Investigating the optimal conditions for expression of GST-APK fusion proteins in E. coli for purification and redox poise.

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Background and aims for the project

Sulfur is an important mineral in the growth and development of all organisms. This project focuses on the pathway involving Adenosine-5'-phosphate kinase (APK) which adds a phosphate group to Adenosine-5'-phosphate (APS) to form 3'-phospho-APS (PAPS). The enzyme's activity is influenced by its quaternary structure. Dimer formation is mediated by the formation of disulfide bridges however the enzyme is active in both monomeric and dimeric form (Kopriva et al., 2012). Changes in the redox environment resulting from oxidative stresses affects APK reaction kinetics and therefor affect flux between the primary and secondary thiol metabolic routes by modulating APK activity (Ravilious et al., 2011). Investigating the response to oxidative stress of all 4 APK proteins aligns with the research group's intentions as they focus on how resource availability affects plant growth and cell division and have extensively studied the expression patterns of various metabolic enzymes, including APK, in the root meristem using reporter genes. This understanding would benefit people who live in countries that experience drought or soil that doesn't contain optimal nutrients for plant growth. Therefore, gaining a comprehensive understanding of the metabolic pathways and enzymes involved in maintaining stem cells and growth at the root tip is essential and will eventually lead to being able to engineer modified enzymes that can control the balance between growth promotion and stress-mitigation states which will have significant effects on the resilience of plants to different abiotic stress conditions such as drought.

Overall, the project aims to investigate how the conformation and efficiency of APK changes in response to oxidative stress by expressing plasmids in E. coli containing the gene for APK and then using affinity purification to cleave the GST-tags before exposing them to differing redox conditions and evaluating their migration through a gel. The aim of the project during my time in the lab was to establish a set of parameters that were optimal for the induction of the GST-APK fusion proteins in E. coli to maximise yields of APK to use in future experiments. These experiments were conducted on all 4 APK proteins as they all have different sets of cysteine residues that will act slightly differently when exposed to oxidative stress.

Methods used

Firstly, we were investigating the length of time which would be optimal to incubate the E. coli to ensure we get the highest yield of protein. This involved inoculating and inducing E. coli that contained the GST-APK fusion plasmid needed to make the 4 different APK proteins. We incubated the culture in a shaker overnight to allow it to grow and then inoculated it with 2yt media and ampicillin and after two hours induced it with IPTG. After induction, we collected aliquots at 1hr, 3hrs and 24hrs and loaded them onto Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to test which incubation time would give us the highest yield. We also conducted a solubility test on all 4 proteins where we flash froze the samples in liquid nitrogen twice as well as sonicating them before centrifugation to separate the proteins and see how much of the protein is soluble in its native state. The first supernatant gives us soluble protein, after treating with Tween-EDTA solution, which stabilises the proteins, the second supernatant gives us the membrane-bound fraction of soluble protein and the pellet gives us the insoluble protein (Figure 1).

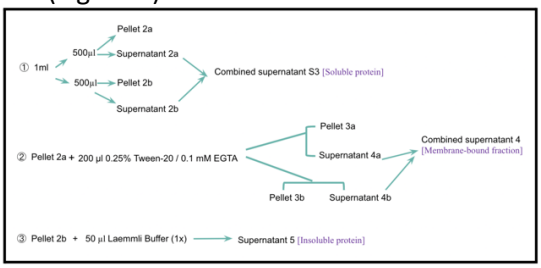

Figure 1. *A Flow diagram detailing the steps involved in separating the protein solution into soluble protein, membrane protein and insoluble protein.*

We then loaded all of these onto SDS-PAGE to investigate the quantity of protein in each. After finding the results of these experiments we moved on

to investigating the optimal concentration of IPTG which was needed to induce the proteins as we had seen low levels of induction with 1000mM concentration. We created dilutions of 500mM and 100mM IPTG and carried out the inoculation and induction with these concentrations. We then loaded them onto SDS-PAGE to compare with our initial concentration. The final experiment we conducted was to test the temperatures at which the E. coli was being induced. Initially, this was 34°C, so we also tested 28°C and 16°C shakers and loaded this on SDS-PAGE to compare all three temperatures.

Results and adjustments from the original plan

The initial experiment was trialling the length of time we incubated the E. coli to ensure the highest yield and we found that 3 hours gave the highest concentration of APK induced in all 4 proteins (Figure 2). We also found that inducing it after 1 hour and 40 minutes gave us a better reading on the spectrophotometer than 2 hours so changed the protocol to accommodate this for future experiments.

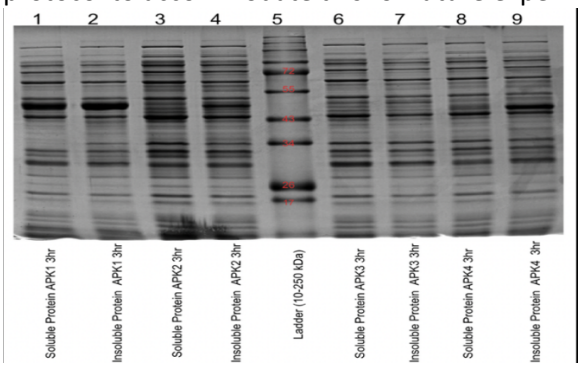

Figure 2. *SDS-PAGE gel displaying insoluble and soluble protein from all 4 APK's at 3hr induction. APK 1 and 4 show larger bands than APK 2 and 3.*

These results showed that 3hr induction gave us the highest concentration of protein, but this was inconsistent between all 4 APK's and there was a higher concentration of insoluble protein than soluble in its denatured state.

Next, we investigated the IPTG concentrations and trialled it with 100mM, 50mM and 10 mM and we found that 10mM gave us a much larger concentration of induced E. coli than the other two concentrations. This may have been due to the high IPTG concentration interfering with the protein formation or the bond between the protein and its GST tag. We adapted the protocol to accommodate this change in concentration.

The final experiment was on the temperature used to incubate the E. coli and we tested 34°C, 28°C and 16°C. The research group intends to continue this experiment in my absence as a new stain is required for the gel which will improve the imaging seen from the SDS-PAGE.

Future directions in which the project could be taken

The next step for the project could be the purification of these fusion proteins using affinity chromatography to separate the GST tag from the APK proteins. Eventually, this would lead to investigating the function of the proteins under oxidative stress using redox poise.

Value of studentship

During my time in the lab, I developed a range of specialized skills, including accurately preparing solutions of varying concentrations, setting up and running SDS-PAGE, safely handling liquid nitrogen, and utilizing a sonicator. Additionally, I gained several transferable skills, such as effective time management while conducting multiple experiments simultaneously and meticulously maintaining a lab journal to document our methodologies for reproducibility. I communicated regularly with my peers, both within my lab and across other teams, which deepened my understanding of research practices. This interaction allowed me to articulate my grasp of the subject matter and to seek clarification on concepts I found challenging. Engaging with different research perspectives enriched my knowledge of current questions being asked in plant science.

This studentship has significantly enhanced my understanding of protein structure and plasmid expression and has increased my eagerness to pursue a career in laboratory research after university.

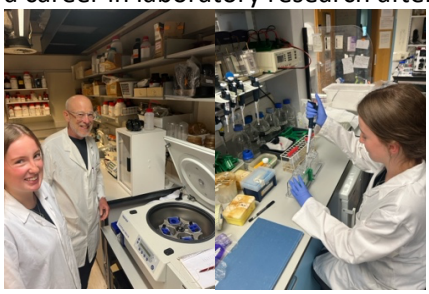

Figure 3. *Photograph of student Rosie Simmonds and supervisor Dr. Peter Doerner in the research lab and Rosie working on SDS-PAGE gel preparation.*

References

- Kopriva, S., Mugford, S.G., Baraniecka, P., Lee, B.-R., Matthewman, C.A. and Koprivova, A. (2012). Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis. *Frontiers in Plant Science*, 3. doi:https://doi.org/10.3389/fpls.2012.00163.
- Ravilious, G.E., Nguyen, A., Francois, J.A. and Jez, J.M. (2011). Structural basis and evolution of redox regulation in plant adenosine-5′-phosphosulfate kinase. *Proceedings of the National Academy of Sciences*, 109(1), pp.309–314.doi:https://doi.org/10.1073/pnas.1115772108.