Molecular Characterization, Functional Analysis and Localization of Polyamine Transporter Pptg_00424 in *P. Sojae*.

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Abstract

Soybeans are a very important plant especially to us back in Kenya as a source of plant protein and animal feed. Soybeans can be cultivated as a sole crop in large plantations but for small scale farmers back in Kenya, it is most often grown with other crops. Since it is a legume, farmers usually plant it together with other crops through intercropping with maize, cassava, sorghum, banana, or even sugarcane. Intercropping is a good planting strategy mostly practiced by Kenyan farmers for the accrued benefits such as soybeans planted together with maize attracts some parasitic wasps that biocontrol the African bollworm (Helicoverpa armigera). However, soybeans are highly susceptible to an oomycete known as Phytophthora sojae which has caused their massive destruction. My research focuses on a polyamine transporter gene known as PPTG_00424 which was isolated from Phytophthora parasitica but has sequence homology in P. sojae and we predict this gene would be performing the same function in P. sojae. Preliminary results show that this gene codes for a polyamine transporter located in the endoplasmic reticulum. This research applies the use of molecular microbiological techniques such as plasmid purification, polymerase chain reactions, gel electrophoresis, transformation reactions, heterologous expression, yeast growth assays, confocal microscopy, and bioinformatic analysis to functionally characterize the suspected polyamine transporter gene. The projected results are that this gene is a putative orthologue that transports polyamines in or out of the endoplasmic reticulum in P. sojae, information crucial for the development of better genetic, chemical or environmental management strategies against this pathogen.

Keywords: Polyamines, soybeans, gene, polyamine transporter, Phytophthora sojae

Introduction

Polyamines are small, positively charged, low molecular weight, aliphatic compounds (Malmberg et al., 2010) ubiquitous in all living things; archaea, bacteria, plants, and human beings (Busse, 2011; Chibucos & Morris, 2006). There are five polyamines; cadaverine with one amine group, putrescine with two amine groups, spermidine with three amine groups, spermine with four amine groups and thermospermine which is synthesized from spermidine through catalysis by thermospermine synthase (Takano, Kakehi, & Takahashi, 2012). Putrescine, spermine, spermidine, and thermospermine are common in plants due to their abundance and involvement in developmental processes (Fujita & Shinozaki 2014; Gill & Tuteja, 2010).

Polyamines play important roles in the biological living system of plants; they are directly related to growth and development, flowering, fruiting and ripening, senescence and aging (Tanguy, 2001). For example, thermospermine has been found to regulate biomass production in woody plants (Takano, Kakehi, & Takahashi, 2012). In addition, polyamines help plants withstand extremely unfavorable abiotic conditions.

Plants are sessile and therefore, more susceptible to abiotic stresses such as drought, changes in pH, floods, changes in levels of salt to unfavorable concentrations, cold, and wind (Liu et al., 2015; Gill and Tuteja, 2010). Polyamines play a role in plants' ability to tolerate the abiotic stresses (Tuteja & Gill, 2010; Ahmad et al., 2012; Minocha, Majumdar & Minocha, 2014; Liu et al., 2015). For example, exogenous application of elevated amounts of polyamines help plants become tolerant to temperature changes, fluctuation in salt levels, osmotic stress, floods, heavy metals and water logging in soil (Gill & Tuteja, 2010). Similarly, polyamines have been found to improve plants resistance to drought (Alcázar et al., 2010). Over expressing arginine decarboxylase in *Arabidopsis thaliana* makes the transformed plant able to withstand dry conditions which otherwise cause stunted growth and consequent death in wild type (Alcázar et al., 2010). Fluctuation in levels of polyamines in a cell at a given time influences plant processes.

Changes in the levels of polyamines in plants produce hormone-like effects hence they can be

considered as signaling compounds in this regard since fluctuation in levels of polyamines have been found to activate or repress plant developmental response pathways (Ahmed et al., 2017). For example, in *Arabidopsis thaliana*, changes in the levels of spermidine by altering the expression of polyamine uptake transporters reveals a role in the stage of flowering and senescence (Ahmed et al., 2017). This study illustrates the role polyamine transporters play in regulating the cellular levels of polyamines and relatively their gene expression. However, the high levels of polyamines present in the cell differentiates them from typical hormones which occur in trace amounts. Oomycetes also require polyamines since polyamine uptake transporters (PUTs) have been characterized from the pathogen.

Oomycetes are a group of eukaryotic microorganisms that are morphologically identical but phylogenetically distinct from true fungi (Meng et al., 2014) and they are commonly referred to as 'water molds'. Plant pathogens of the genus *Phytophthora* belongs to the oomycetes. *Phytophthora* is a soil-borne pathogen of economic importance costing the agricultural industry millions of dollars in losses annually and leading to famine in other parts of the world (Tyler, Tripathy, Zhang et al., 2006). This genus of pathogen has many species including *Phytophthora ramorum*, a new species causing sudden oak death and *Phytophthora infestans* which causes late blight in potatoes and tomatoes (Tyler, Tripathy, Zhang et al., 2006).

Phytophthora sojae infects all the stages and parts of the soybeans starting with pre and post-emergence 'damping off' of seedlings, as well as stem and root rots (Tyler, 2007). *P. sojae* produces various asexual spores namely; chlamydospores, sporangiospores and zoospores (Lannon, 2010). During wet season, zoospores swim in water and therefore cause infection to plants. Zoospores are thus, the dispersal and infective stage of *Phytophthoras sojae* (Lannon, 2010). The zoospores are chemically attracted to plant surfaces by isoflavones and can also be attracted to plant surfaces by minute concentrations of ethanol and amino acids present in plant roots or surfaces of the plant in close contact with the soil (Morris & Ward, 1992).

The study

Problem statement

The world's population growth continues to increase exponentially, according to the Census Bureau the current population is about 7.7 billion people and it is postulated that by 2050 the world's population will increase to approximately 10 billion people. Interestingly, statistics illustrate that most of these population will be concentrated in developing countries. As it is, developing countries face a lot of challenges with food security being one of the major problems. An increase in population could only intensify this challenge hence there is a need to create food security. This study's contribution to alleviating the food security challenge is by interrupting the soybean pathogen that cause massive destruction of soybeans causing huge losses to farmers.

Rationale & Hypothesis

Soybean is one of the most important food crops in the world; it is the second largest source of vegetable oil and the largest source of plant protein to both human beings and in animal feed in the world. (Sugiyama et al., 2015). The soybeans produce polyamines and then secrete them into the rhizosphere (Chibucos & Morris, 2006). The secretion of polyamines may enable the colonization of selective microorganisms in the rhizosphere such as *Bradyrhizobium* or confer other unknown biotic or abiotic effects (Sugiyama et al., 2015). Previous studies in our lab identified that there is high accumulation of polyamines; specifically, putrescine and spermidine at the zoospore stage of *P. sojae*. The study also that illustrated that swimming zoospores actively acquire putrescine from the media, but polyamines do not function as chemo attractants (Morris & Chibucos, 2006).

Since polyamines are accumulated in large amounts in the dispersal stage of *P. sojae* preceding infection, we hypothesize that the accumulation of polyamines may contribute to their ability to colonize plant tissues of soybeans or they may be needed for growth after infection or they could possibly facilitate uptake of secreted virulence infectors inside the soybean plants. PPTG_00424 is a predicted polyamine uptake transporter known to be expressed by swimming zoospores of *P. parasitica*. A sequence homologue has been found in *P. sojae* and we suspect it is a polyamine transporter highly likely to perform the same function. If it is localized to the plasma membrane it may be responsible for the uptake of polyamines from the media. However, if it is localized internally, then this transporter, might be involved in sequestering polyamines within a compartment in the swimming zoospores.

Methods

The complete nucleotide sequence of PPTG_00424 was codon optimized for its expression in yeast cells and obtained from GenScript, China in a

puc57 vector. The PPTG_00424 gene was then transformed into *E. coli* Top 10 competent cells using the heat shock protocol. The transformants were then plated on LB Amp plates to confer selection of the bacterial cells that have taken up the gene through Ampicillin antibiotic resistance. Subsequent streaking followed to obtain single colonies which were grown overnight in broth. The broth cultures were centrifuged to obtain a pellet that was used in the plasmid purification step. Plasmid purification was conducted using the specification and protocol in the Zyppy™ Plasmid Miniprep Kit. NanoDrop™ 1000 Spectrophotometer from Thermo Fisher Scientific was used to measure the concentration of the purified plasmid.

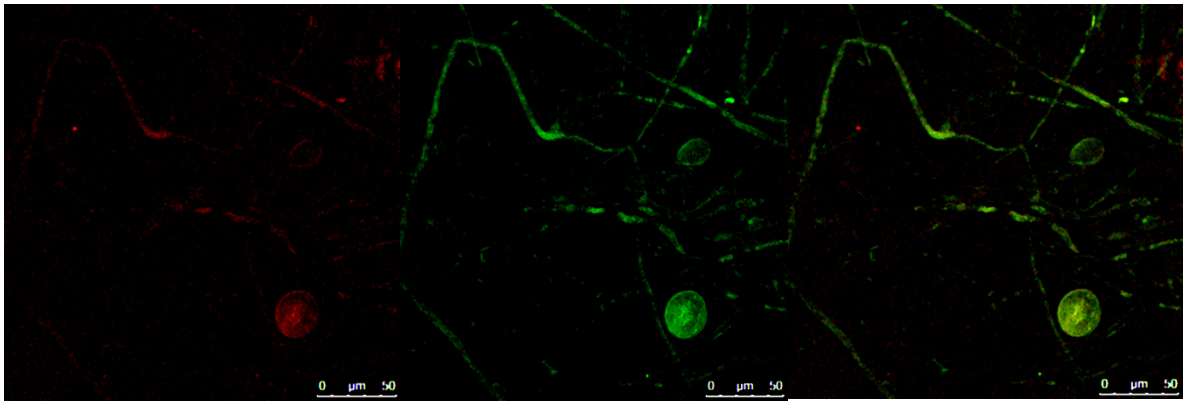
Primers flanking the restriction sites for Spe I enzyme were designed using the Primer3 online tool with the following sequences: forward primer sequence (5' to 3') PPTG_00424F- (DNA) ATAACTAGTCACCATGTTGGAAGAAGGTGAC and reverse primer sequence (5' to 3') PPTG_00424R- (DNA) TATAACTAGTAGCCAAACTTCTTCTGTA synthesized by Invitrogen. They were then used for subsequent PCR-based cloning followed by gel electrophoresis to confirm correct band sizes. Restriction digests of the gene PPTG_00424 and the destination vector PYF3_GFP followed by the enzyme Spe I. The gene and vector were ligated using NEB ligation protocol and transformed into *E. coli* Top 10 competent cells. Colony PCR followed to check for colonies with the gene inserted in the correct orientation. The positive screen colonies for correct orientation were grown in broth cultures and purified using the midi-prep kit from Invitrogen by Thermo Fisher Scientific using the manufacturer's protocol.

P. sojae was grown for five days and the PYF3_GFP vector with insert (PPTG_00424) was transformed into the mycelia of growing *P. sojae* (Fang et al., 2017). By employing fluorescent tagging of proteins, the YFP was used as the organelle marker for endoplasmic reticulum while the Green Fluorescent Protein (GFP) present in the vector was used as the marker for the gene. The localization of the gene was done through confocal microscopy which uses laser at a single wavelength to activate fluorescent proteins in the markers which then light up to visualize color.

Results & Conclusion

Transformation was successful with integration of both the GFP-tagged PPTG_00424 sequence and YFP-ER organelle marker on the same transformant. From confocal microscopic analysis, images of the mycelium of *P. sojae* transformed with a GFP-tagged PPTG_00424 fluoresced green while the YFP-ER marker fluoresced red. A merged image showed that the red color of the organelle marker and the green color of GFP-tagged PPTG_00424 gene superimposed on each other. It was concluded that the polyamine transporter gene PPTG_00424 is localized to the endoplasmic reticulum on the hyphae of *P. sojae* as shown in the confocal microscopy images below.

Fig 1. Confocal microscopy images of *P. sojae* mycelia transformed with GFP tagged PPTG_00424 and YFP tagged ER marker (shown in red signal)



YFP- ER marker GFP marker **Merged image**

Future projections

This study continues with heterologous expression experiments and radioactive assays in the next steps to determine the direction of polyamine transport; whether the gene transports polyamines in or out of Endoplasmic Reticulum (ER). Also, genetic knockouts by CRISPR-cas9 system are suggested to determine the significance of the probable transporter gene in the virulence of *P. sojae* to soybeans.

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