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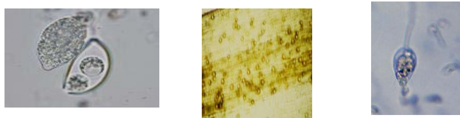
Cloning of a myb transcription factor from Phytophthora sojae, a soybean pathogen

Soybeans (Glycine max) are an important agricultural staple used in various products. Not only are they used in foods such as chocolate and soy milk, they are also found in cosmetics, animal feed, inks and waxes (Soybeans: A to Z). Also, they are being used in biodiesel fuel. They are grown all over, including Minnesota, Missouri, Kansas, Michigan, Nebraska, Iowa, and right here in Ohio.

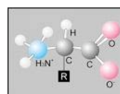
Phytophthora sojae is a common pathogen that infects soybeans (Erwin and Ribeiro, 1996). It is one of the most harmful pathogens that exist in soybeans (Chen, et al.). It is an oomycete, a type of water mold that is similar to fungi (Erwin and Ribeiro, 1996). It causes Phytophthora root and stem rot (Tyler). *P. sojae* has the ability to reproduce by asexual or sexual means. The oospores can live in the soil for many years even after the plant has decomposed. When the moisture in the soil is high, the oospores germinate and form sporangia. Asexual reproduction occurs by the production of zoosporangia which release motile zoospores, with two flagella. Sexual reproduction occurs via mating of male and female gametangia located on the same mycelium (homothallic or self fertile) to produce oospores (Erwin and Ribeiro, 1996). These sporangia either make small motile zoospores, or they directly infect the plant roots (Erwin and Ribeiro, 1996). The zoospores latch on to the root, get inside it, and then grow up through the root as mycelium.

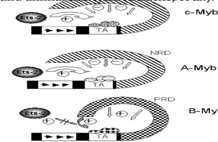


P. sojae causes root and stem rot in mature soybeans and "damping off" in seedlings (Tyler). Damping off is the general name for any disease that kills seedlings or seeds before or after they germinate. Infection commonly occurs when the temperature is over 25-30 degrees F and the soil is very wet for 7 to 14 days (Tyler). Most often, *P. sojae* infection is seen in fields with poor drainage, heavy rains, or over irrigation. Symptoms of *P. sojae* root and stem rot infection include brown spots that go up the stem (Phytophthora sojae phases). Other symptoms include dead and wilted plants, stand reduction, yellow plants (due to insufficient Nitrogen) and brown leaves (Erwin and Ribeiro, 1996). *Phytophthora* infections amongst plants cost $1-2 billion dollars a year (Tyler).

Myb genes are found in humans, animals, and plants such as Maize, Arabidopsis, and soybeans (Phan Tran, et al.). Also, they exist in fungi and molds, including *P. sojae*. There are three members of the Myb gene family. They are A-Myb, B-Myb, and C-Myb. C-Myb is involved in hematopoiesis. A-Myb takes part in neurogenesis. Both A-Myb and C-Myb show a pattern of restricted expression in cell and tissue type (Ansieau, et al.). Also, both A-Myb and C-Myb activate the expression of genes. B-Myb shows activity as both a transactivator and a repressor (Masselink, et al.). It is also necessary for the progression of the cell cycle. All three members of the Myb family are transcription factors.

Members of the Myb gene family bind to DNA (Stracke, et al.). Also, they regulate gene expression. The Myb domain, a series of approximately 50 amino acids, defines this gene family. Multiple copies of the Myb domain show up as tandem repeats. The 3 members of the Myb gene family (A, B, and C) encode nuclear proteins that act as transcriptional transactivators. Viruses encode these transactivators to enhance their own gene expression. Myb genes are also involved in the identity and fate of plant cells, as well as plant secondary metabolism (Stracke, et al.). "Classical" Myb transcription factors are involved in controlling the plant and animal cell cycle (Stracke, et al.). They have also been found to activate the circadian clock.





The goal of this project was to clone a myb transcription factor gene into the bacteria..

Methods

-*Phytophthora sojae* was grown on V8 plates for a week. Next, it was scraped from the plates, frozen using liquid nitrogen, and ground into a fine powder.

- Next. The *P. sojae* was used in the Dneasy Plant Mini Kit (Qiagen, California) to extract the DNA. Approximately 100 mg of finely ground *P. sojae* was put into each tube (2 tubes were used). Next, 400 microliters of Buffer AP1 and 4 microliters of RNase A were added to each sample. Then, the tubes were incubated at 65 degrees C for 10 minutes. During the incubation period, each tube was inverted 2 times to lyse the cells. Next, 130 microliters of buffer AP2 was added to each tube, the tubes were mixed, and they were incubated on ice for 5 minutes. Then, the tubes were centrifuged on 14000 rpm for 5 minutes. Next, the lysate from each tube was pipetted into its own QIAshredder Mini spin column and centrifuged at 14000 rpm for 2 minutes. Next, the supernatant from each spin column was removed and placed in new tubes. Then, each tube received buffer AP3 that was 1.5 times the volume of lysate. Next, 650 microliters of the lysate mixture from each tube was added into its own DNeasy Mini spin column. Then, the tubes were centrifuged at 13000 rpm for one minute, and the flow-through was discarded. This process was repeated with the remaining volume of lysate in each tube. Next, the Dneasy Mini spin columns were placed into new collection tubes, 500 microliters of buffer AW was added to each tube, and the tubes were centrifuged for one minute at 13000 rpm. The flow-through was then discarded. Next, 500 microliters of buffer AW was again added to each Dneasy Mini spin column, then the tubes were centrifuged for two minutes at 14000 rpm. The tubes were then transferred into new microcentrifuge tubes, and 100 microliters of buffer AE was added directly to the Dneasy membrane. Next, the tubes were allowed to incubate for five minutes at room temperature, and then centrifuged for one minute at 13000 rpm. This step was repeated once. Extracted DNA was the result of this process. The concentration of the extracted DNA was tested on the Nanodrop Spectrophotometer and ran on an agarose gel to test the quality of the sample. The extracted DNA was then kept on ice or in the freezer.

- Next, the purified DNA of *P. sojae* was used as a template to amplify a myb gene by PCR reaction. One microliter of DNA was mixed with one microliter of the forward and reverse Myb primers, ten microliters of Taq 2x master mix (Taq Polymerase), and 13 microliters of Nuclease-free water. Primers that were specific for the myb gene were used. The sequence of the forward primer read 5'-GGA-GAT-ATA-CAT-ATG-ATG-AGC-AAG-TCG-AGC-GGC-TTC-3', and the sequence of the reverse primer read 5'-CGT-GAT-GCA-GGA-AGA-TTA-GTT-GGC-GGG-CGT-CTT-TTT-C-3'. It was set in the Bio-Rad DNA Engine Peltier Thermal Cycler PCR machine and run on program Stacey5. Stacey 5 PCR program was 5 minutes at 95 degrees C to initiate, then 10 seconds at 95 degrees C to denature the bonds, then 20 seconds at 60 degrees C to anneal, then 45 seconds at 72 degrees C to amplify. Steps 2 through 4 were repeated 30 times, then the program continued for 2 minutes at 72 degrees C, and final step was 4 degrees C forever for storage. Each tube contained 25 microliters of liquid, and the lid of the PCR machine was heated. The concentration of the PCR'ed Myb was tested using a Nanodrop Spectrophotometer and then loaded on an agarose gel. .

- After the Myb gene showed on the gel, the PCR product of Myb gene was cloned into the TOPO TA vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen, CA). First, I mixed one microliter of PCR product, one microliter of salt solution, one microliter of TOPO vector, and 3 microliters of water. Next, I incubated the mixture at room temperature for five minutes, and then placed the reaction on ice. Then, I added 2 microliters of the cloning reaction to a vial of chemically competent E. coli and mixed gently. Next, I incubated that mixture on ice for 30 minutes, heat shocked the cells for 30 seconds at 42 degrees C in the Labnet AccuBlock Digital Dry Bath, then immediately transferred the reaction to ice. While the tube was on ice, I added 250 mL of SOC medium, and then I capped the tube and put it in the shaker horizontally at 200 rpm at 37 degrees C for one hour. Next, I spread approximately 50 microliters from each transformation onto a prewarmed LB+amp+X-gal plate and allowed it to incubate overnight at 37 degrees C. Then I picked about 10 colonies from the plates, placed them in tubes with 5 microliters of water, 5 microliters of mastermix, and 0.5 microliters of the M13 primers. Ten colonies were used to insure that at least one insert got into the plasmid. My M13 forward primer read 5'-GTA-AAA-CGA-CGG-CCA-GT-3', and my M13 reverse primer read 5' -CAG-GAA-ACA-GCT-ATG-AC-3'.

Next, this mixture was used in 10 PCR reactions. Each tube represented a clone of the bacterium. It was set in the Bio-Rad DNA Engine Peltier Thermal Cycler PCR machine and run on program Stacey5. Each tube contained 10 microliters of liquid, and the lid of the PCR machine was heated. The PCR product of the Myb was loaded on a gel. According to the size of the inserts in the gel, 4 of the clones suggested that the myb gene was successfully inserted into the plasmids.

Discussion

The Myb gene was successfully cloned into the vector. In the future, the gene will be sub-cloned into the expression vector to produce protein and will be used for functional analysis

Works Cited

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