Investigating the Role of Dynamic Ubiquitin Signaling in Orchestrating Plant Immunity

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Introduction

As the world's population is expected to exceed 9.7 billion in the year 2050, agricultural yields must increase significantly to accommodate a growing population, while overcoming the challenge of limited arable land, and the persistence of P&P's (pathogens and pests). Therefore scientists are looking into improving crops genetically. Over the course of evolution, plants and P&Ps have developed genetic strategies against each other. Today, P&Ps are known to reduce crop yields by up to 40% (Sevary et al., 2019). Upon recognition of pathogen associated molecular patterns (PAMPs), an immune response is triggered. A key player in this response is the highly-conserved, small protein, ubiquitin, which attaches to a variety of substrates via the ubiquitination pathway consisting of: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). Once attached, ubiquitin acts as a signaling molecule, affecting the fate of the substrate. It has the ability to form at least eight different chain topologies, enabled by its seven lysine residues (K6, K11, K27, K29, K33, K48, K63) or its N-terminal methionine (M1). Although much is known about the role of K48 topologies in targeting substrates for proteasome-mediated degradation, little is known about the remaining seven topologies. Spoel research group recently identified an increase in different ubiquitin chain topologies upon pathogen infection, suggesting they are important drivers of plant immunity. We sought to learn more about the substrates affected by ubiquitination, and discover if there is a relationship between substrate type and chain topology.

Aims

To identify the substrates of various ubiquitin chain topologies by designing mutant ubiquitins which contain a single lysine residue, therefore can only be incorporated in single chain types. To do this, we

i) Clone the gene in E.coli to express and purify our mutant proteins.

ii) Add the purified mutant ubiquitin to plant cell extract for *in vitro* chain formation.

Method/description of work

Expressing ubiquitin in E. coli-

- Mutant ubiquitin was produced by my supervisor earlier on. We use Wild Type (WT) Ubiquitin which has all linkage sites in-tact, and 7KR ubiquitin which has all seven lysine (K) (K) residues changed to arginine (R). Using this 7KR ubiquitin we would be able to observe and identify M1 linked ubiquitin chains since it can only be incorporated in that specific chain type. Both constructs contain an internal STREP II tag for purification. (Fig 1).
- We inserted the sequences of WT and 7KR ubiquitin into pET-28a plasmid backbones, then transformed those plasmids into *E. coli* strain BL21(DE3)pLysS for protein expression.
- We standardized protein induction by varying the temperature and concentration of the inducer (IPTG).

Optimisation of an *in vitro* ubiquitination reaction- To identify the substrates of different ubiquitin chain topologies, we aim to see the incorporation of mutant ubiquitin by the plant cell extract (PCE). We hypothesize that the present

Fig 1. Insert sequences

WT ubq-

atg cag atc ttc gtc **aag** act ttg acc gga **aag** acc atc acc ctt gaa gtg gaa agc tcc gac
acc att gac aac gtc **aag** gcc **aag** atc cag gac **aag** gaa ggt att cct ccg gac cag cag cgt
ctc atc ttc gct gga **aag** cag ctt gag gat gga tcggcctggtcgcatccgcagttcgagaagtcggccact
ttg gcc gac tac aac atc cag **aag** gag tct act ctt cac ttg gtc ctg cgt ctt cgt ggt ggt
ttc taa

7KR ubq-

atg cag atc ttc gtc **aGg** act ttg acc gga **aGg** acc atc acc ctt gaa gtg gaa agc tcc gac
acc att gac aac gtc **aGg** gcc **aGg** atc cag gac **aGg** gaa ggt att cct ccg gac cag cag cgt
ctc atc ttc gct gga **aGg** cag ctt gag gat gga **tcggcctggtcgcatccgcagttcgagaagtcggcc** act
ttg gcc gac tac aac atc cag **aGg** gag tct act ctt cac ttg gtc ctg cgt ctt cgt ggt ggt
ttc taa

These are the sequences of our ubiquitin inserts: Wild type (WT) and mutant (7KR). Mutations are bolded in both WT and 7KR, showing the seven lysine's that were changed to arginine's. The strep tag (area of sequence with no spaces) is bolded in 7KR's sequence, and red in color for both sequences.

Reference

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N. and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, 3(3), pp.430–439. doi:https://doi.org/10.1038/s41559-018-0793-y.

ubiquitination machinery in the PCE will be able to incorporate the added ubiquitin to make ubiquitin chains *in vitro*. First we had to check the feasibility of this idea and optimize the experiment conditions.

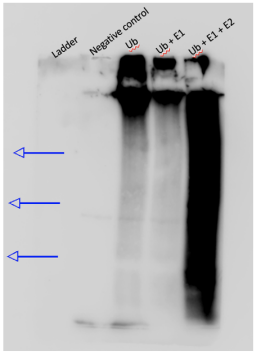
- We added FLAG- tagged WT ubiquitin to PCE in three different conditions, containing no added enzymes, or additional E1 enzyme, or additional E1 and E2 enzymes..
- We tested the incorporation of FLAG-Ubiquitin by doing an α-FLAG immunoblot.
- In subsequent reactions, we would then put STREP-tagged ubiquitin (WT and mutants) into PCE to replace the commercial FLAG-Ubiquitin..)

Assessment of results and outcomes of the studentship

We saw the incorporation of ubiquitin increase with the addition of both E1 and E2 enzymes (Fig 2). Although we see the most incorporation with the addition of both E1 and E2 enzymes, we observed that PCE on its own can drive ubiquitination without the addition of enzymes.

We found that it was difficult to detect STREP-tagged ubiquitin in *E. coli* to confirm expression, due to the presence of ubiquitin-like proteins and endogenous sequences that exhibited similarity to the STREP tag. We tried two antibodies (FK2 and P4D1), and saw nonspecific binding in both cases (Fig 3.) Upon a BLAST search, we discovered significant similarity between our insert and endogenous *E. coli* proteins. Overall, we discovered that this experiment had potential, however we would need to improve our method of detecting ubiquitin. Once that is made possible, we will be able to implement it.

Fig 2. Addition of enzyme to PCE

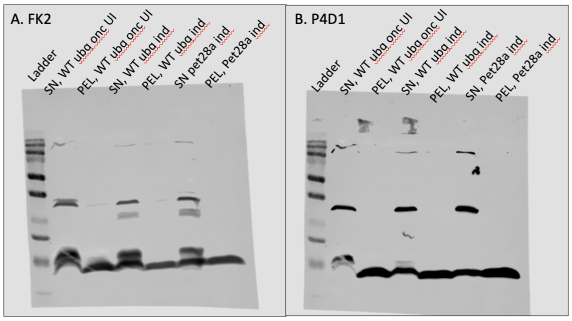


Western blot showing the incorporation of ubiquitin in 4 different conditions: negative control (no ub added), Ub (no added enzyme), Ub+E1 (added E1 to PCE), Ub+E1+E2 (added E1 and E2). Blue arrows denote ladder. Ub+E1+E2 showed the highest binding capacity

Further steps

The research done this summer provides a good basis for further development of this project. We were able to transform our constructs into our plasmids and our plasmids into *E. coli*, and were able to successfully carry out an *in vitro* ubiquitination reaction using plant cell extract.

Fig 3. Western blots



Western blots of induced (Ind) and uninduced (UI) cultures. We took samples from supernatants (SN) and pellets (PEL). Although P4D1 showed less nonspecific binding than FK2, we were not able to identify ubiquitin on these blots.

Departures from the original proposal

As the STREP-tag proved unsuccessful for this experiment, we discussed using a different tag, such as polyhistidine, to visualize ubiquitin. This tag would not show too much similarity to *E. coli* endogenous sequences, allowing us to clearly observe ubiquitin, and we would be able to cleave it off later- leaving ubiquitin intact.

Value of studentship

As this was my first lab experience, it was equally as informative as it was fun. I had a lovely time working with the people in the lab and learning about their research. I learned a lot about PCR (a lot!) and western blots, and how you can change the conditions to suit your experiment. Not only did I learn techniques relevant to my project, I was also able to participate in other experiments of interest. I looked into the effects of DNA damage on *Arabidopsis* phenotypes in different growing conditions, and learned how to take and represent quantitative data. I got to peek into various other projects going on in the lab, and meet some incredible people. I learned how to do qPCR, agroinfiltration, seed sterilization, primer design, and so much more. Furthermore, my supervisor provided informative explanations that aided me throughout the project. My knowledge of ubiquitin and plant science has grown immensely. I miss being in the lab, but I am excited to see what I can do with everything I learned this summer! The work performed supported an ongoing PhD project. The generated clones of a protein of interest for the lab will be used in further experimentation to discover the functions of various ubiquitin chain topologies.

Reference

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N. and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, 3(3), pp.430–439. doi:https://doi.org/10.1038/s41559-018-0793-y.