Identifying the Highest Affinity Nanobody Binder for RHC-1 To Investigate Its Effect on Plants Stomatal Movement

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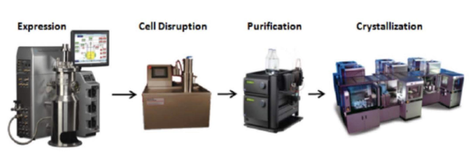
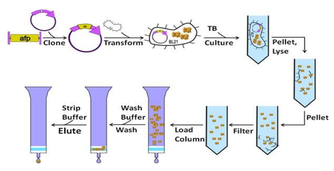
Introduction

RHC-1 is a member of the Multidrug And Toxin Extrusion protein (MATE) family of transporters. Its duty is to restrain HT1, which is a protein that hinders CO2-initiated stomatal closing. Stomatal opening and closing is an imperative piece of a plant's metabolism since stomata open in response to low CO2 concentrations and close in response to high CO2 concentrations. With CO2 levels continually rising, stomatal motion is constantly being influenced. Unfortunately, very little is known about the manner by which CO2 levels impact stomatal movement. Given that RHC-1 is critical to stomatal action, studying it is essential. By cultivating yeast cells containing the protein, screening for the best expressors, and purifying the protein, we can guarantee there will be a wealth of protein available to examine. We are using RHC1 and finding a binder to modulate RHC1's function and to help facilitate structural studies.

Transformation and Purification

To get our gene of interest to express in a yeast cell, transformation of our DNA construct (plasmid) into *E. coli* was first needed. Then transformed *E. coli* was grown in culture to amplify the amount of our vector containing our gene of interest. A mini prep was then done to obtain DNA and to further transform into yeast cells using electroporation. Carefully controlling factors such as dissolved oxygen, agitation and pH, the yeast was successfully fermented in a BioFlo 415 Bioreactor and protein expression was induced with methanol. These cells were then harvested and had their cell membranes disrupted. The cells were spun in an ultracentrifuge, the supernatant was discarded and the pellet was kept ensuring only the membrane fraction was left. To isolate the protein, affinity chromatography was carried out by utilizing a His-60 gravity nickel column. In order to further purify the protein, size exclusion chromatography was carried out using fast protein liquid chromatography (FPLC).



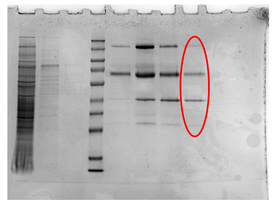


Cell Sorting and Recombination

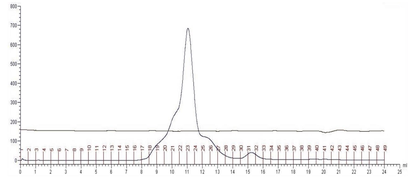
Fluorescent Activated Cell Sorting (FACS) utilizes flow cytometry to provide a fast, objective, quantitative measurement of intra- and extracellular properties, for sorting heterogeneous mixtures of cells. Our nanobodies bind to RHC-1 which is fused to green fluorescent protein (GFP). The bacteria bound to RHC1-eGFP were sorted and collected, while everything else was discarded. The sorted bacteria was grown in a culture, validated using a NovoCyte cell analyzer, and mini prepped to obtain DNA. That DNA was then recombined using a PCR machine and ligated into the pBADk vector which was then transformed into ShuffleT7 competent cells. Then that bacteria was grown overnight to produce another generation of library.

Results

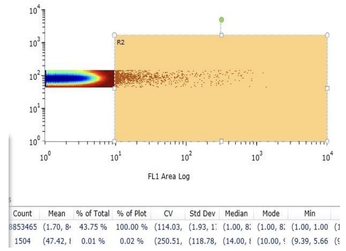
Protein purification of RHC1 using a His60 gravity nickel column



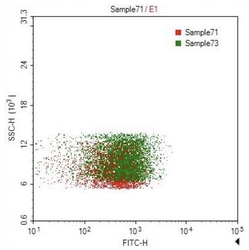
Further purification of RHC1 using size exclusion chromatography (SEC) using FPLC



The initial sort shows the gate, R2 which contains the (bacteria) cells that were fluorescing the most when their displayed nanobody bound to the RHC1-eGFP. We then re-sorted those cells to collect the ones with the strongest affinity to our protein.

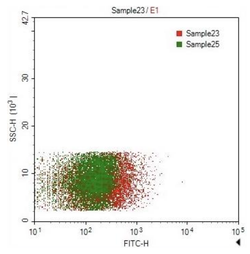


Count	Mean	% of Total	% of Plot	CV	Std Dev	Median	Mode	Min
853465	(1.70, 8	43.75 %	100.00 %	(114.03,	(1.93, 1	(1.00, 8	(1.00, 8	(1.00, 1.00
1504	(47.42,	0.01 %	0.02 %	(250.51,	(118.78,	(14.00,	(10.00,	(9.39, 5.66

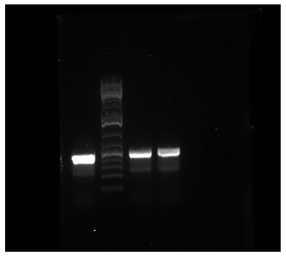


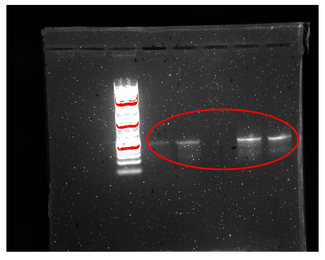
The scatter plot from a cell analyzer (left) shows the bacteria which were incubated with a different protein and shows no shift, which was expected, because the antibody was combined with a protein that was not RHC1-eGFP. This was done to see if our bacterial nanobodies are specific enough to resist binding to another protein similar to ours.

The analyzer to the right showed a shift because the bacteria cells were bound to our protein, RHC1-eGFP.



On the left: amplification of our insert (our nanobody)
On the right: our recombined inserts



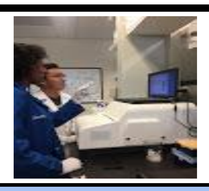


Conclusion

RHC1 is a transporter protein that controls the stomatal closing of plants by hindering another protein known as HT1. Our purpose in this experiment was to grow, and subsequently select for, cells displaying a library of nanobodies that bind strongly to our protein, RHC1. To express RHC1 in yeast, bacteria containing our expression vector was first propagated, mini-prepped, linearized and then and electroporated into competent *P. pastoris* cells. After picking a high expressing colony, the yeast was fermented and induced with methanol. We then harvested them and disrupted their cell membranes. They were then spun in a centrifuge and the pellet was kept while the supernatant was discarded. We isolated the protein using affinity chromatography and further purified it using size exclusion chromatography. Our GFP tagged protein was then mixed with a cell surface expressing bacterial library and put through a cell sorter to find any potential binders. The cell sorter then sorted out the bacteria that showed the highest amount of fluorescence. Following that, we re-sorted the cells to only capture those with the highest affinity to the protein. Those cells were validated through a cell analyzer and recombined with a PCR machine to be ligated back into the vector. Finally, we grew those new cells to make another generation of enriched library that should have a higher affinity to the protein than its parents since.

Future Directions

Future directions will focus on crystallization of our protein, RHC-1, with and without our generated nanody binders. The process would start with performing crystallization trials to determine conditions for the crystallization of the protein. Then we would use the X-ray crystallography which allows us to conceptualize protein structures at the atomic level and strengthen our understanding of the protein function. We can study how proteins interact with other molecules, how they undergo conformational changes, and how they mediate transport in the case of RHC1.



References

Tian, Wang, et al. A Molecular Pathway for CO2 Response in Arabidopsis Guard Cells." Nature Communications, vol. 6, 23 Jul 2014.
Lerner, Richard. Combinatorial Antibody Libraries; New Advances, New Immunological Insights. Nature Reviews, vol. 6, Aug 2016.

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