

Cloning, expression, and purification of the kinase domain from the *M. truncatula* symbiotic receptor kinase NFP

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Abstract

The rhizobium-legume symbiosis requires plant receptors for the transduction of rhizobial signals, named nod factors, that lead to nodulation. Genetic and genomic analysis in the model legume *Medicago truncatula* has revealed some of the genes involved in nod factor perception. Genetic screening has shown that *NFP*, *NORK*, and *LYK3* are required to form root nodules. These genes encode receptor like kinases that transmit the Nod factor signal. This project is developing resources to investigate the roles *NORK*, *NFP*, and *LYK3* kinase domains play in the nodulation process. Here we describe the cloning and expression of epitope tagged NFP. We added c-myc epitope tag onto the NFP kinase domain and cloned it into the bacterial expression vector pGEX to make a glutathione S-transferase fusion protein. We screened for the cloned gene with a colony PCR assay. Then we induced the positive colonies with IPTG to verify if GST-NFP-myc was expressed. Expressed proteins were purified by batch affinity chromatography. Tests for auto-phosphorylation activity and for protein-protein interactions will be performed.

Introduction

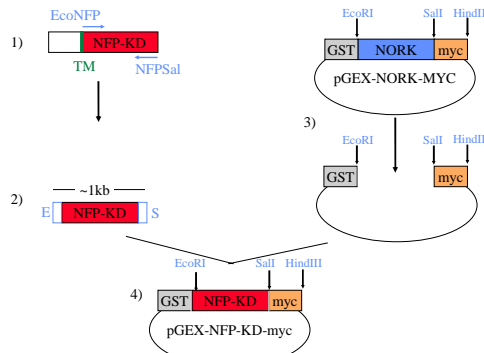
Bacteria called rhizobia colonize legume roots resulting in the formation of a root nodule. This is where nitrogen fixation takes place. Bacteria convert atmospheric N_2 to ammonia (NH_4^+), a form that the plant can use. This association involves a "molecular dialog". The legume roots secrete flavonoids that trigger the gene expression and synthesis of Nod factors by the bacteria. Nod factors facilitate infection of the host's roots by the bacterium and also initiate root nodule development. This dialog is specific to each rhizobia and legume species, and is required for the symbiosis.

The *NORK*, *NFP* and *LYK3* genes encode transmembrane receptors believed to reside on the root hairs of the legume. *NFP*, *NORK*, and *LYK3* deficient plants do not form root nodules indicating that Nod factor signaling requires these receptors. *LYK3* and *NFP* have extra cellular LysM domains. Bacterial LysM domains bind peptidoglycans that resemble the Nod factor backbone suggesting that the LysM domain of *NFP* and *LYK3* bind to Nod factors. *NORK* encodes a receptor like kinase containing an extracellular domain with three leucine-rich repeats (LRR). LRR's participate in protein-protein interactions and *NORK* may therefore reside in a protein complex, possibly with *NFP* and *LYK3*. Recent studies have suggested that phosphorylation by the intracellular kinase domains may regulate perception and transduction of the Nod factor signal. Our project is to express and purify the *NORK*, *NFP*, and *LYK3* receptor kinase domains in order to elucidate their roles in the intracellular symbiotic signaling. Purified kinase domains will be used to investigate protein-protein interactions and *in vitro* phosphorylation of the symbiotic receptor kinases. Investigating the interaction between *NORK*, *NFP* and *LYK3* will expand our understanding of how Nod factors are perceived and transduced.

Methods

pGEX is a plasmid for the regulated expression of a gene as a fusion to glutathione S-transferase (GST). The GST tag allows the purification of the protein using agarose beads that are bound to glutathione. The lac repressor (*lacI*) binds to P_{lac} promoter repressing the expression of GST fusion protein. Induction of the lac operon with isopropyl- β -D-thiogalactoside (IPTG) inhibits repression and the GST fusion protein is expressed. c-myc is an epitope tag. An epitope is an antigenic determinant of amino acid residues. Epitope tagging is a technique that helps study structure and functions of new protein. Attaching a known epitope tag to a cloned DNA allows the detection of the fusion protein with commercially available antibodies. Tagged proteins can be purified by affinity chromatography, or studied by Western Blot, immunoprecipitation or immunofluorescence.

We amplified the NFP kinase domain using primers that contained EcoRI and SalI restriction sites. We used these sites to replace the NORK gene in pGEX-NORK-myc with NFP. We used similar techniques to clone tagged kinase domains for NORK, and LYK3 (data not shown).

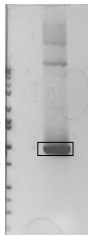


- 1) PCR amplification of pCR2.1-NFP with EcoNFP/NFPsal primers and *Pfu* high-fidelity polymerase.
- 2) NFP is digested with EcoRI/SalI and cleaned up in QIA-quick column to remove the restriction enzyme.
- 3) pGEX-NORK-MYC is digested with EcoRI and SalI and gel extracted to remove NORK and create pGEX with EcoRI and SalI overhangs.
- 4) pGEX is ligated to NFP and transformed in One shot competent *E. coli* cells

Results

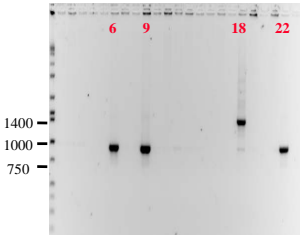
NFP-KD amplification

Agarose gel showing NFP-KD amplified using *pfu* high fidelity polymerase and primers designed with EcoRI and SalI restriction sites. The predicted of NFP-KD PCR product (black box) is approximately 1000bp.



We gel extracted the NFP-KD per product, digested it with EcoRI and SalI, ligated it to EcoRI and SalI sticky pGEX, and transformed *E. coli*.

Colony PCR of pGEX-NFP-KD-MYC



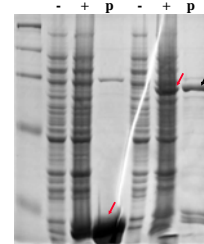
With Colony PCR we screened colonies for the GST-NFP-KD-MYC fragment using the same primers EcoNFP/NFPsal. Colonies #6, #9, #22 amplified the predicted PCR product of ~1kb.

Diagnostic Digests of pGEX-NFP-KD-myc #9



We did diagnostic restriction digests with EcoRI and HindIII, and we found that pGEX-NFP-KD-myc#9 contains both the 1kb NFP fragment and the 5kb pGEX vector.

Induction of GST and GST-NFP-KD-myc with IPTG

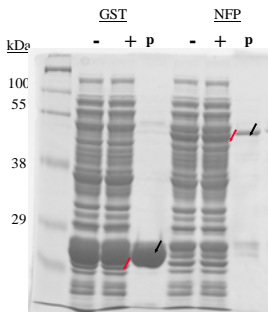


- = crude bacterial extract before IPTG induction
+ = crude bacterial extract after IPTG induction
p = Proteins purified with glutathione agarose

We grew a culture of pGEX and pGEX NFP-KD-myc and took samples before and after induction with IPTG. We purified protein from the IPTG induced sample using glutathione agarose beads. We loaded samples on an SDS-PAGE gel, separated the proteins, and stained them with Coomassie brilliant blue.

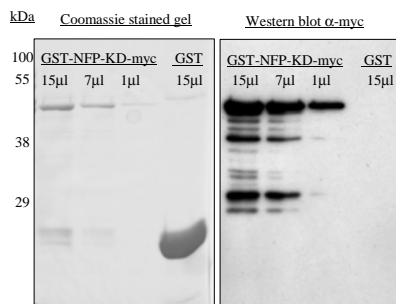
Results

SDS-PAGE of GST-NFP-KD-MYC purification



We repeated the same protocol as above and sampled 1/100th of total protein before and after incubating with glutathione agarose resin. We separated these proteins with ~1/3 of the purified protein. There is still a lot of protein after incubation with the resin indicating that we need to change our protocol to be more efficient.

Detection of NFP-KD-myc with α -c-myc antibodies



We separated purified, diluted GST-NFP-myc or GST by SDS-PAGE on two identical gels. One gel was stained with coomassie brilliant blue. Proteins from the other gel were transferred to PVDF, and probed with α -c-myc monoclonal antibodies and detected using HRP conjugated secondary antibodies and ECL kit. The α -c-myc antibodies detected the purified GST-NFP-myc protein but not GST alone.

Conclusions and future work:

- 1) We have generated an IPTG inducible GST-NFP-KD-myc construct for protein expression in *E. coli*
- 2) Glutathione agarose beads purify this protein from crude *E. coli* protein extracts
- 3) The protocol to express and purify this protein should be optimized for more efficient purification.
- 4) This construct can be used to generate protein for investigating interactions between NORK, NFP and LYK3 *in vitro*.