TEXAS WOMAN'S UNIVERSITY™

Department of Biology

# Exploring lipid binding capacity of the *Medicago truncatula* nodule-specific PLAT domain 1 (MtNPD1) protein

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## Introduction

Biological nitrogen fixation is an environmentally-friendly process in which nitrogen-fixing bacteria covert atmospheric nitrogen ($N_2$) into fixed forms such as ammonium, which can be used by host leguminous plants without the need for nitrogenous fertilizers[1]. Exchanging signaling molecules during Symbiotic $N_2$ Fixation (SNF) results in new organs, root nodules, where rhizobia fix $N_2$. To explore SNF, the genetic model *Medicago truncatula* (a close relative to alfalfa) and its symbiont *Sinorhizobium meliloti* are widely used[2]. Earlier, a nodule-specific Medicago gene (*MtNPD1*) encoding a PLAT domain protein, was identified and linked to a symbiotic defect. Disruption of *MtNPD1* causes the development of small, white nodules where rhizobia do not fix $N_2$ and are quickly degraded[3] (**Fig. 1**). Several single PLAT domain proteins have been linked to biotic and abiotic stress responses in plants, but have not been functionally characterized[4,5,6]. The PLAT domain is predicted to be involved in protein-protein and protein-lipid interactions (InterPro). Enormous membrane biogenesis occurs during nodule development and at the interface between the plant cells and nitrogen-fixing forms of rhizobia (bacteroids). The *npd1* mutant is characterized by extensive membrane degradation[3]. Therefore, based on the InterPro prediction and the *npd1* phenotype, we hypothesize that the PLAT domain interacts with lipid molecules. Thus, we aim to explore lipid binding capacity of MtNPD1 by determining if it interacts with membrane lipid substrates.

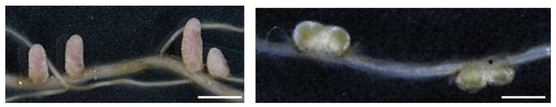

**Fig . 1: Root nodules of Wild Type (R108) and *npd1* mutant after inoculation with Sm1021.** Bars = 1 mm.

## Objectives

- Cloning of *MtNPD1 PLAT* domain into the expression vector pDEST-17 as fusion to N-terminal 6XHis tag.
- Introduce the appropriate negative control, Glutathione S-Transferase (GST) tag into expression vector pDEST-24.
- Express and purify the tagged proteins into *Escherichia coli* strain BL21-AI.

## Methods

tagged proteins.

- The Gateway cloning technique was used to generate 6XHis-MtNPD1 PLAT and GST in pDEST-24. All constructs were confirmed by sequencing.
- GST and 6XHis-MtNPD1 PLAT proteins were induced with 0.2% L-arabinose at 16 °C for overnight.
- The Pierce Glutathione Agarose (ThermoFisher) and Nickel (Ni)-NTA Agarose Resin (GoldBio) was used to purify the GST and 6XHis-MtNPD1 PLAT proteins respectively.
- Lipid strips (Echelon Bioscience) were hybridized with the purified recombinant tagged proteins and the bound protein were detected using an primary antibody against the tag, followed by HRP-conjugated secondary antibody.
- Lipid-protein binding signal detected using the Chemidoc

## Methods

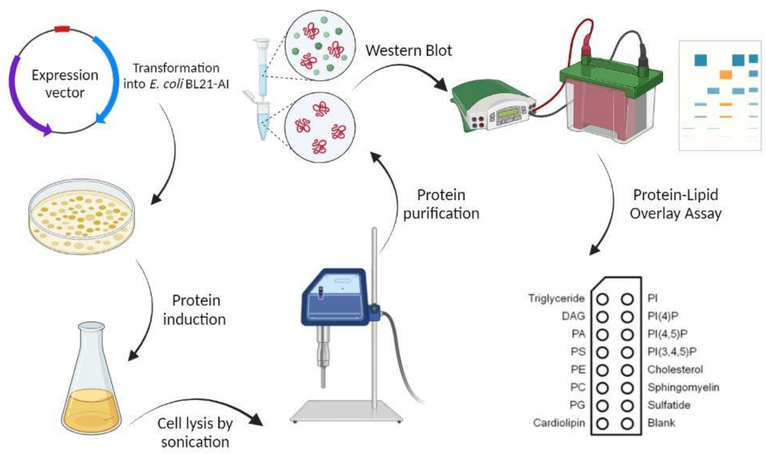


**Fig. 2: Protein expression, purification and PLO assay workflow.**

## Results

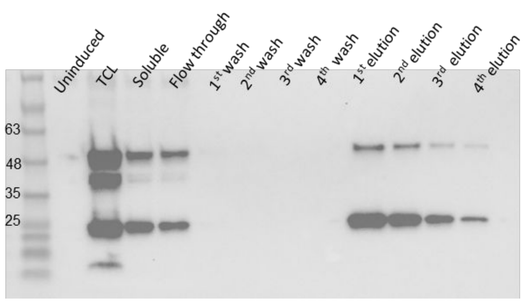


**Fig. 3: GST-tagged glutathione protein purification.** The GST-tagged protein was purified from the soluble fraction using Pierce Glutathione Agarose. The membrane was probed with anti-GST primary antibody followed by HRP-conjugated secondary antibody. TCL: Total cell lysate.

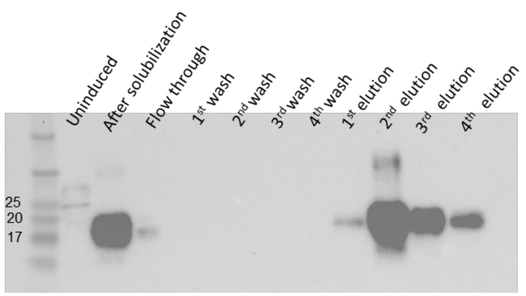


**Fig. 4: Purification of 6XHis-MtNPD1 PLAT.** The recombinant protein was solubilized in 2% sarkosyl, and after dialysis, it was purified using Ni-NTA agarose beads. The membrane was probed with anti-His tag antibody and HRP-conjugated secondary antibody

The 6XHis-MtNPD1 PLAT and GST proteins were expressed well in *E.coli* BL-AI after induction with 0.2% L-arabinose. The GST protein was found in the soluble fraction (~50 KDa) and was successfully eluted from Pierce Glutathione Agarose using 10mM reduced glutathione (**Fig. 3**). The band of nearly 26 KDa indicates the free GST tag (**Fig. 3**). Expression of 6XHis-MtNPD1 PLAT led to the formation of inclusion bodies (IBs). To retrieve the protein from IBs, 2% sarkosyl was used to solubilize it, and, after incubation with Ni-NTA agarose beads, a step gradient of imidazole elution buffer [ 100mM (1x), 250mM (2x), and 400 mM (1x) imidazole] was used to elute the purified and functional protein (**Fig. 4**).

## Results

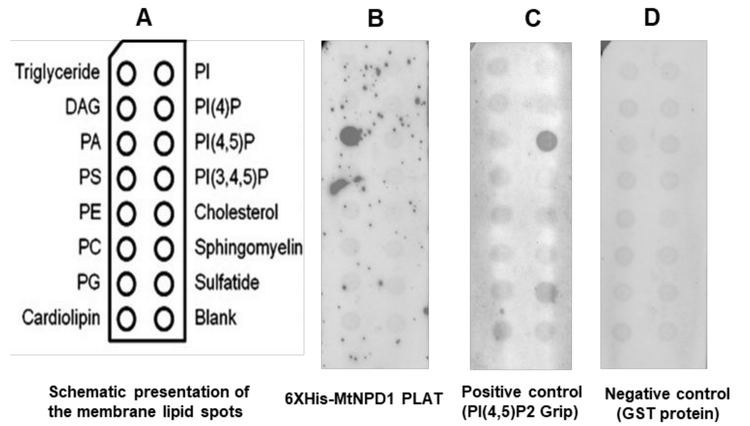


**Fig. 5: Hybridization of recombinant His-tagged PLAT domain of MtNPD1 to lipid strips revealed binding with phosphatidic acid.** Membrane strips with a range of spotted lipids as illustrated in panel A were blocked with 1% non-fat dry milk in TBST buffer, then incubated with the recombinant proteins for 4 hours. The membranes were incubated with tag-specific primary antibodies (anti-His in B and anti-GST in C and D), followed by HRP-conjugated secondary antibody. Binding signals were amplified with clarity western ECL substrate kit (Bio-Rad).

Hybridization of recombinant 6XHis-MtNPD1 PLAT with the lipid strips revealed a specific association with phosphatidic acid (PA) (**Fig. 5 B**). The positive control, PI(4,5)P2 Grip, was provided by the manufacturer where a signal was detected in the PI(4,5)P lipid spot (**Fig. 5 C**). No binding activity was detected with the negative control (GST protein) (**Fig. 5 D**).

## Conclusion and Future Directions

- We expressed the 6XHis-MtNPD1 PLAT and GST recombinant proteins on *E. coli.*
- The recombinant proteins were purified and hybridized to lipid strips.
- 6XHis-MtNPD1 PLAT strongly bound to PA, which is a signaling molecule that is activated in response to rhizobia recognition[7].
- MtNPD1 PLAT fused to C-terminal His tag will also be used to reproduce the PLO assay.
- Calcium-dependent binding of the MtNPD1 PLAT domain to lipids will be assessed.

## References

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