and the Bgh effector were cloned into pDEST15, which confers a N-terminal GST-tag fusion. The clones were also checked by sequence with a primer upstream the GST fusion site. The vectors were then transformed into DE3 *E.coli* expression strains.

Expression and purification: The first part of the work comprised of determining ideal expression conditions for the 3 genes being tested. In order to avoid troubleshooting for solubility of the recombinant protein expression, we started directly with the induction conditions previously tested by Gary's lab for the MBP-AtCSI1. Overnight 5 mL starter cultures were diluted 1:100 in 50 mL LB media and incubated at 37^{o}C with vigorous shaking until OD_{600} was 0.8. Adding IPTG induced soluble expression at the following concentrations: 50 µM for HvCSI1 and BghCSEP, 5 µM for HvPCS. Induction was made from 6 h to overnight at room temperature (around 23^{o}C) slow shaking at 150 rpm. Cells were pelleted for 10 min at 4^{o}C and 4000 x*g*, washed 2x 50 mL PBS and re-suspended in 1 mL PBS. Cells were lysed by sonication on ice at 6 µm amplitude for a minimum of 6x 10 sec (with 10 sec intervals) or until the solution changed color from very milky to slightly more translucent. Cell debris were pelleted at >15000 x*g* 4^{o}C for 10 min. Purification of the proteins was done using amylose magnetic beads (NEB E8035S) for MBP-fused proteins and Glutathione Sepharose 4B (GE Healthcare) following the manufacturers recommendations.

Ubiquitination of HvPCS from Total Barley Extract: Total barley protein extracts were prepared from 0.3 g of fresh barley leaves in 300 µl of extraction buffer (20 mM Tris-HCl, 25 mM NaCl in the presence of proteinase inhibitor (SIGMA)). The reaction was set to 50 µl barley extract, 50 µl reaction buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, 2

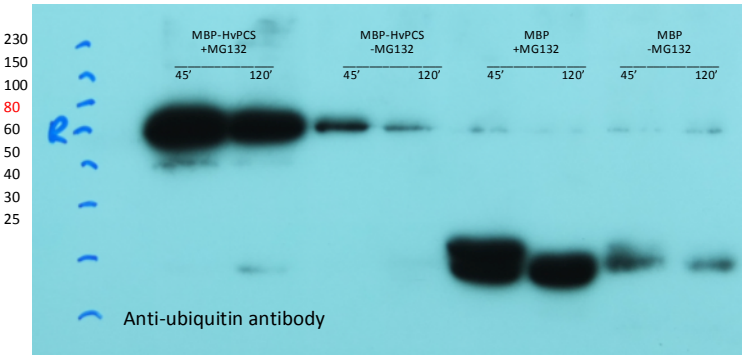
mM ATP), 10 µg ubiquitin (SIGMA), 15 µl MBP-HvPCS (or only MBP as control), 2 µl MG132 (or DMSO as control). Reaction was incubated at 30°C for 2 h with an aliquot collection after 45 min. Reaction was stopped by addition of 5x SDS loading buffer.

Ubiquitination activity of HvCSI1 and HvPCS as substrate: the ubiquitination activity reaction was performed as described in Stone et al. (2005) with some adaptations. We used Human ubiquitin-activating enzyme (E1) (Calbiochem cat# 662072) together with a total *E.coli* lysate of recombinant AtUBC8. Purified recombinant AtPCS and HvPCS were used as tested substrates in the reactions.

Stone et al. (2005) Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. *Plant Physiology* 137:13-30.

- **Main results:**

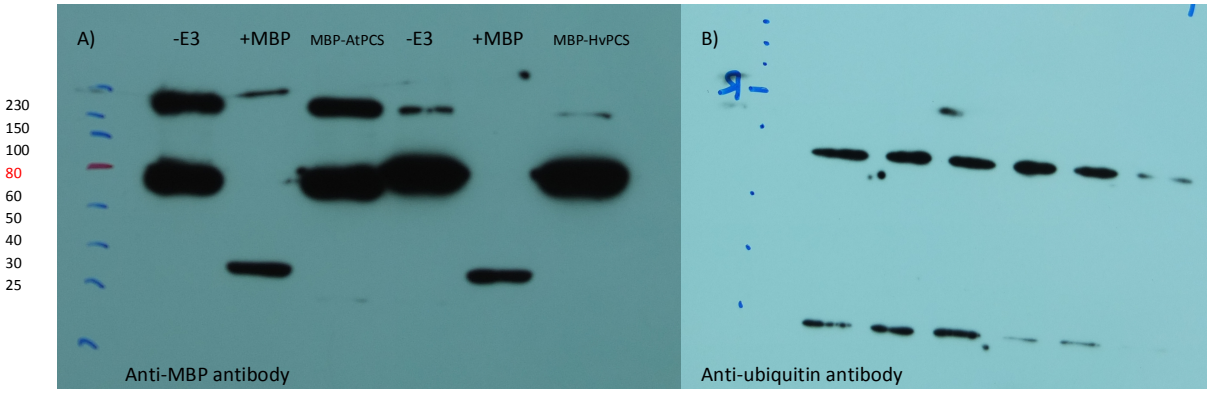
  Ubiquitination of HvPCS from total barley extract: the goal of this experiment was to determine whether HvPCS can be ubiquitinated and degraded in a proteasome-dependent manner. Under the conditions analyzed here, there was no clear ubiquitin chain formation in neither MBP-HvPCS nor MBP-AtPCS when using anti-MBP antibody (Figure 1). Interestingly, we were able to see a clear proteasome-dependent degradation of both MBP-HvPCS and MBP-AtPCS when using the total barley protein extract. Already at 45 min after reaction start a decrease in the band signal was already noticeable in the absence of the proteasome inhibitor MG132. At 2 h after reaction start there was a larger degradation of these proteins, which was only observed in the absence of the proteasome inhibitor. It is yet to be determined if MBP-AtPCS can be similarly degraded by a total Arabidopsis extract. These results indicate that these proteins might somehow be targeted to proteasome degradation even though no obvious ubiquitin chain



Figur 1: Ubiquitination from total barley extract. Degradation of MPB-Is observed only in the absence of the proteasome inhibitor MG132, already at 45' after reaction start.

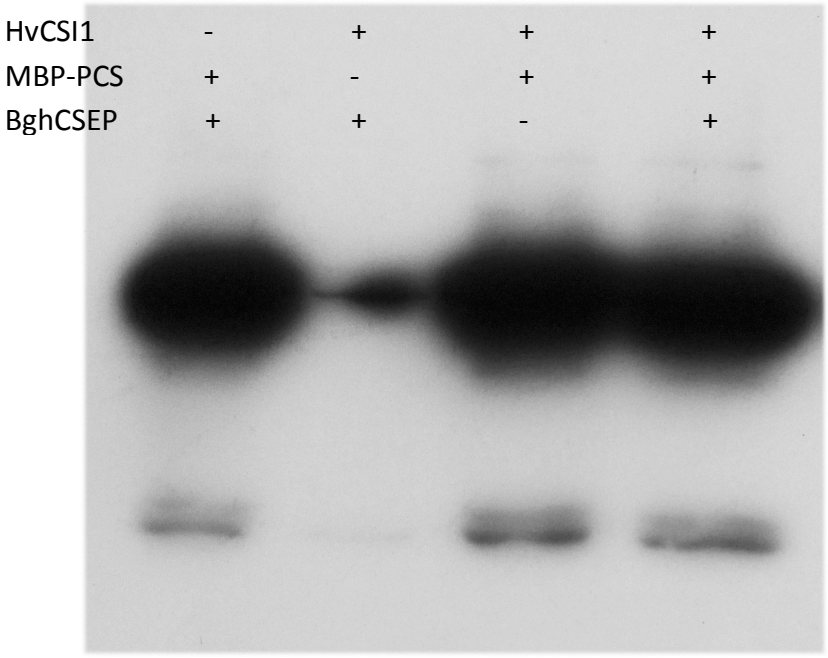
formation was observed. The lack of a 0 h time point makes it difficult to validate, in this experiment, the initial presence of ubiquitin when using the anti-ubiquitin antibody. However, the same ubiquitin stock was used in different experiments (Figure 2b) where it then had a clear visible band under similar Western Blotting conditions. Overall, these results indicate proteasome-dependent degradation even though no ubiquitin chain formation was observed in the given conditions.

E3 ligase activity of HvCSI1 in the presence of HvPCS: the purpose of this experiment was to determine whether HvCSI1 shows E3 ligase activity *in vitro* and if this activity can be interfered by the addition of recombinant GST-BghCSEP. The first experiment was made using glycerol stocks of purified proteins whilst a second experiment was made from freshly purified proteins. In the first experiment, it was not possible to observe any differential ubiquitination activity of HvCSI1 using both AtPCI and HvPCI as substrates (Figure 2a). The presence of glycerol, although at generally tolerable levels (<10%), might have affected the reaction since no ubiquitin chain was observed at all when using the anti-ubiquitin antibody (Figure 2b). Interestingly, there was formation of a high molecular weight complex containing the MBP-AtPCS, suggesting the existence of protein-protein interaction between components of the reaction. MBP-HvPCS showed relatively less amount of this high molecular weight complex, somehow indicating that although these two proteins are close homologues they show differential interaction under similar conditions.



Figur 2: Ubiquitination activity of HvCSI1 using AtPCS and HvPCS as substrates. The enzymes used in this experiments were from glycerol stocks prepared in the first protein expression. There was no observed ubiquitination activity as observed by a ladder of increasing bands due to polyubiquitin chains of different sizes. It is possible that the storage of the proteins in glycerol has affected the enzymatic activity.

The second ligase activity experiment was made using freshly purified proteins. In this experiment it is possible to see some ladders of increasing molecular weight that are different from the negative control (Figure 3). This indicates that HvCSI1 has ubiquitination activity *in vitro*. It was possible though to see faint bands in the negative control, suggesting that the experimental set up still needs to be further optimized. Formation of faint bands in the absence of the E3 ligase HvCSI1 could be due to the presence of BghCSEP in the reaction. A proper control lacking the BghCSEP and including MBP needs to be added in order to determine if the BghCSEP can show E3 ligase activity as well. Additionally, presence of BghCSEP neither interfered with the pattern nor intensity of the bands formed (Figure 3). This preliminary but not conclusive result indicates that BghCSEP does not interfere with the *in vitro* E3 ligase activity of HvCSI1. Further replicates using optimized conditions and additional controls need to be made in order to confirm these results.



Figur 3: Ubiquitination activity of HvCSI1 in the presence of BghCSEP. Presence of HvCSI1 seems to increase the intensity of the laddering, indicating ubiquitin ligase activity.

Summary of results:

1) evidence for proteasome-dependent degradation of HvPCS and AtPCS;
2) *in vitro* formation of a high molecular weight complex containing HvPCS;
3) preliminary evidence for ubiquitination of HvPCS;
4) no indication that presence of BghCSEP alters the ubiquitin laddering pattern of HvPCS.

- **Future collaboration with the host institution (if applicable):**
  This project is an ongoing collaboration between the two groups and currently other experiments are being done in both labs in order to obtain a complete and cohesive publishable story.

- **Projected publications/articles related to or resulting from the STSM:**
  Once confirmed, this data will be put together as part of a conjoint paper with authors from the two groups.

- **Confirmation by the host institution of the successful execution of the STSM (this part should be written by the host institution):**
  We confirm that Geziel Aguilar has successfully completed his Short Term Scientific Mission during the period from 31.03.2014 to 14.04.2014.

- Other comments (if any):